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Catalytic reductive dehalogenation of hexachloroethane by molecular variants of cytochrome $P450_{cam}$ (CYP101)

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CYP101 (cytochrome $P450_{cam}$) catalyses the oxidation of camphor but has also been shown to catalyse the reductive dehalogenation of hexachloroethane and pentachloroethane. This reaction has potential applications in the biodegradation of these environmental contaminants. The hexachloroethane dehalogenation activity of CYP101 has been investigated by mutagenesis. The effects of active-site polarity and volume were probed by combinations of active-site mutations. Increasing the active-site hydrophobicity by the Y96A and Y96F mutations strengthened hexachloroethane binding but decreased the rate of reaction. Increasing the polarity with the F87Y mutation drastically weakened hexachloroethane binding but did not affect the rate of reaction. The Y96H mutation had little effect at pH 7.4, but weakened hexachloroethane binding while increasing the rate of dehalogenation by up to 40% at pH 6.5, suggesting that the imidazole side-chain was partially protonated at pH 6.5 but not at pH 7.4. Substitutions by bulkier side-chains at F87, T101 and V247 weakened hexachloroethane binding but increased the dehalogenation rate. The effect of the individual mutations was additive in multiple mutants, and the most active mutant for hexachloroethane reductive dehalogenation at pH 7.4 was F87W–V247L (80 min^{-1} or 2.5 $\times$ the activity of the wild-type). The results suggested that the CYP101 active site shows good match with hexachloroethane, the Y96 side-chain plays an important role in both hexachloroethane binding and dehalogenation, and hexachloroethane binding and dehalogenation places conflicting demands on active-site polarity and compromises were necessary to achieve reasonable values for both.

Keywords: biodegradation; CYP; mutagenesis; P450; reductive dehalogenation.

Halogenated C_2 compounds are ubiquitous in the environment due to their widespread use as solvents and chemical feedstocks in industry. They are bioconcentrated in lipids because of their hydrophobic nature and, although chemically unreactive, small amounts are toxic to humans [1,2]. As such they are classified as priority pollutants by the US and European Environment Agencies. Naturally occurring microorganisms slowly attenuate the concentration of these compounds in the soil via initial reductive dehalogenation. Thus hexachloroethane (HCE) is converted to tetrachloroethene, and pentachloroethane to trichloroethene, which could then be degraded further. This process is slow, and the development of more rapid and efficient degradation systems could have significant applications in environmental biotechnology [3].

Although reductive dehalogenation of haloaliphatic compounds is well established, the mechanisms are not fully understood, and the enzymes involved are poorly characterized. Nickel- and cobalt-containing enzymes and respiratory *c*-type cytochromes have been shown to catalyse the reaction but no details are available [4,5]. CYP101 (cytochrome $P450_{cam}$) from *Pseudomonas putida*, which naturally catalyses the oxidation of camphor [6], has been shown to catalyse the reductive dehalogenation of haloaliphatic compounds [7,8]. Remarkably, HCE was found to induce $>95\%$ shift of the haem spin state to high spin [8]. Although the rates of reductive dehalogenation are much slower than the camphor oxidation rate, a novel bioremediation system has been described in which polyhalogenated aliphatic compounds are mineralized via reductive dehalogenation by CYP101 followed by an oxidative pathway based on a toluene dioxygenase system genetically introduced into the *Ps. putida* host [9]. This genetically engineered microorganism has been shown to be viable for HCE reductive dehalogenation in a soil slurry under different growth conditions for periods of months [10].

The rate-limiting step for reductive dehalogenation of polyhalogenated C_2 compounds by CYP101 (Fig. 1) has been proposed to be the transfer of the first electron from the ferrous haem to the substrate [11,12]. The F87W mutant of CYP101 has been reported to dehalogenate pentachloroethane at three times the rate of the wild-type [13]. Molecular dynamics simulations suggested that this mutation increased the rate by sterically limiting the substrate to regions close to the haem, thus shortening the donor–acceptor distance for the rate-limiting electron transfer reaction [14]. This step should also be accelerated by stabilizing the developing negative charge on the chloride leaving group, and calculations predicted that the Y96H mutation could increase the rate by as much as 400-fold if the imidazole side-chain was protonated [12].

Protein engineering of CYP101 has been investigated extensively because the mechanism and biochemistry of this enzyme have been studied in detail [6,15], and the crystal structures of different forms of the enzyme are available [16]. Significant insights have also come from molecular dynamics simulations [12,17–19]. Here, we report the active-site engineering of CYP101 with the aim of increasing the rate of

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Abbreviation: HCE, hexachloroethane.

Enzymes: cytochrome $P450_{cam}$ (CYP101) (EC 1.14.15.1); putidaredoxin reductase, P16640; putidaredoxin, P00259.

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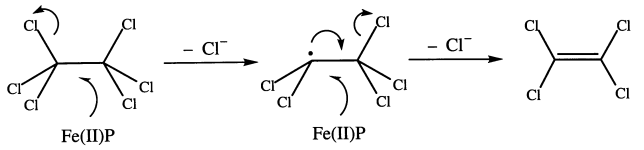

Fig. 1. The two-step mechanism of reductive dehalogenation of hexachloroethane (HCE) to form tetrachloroethene catalysed by CYP101, where Fe(II)P is the ferrous form of the haem.

reductive dehalogenation of HCE by shortening the donor–acceptor distance and stabilizing the developing negative charge on chlorine. Combinations of hydrophobic substitutions at F87 (F87L, F87W), Y96 (Y96A, Y96F), T101 (T101L), V247 (V247L) and V396 (V396L), and the polar substitutions F87Y and Y96H were used to investigate the role of these two factors. HCE binding and reductive dehalogenation by the CYP101 variants, and the effect of pH on the reaction, are described.

MATERIALS AND METHODS

General

HCE and tetrachloroethene were from Aldrich Chemical Co. Molecular biology grade buffer components were from Anachem (UK). NADH was from Boehringer-Mannheim. UV/Vis spectra were recorded on a CARY 1E spectrophotometer equipped with a Peltier cell temperature controller (± 0.1 °C). GC analysis was performed on a Fisons Instruments 8000 Series gas chromatograph using flame ionization detection and a DB-1 fused silica capillary column (30 m × 0.25 mm i.d.).

Enzymes and molecular biology

Wild-type CYP101 and the mutants, and the electron transfer proteins putidaredoxin and putidaredoxin reductase were expressed in *Escherichia coli* and purified to homogeneity following literature methods [20–22]. General DNA manipulations followed standard methods [23]. Enzymes for molecular biology, and the appropriate buffers, were from New England Biolabs (UK). Site-directed mutagenesis of CYP101 was carried out by the Kunkel method on a M13mp19 subclone using the Bio-Rad Mutagene kit [24], or by PCR methods using the QuikChange kit from Stratagene. The mutagenic oligonucleotides were designed according to guidelines provided by the manufacturers. The entire nucleotide sequence of all the mutant genes was determined by automated DNA sequencing on an ABI 377 Prism DNA sequencer in the Department of Biochemistry, University of Oxford, to confirm that no spurious mutations were present. All the CYP101 enzymes described in this work also contained the C334A base mutation which has been shown to maintain the enzyme in the monomeric form by preventing dimerization via cysteine disulphide bond formation [25]. For simplicity, this C334A base mutant is referred to as the 'wild-type' protein.

Binding constant determinations

The binding constants for HCE by the CYP101 variants were determined at 30 °C by following the spectral changes between 350 and 450 nm on addition of increasing amounts of HCE. The protein was equilibrated into 50 mM Tris, pH 7.4 using a PD10 (Pharmacia) gel-filtration column. KCl was added as a 2 M stock to a final concentration of 200 mM, and the solution was diluted with buffer to 1 μM CYP101. Aliquots of a 1-mM solution of HCE in ethanol were added to final concentrations of 1–400 μM and the spectra recorded. The binding constants were determined from the spectra as described previously [26]. Mops (50 mM) was used for titrations at pH 6.5. Ethanol did not induce any changes in the haem spin state at concentrations up to 20% v/v.

Kinetic studies of substrate transformations

Substrate transformations were studied in 4-mL UV cells equipped with a Teflon-lined rubber septum held tight by a screw top (Helma, UK). Incubation mixtures were prepared in an argon-filled Bell Technology glove box ($O_2 < 4$ p.p.m.), and contained 1 μM CYP101, 1 μM putidaredoxin reductase, 16 μM putidaredoxin and 200 mM KCl in 50 mM Tris, pH 7.4. Reducing equivalents were provided by the addition of 300 μM NADH in the glove box. Before initiating the reaction by adding the substrate, the absorption of the incubation mixture at 340 nm was monitored for 10 min to ensure that there was no consumption of NADH due to traces of O_2. The reaction was then initiated by the addition in the glove box of 400 μM HCE (100 mM stock in ethanol). We noted that incubation mixtures containing HCE at concentrations > 400 μM were cloudy. The decrease in absorption at 340 nm indicated consumption of NADH due to substrate transformation, the rate of which was calculated using $\varepsilon_{340nm} = 6.22\ mM^{-1}{\cdot}cm^{-1}$ and expressed in nmol NADH consumed per nmol CYP101 per min. The rate of NADH consumption was not affected by the presence of up to 20% v/v ethanol. The activity of the wild-type and some mutants were determined at pH 6.5 in 50 mM Mops buffer. All three proteins were buffer exchanged into Mops by gel filtration, and concentrations of all components identical to measurements at pH 7.4 were used

After all the NADH had been consumed, the sealed UV cell was chilled on ice to minimize volatilization of the tetrachloroethene product, and all subsequent manipulations were also carried out at 4 °C. The mixture was extracted with 1 mL of $CHCl_3$ and the phases separated by centrifugation at 4000 ***g*** for 20 min. The organic phase was removed, a 30-μL aliquot of a 1 mM stock of 4-heptanol in $CHCl_3$ was added as an internal standard for the analysis step, and a 1 μL aliquot of this mixture was analysed by GC. The injector was set at 150 °C and the flame-ionization detector at 250 °C. The DB-1 column was held at 80 °C, and the retention times were: tetrachloroethene 3.3 min, 4-heptanol 4.2 min and HCE 11.2 min. The tetrachloroethene product was identified by co-elution experiments under different column temperature profiles.

The concentration of the tetrachloroethene product formed, and therefore the stoichiometry, i.e. the coupling efficiency, of the reactions was determined by extraction of mixtures containing different concentrations of the product and all the incubation components except NADH and HCE. The calibration plot thus obtained was linear and passed through the origin. At random intervals the calibration was checked by further extractions and consistent results (within 5%) were obtained. We noted that the results from both the calibration checks and the incubations were variable unless all the solutions were kept on ice and the extraction procedure carried out at 4 °C, presumably due to volatilization of the product.

RESULTS AND DISCUSSION

Molecular variants of CYP101

Figure 2 shows the CYP101 active site residues including all those targeted for mutagenesis. The molecular variants constructed and purified are listed in Table 1. There are three subgroups of variants. Firstly, the effect of active site polarity and specific stabilization of a developing negative charge was investigated by the F87Y, Y96A, Y96F and Y96H substitutions. Tyr96 is the most prominent polar residue in the active site and may be important in both HCE binding and dehalogenation. The side-chains of F87 and Y96 are in van der Waal's contact with each other and at the same height above the haem plane. The F87Y mutation could inform on whether two polar phenol side-chains in close proximity could enhance the dehalogenation activity, while the Y96H mutation was used to study the potential benefit of a side-chain which may be protonated.

Secondly, amino acid residues with nonpolar side-chains were replaced by others with bulkier side-chains to engineer a smaller substrate binding cavity that could sterically constrain HCE closer to the haem. The target residues included F87, F98 and V247, which are high up in the substrate pocket, as well as T101, which is close to the haem. Mutants F87W [13,27], T101L, V247L [28], Y96F–F87W, Y96F–V247L [29], F87W–V247L, Y96F–F87W–V247L and F87W–V247L–V396L were prepared. F87L, Y96F–F87L [30], F87L–V247L and Y96F–F87L–V247L mutants were employed as controls for the effect of the F87W mutation. Finally, the sets of mutations were combined in the Y96H–F87L and Y96H–F87W–V247L mutants to investigate whether the effects of active site polarity and volume were additive.

The introduction of multiple active site mutations did not significantly affect the stability of the CYP101 enzyme. All the

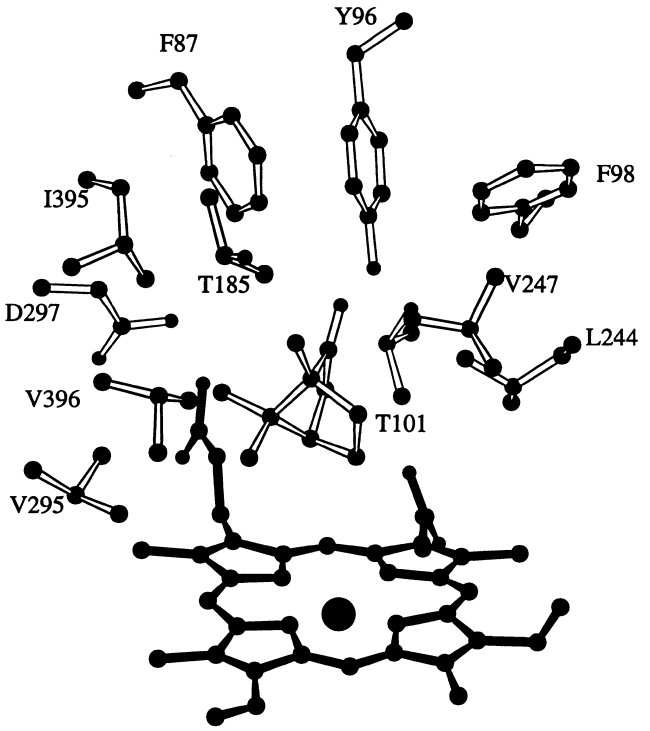

Fig. 2. The structure of the active site of CYP101 with bound camphor showing the position of residues F87, Y96, T101, V247 and V396 which were targeted for mutagenesis.

Table 1. High-spin haem content, hexachloroethane-binding constant and reductive dehalogenation rate of wild-type and molecular variants of CYP101 (cytochrome $P450_{cam}$) at pH 7.4. Data at pH 6.5 are given in brackets.

CYP101 enzyme	% High-spin haem	K_d (μM)	Rate of reaction k_2 [nmol (nmol CYP101)$^{-1}$·min^{-1}]	$10^5 \times k_2/K_d$ ($M^{-1}·s^{-1}$)
C334A (wild-type)	85 ± 5	1.09 ± 0.16	32 ± 4	4.9
		(1.98 ± 0.27)	(35 ± 5)	
Y96A	90 ± 5	0.68 ± 0.20	33 ± 2	8.1
Y96F	90 ± 5	0.16 ± 0.08	19 ± 5	20
Y96F–F87W	90 ± 5	0.51 ± 0.20	32 ± 5	10
Y96F–V247L	90 ± 5	0.22 ± 0.05	45 ± 7	34
Y96F–F87L	85 ± 5	0.77 ± 0.03	25 ± 2	0.5
Y96F–F87W–V247L	90 ± 5	0.79 ± 0.30	46 ± 4	9.7
Y96F–F87L–V247L	85 ± 5	0.46 ± 0.06	47 ± 2	17
Y96H	85 ± 5	1.38 ± 0 31.	40 ± 2	4.8
		(5.53 ± 2.16)	(56 ± 4)	
Y96H–F87L	50 ± 5	1.93 ± 0.10	54 ± 2	4.7
Y96H–F87W–V247L	90 ± 5	8.91 ± 2.10	76 ± 5	1.4
			(101 ± 3)	
F87L	95 ± 5	4.17 ± 0.10	50 ± 2	2.0
F87Y	40 ± 5	42.3 ± 5.0	34 ± 7	0.13
F87W	85 ± 5	3.10 ± 0.20	55 ± 4	2.9
F87L–V247L	95 ± 5	2.26 ± 0.15	67 ± 3	4.9
F87W–V247L	95 ± 5	2.77 ± 0.20	80 ± 5	4.8
F87W–V247L–V396L	60 ± 5	–	23 ± 4	–
T101L	85 ± 5	4.73 ± 0.50	49 ± 2	1.7
V247L	95 ± 5	1.98 ± 0.12	52 ± 5	4.4

mutants were expressed in *E. coli* at levels comparable with the wild-type and were readily purified to homogeneity.

Binding of hexachloroethane by the CYP101 variants

Haem spin-state shifts are primarily due to changes in the iron coordination sphere. In the low spin ferric resting state of CYP101 a water molecule is bound to the haem iron. When substrates bind in the active site, this water molecule could be displaced and the haem becomes high spin. HCE binding to the wild-type enzyme has been shown to induce $> 95\%$ high spin haem content. The majority of CYP101 mutants studied also showed $> 80\%$ spin state shifts when HCE was added. The exceptions were the F87Y, Y96H–F87L and F87W–V247L–V396L mutants.

The dissociation constant of HCE binding by the CYP101 enzymes (K_d, Table 1) showed interesting variations. The hydrophobic substitutions Y96A and Y96F were the only ones to strengthen HCE binding, with the larger phenyl side-chain of the Y96F having a greater effect. The Y96H mutation affected HCE binding only slightly at pH 7.4, while the polar substitution F87Y drastically weakened HCE binding. These observations were almost certainly due to the more hydrophobic nature of HCE compared with camphor. The important effect of the hydrophobic phenyl side-chain at position 96 was clearly evident in the multiple mutants. HCE binding by the Y96F–F87L, Y96F–F87W and Y96F–V247L mutants was much stronger than the corresponding single-site mutants without the Y96F mutation, so that these double mutants were able to bind HCE tighter than the wild-type. Similarly, multiple mutants containing the Y96F mutation showed tighter binding than analogues with the Y96H mutation.

The hydrophobic substitution T101L weakened HCE binding, probably because steric hindrance between HCE and the bulkier leucine side-chain outweighed any gain due to increased hydrophobicity. Steric hindrance would also account for weaker HCE binding by the F87W and V247L mutants. Interestingly, the F87L mutation, which should increase the activity site volume slightly, also weakened HCE binding. Therefore, the binding data indicated that there was already a good match between HCE and the active site of wild-type CYP101, and only the increased the hydrophobicity engendered by the Y96A and Y96F mutations strengthened HCE binding. The K_d value of 0.16 μM for the Y96F mutant was in fact slightly smaller than that for camphor binding by the wild-type ($K_d = 0.24$ μM).

The product and rates of HCE dehalogenation

The product of HCE reductive dehalogenation catalysed by all the CYP101 variants was tetrachloroethene. No further transformation of tetrachloroethene was observed although all the enzymes showed binding with this compound, as detected by haem spin-state shifts of 20–40% (data not shown). The coupling efficiency for all the CYP101 variants was shown by quantitative GC analysis to be 100%, i.e. all the reducing equivalents from NADH were channelled to form the product.

The rates of HCE dehalogenation by the different CYP101 variants are given in Table 1. It was clear that the rate of HCE dehalogenation by all CYP101 variants with a hydrophobic substitution at position 96 was lower than those in which a polar residue, either tyrosine or histidine, was at this position. Thus the Y96F mutation retarded the dehalogenation reaction, whereas the Y96A mutation had little effect, but both had lower activity than the wild-type and the Y96H mutant. The data were entirely consistent with the phenol side-chain of Y96 stabilizing the developing negative charge on the chloride leaving group (Fig. 1), thus lowering the activation energy for the reaction. Stabilization of charge to promote the loss of chloride had been observed in the haloacid dehalogenase from *Pseudomonas* sp. [32] and the haloalkane dehalogenase from *Xanthobacter autotrophicus* [33], in which Arg and Trp, respectively, were the residues involved. Substitution of critical Trp residues in haloalkane dehalogenase significantly reduced the activity [34]. Such considerations of the effect of polar residues were the basis for modelling work on the potential effect of the Y96H substitution in CYP101. It was predicted that a protonated imidazole side-chain could increase the rate of pentachloroethane dehalogenation by 400 ×, but no effect was expected if the imidazole was not protonated [12]. Our results showed that the Y96H substitution did not alter the activity at the standard incubation pH of 7.4, suggesting the imidazole side-chain was not protonated at this pH. Surprisingly, the polar substitution F87Y did not change the dehalogenation activity.

The F87W mutation increased the activity, consistent with the previously reported effect on pentachloroethane dehalogenation [6,13]. The T101L and V247L mutations, which also introduced a bulkier side-chain, had similar effects. It has been suggested that the F87W mutation accelerated the reductive dehalogenation of pentachloroethane threefold by placing the substrate closer to the haem, thereby shortening the donor–acceptor separation for electron transfer [14]. A similar mechanism is probably operating here for HCE. However, it should be noted that the F87L mutant had the same rate of HCE dehalogenation as the F87W mutant. Indeed, this similarity extended to the multiple mutants in which these two mutations were combined with the Y96F, Y96H and Y96F–V247L mutations. Further work is required to establish whether the F87L mutation increased the rate by changing the distance of electron transfer or by facilitating better interaction between the chloride leaving group and the Y96 side-chain.

In general, mutants containing multiple mutations showed good additivity of the effects of the individual mutations. For instance, the HCE dehalogenation rate of ≈ 80 min^{-1} for the F87W–V247L and Y96H–F87W–V247L mutants was higher than the corresponding single site mutants and 2.5× that of the wild-type. In an attempt to increase the activity further, the active site volume was reduced by adding the V396L mutation. However, the activity of the triple mutant F87W–V247L–V396L was in fact significantly lower than that of the wild-type.

The rate of dehalogenation k_2 and the substrate binding constant K_d shown in Table 1 could be approximated to the Michaelis–Menten parameters k_{cat} and K_M, respectively [31]. The k_2/K_d ratios for the CYP101 variants are also listed in Table 1. The value of 4.9×10^5 M^{-1}·s^{-1} for HCE reductive dehalogenation by wild-type CYP101 under experimental conditions was two orders of magnitude lower than that for camphor oxidation ($k_2 = 17.5$ s^{-1}, $K_d = 0.24$ μM, $k_2/K_d = 7.3 \times 10^7$ M^{-1}·s^{-1}), and this arose from a combination of slower turnover rate as well as weakened substrate binding.

The data in Table 1 show that, despite the higher values of k_2 for HCE dehalogenation for many mutants, few had higher k_2/K_d ratios than the wild-type due to weaker HCE binding. The Y96F mutant had a high k_2/K_d ratio of 2.0×10^6 M^{-1}·s^{-1}, but this was due entirely to very tight HCE binding. The other mutants with high k_2/K_d ratios were Y96F–V247L and Y96F–F87L–V247L, primarily because these also showed tighter HCE binding. The F87W–V247L mutant had the highest k_2 but significantly weaker binding, and so the k_2/K_d ratio was identical to the wild-type. The best compromise was the

Y96F–V247L double mutant ($k_2/K_d = 3.4 \times 10^6$ M^1·s^{-1}) because of the 40% higher k_2 and fivefold tighter HCE binding compared with the wild-type.

The effect of pH on HCE binding and dehalogenation

HCE binding and reductive dehalogenation by some CYP101 mutants were studied between pH 5.5 and 7.4. Reliable dehalogenation rates could not be obtained at and below pH 6.0 because of the onset of denaturation of both CYP101 and putidaredoxin, the Fe_2S_2 electron transfer protein. Therefore the pK_a of the imidazole side-chain in mutants containing the Y96H substitution could not be determined.

The wild-type enzyme showed identical HCE binding and turnover at pH 6.5 and 7.4 (Table 1). In contrast, all mutants containing the Y96H mutation showed weaker HCE binding but higher dehalogenation activity at pH 6.5 than at pH 7.4. The data were entirely consistent with the imidazole side-chain not being protonated at pH 7.4. A charged side-chain, such as a protonated imidazole, would be expected to weaken substantially the binding of a highly hydrophobic molecule such as HCE. However, at pH 6.5 HCE binding by the Y96H-containing mutants was tighter than the F87Y mutant, and so it was most unlikely that the imidazole nitrogen was fully protonated at this pH. The generally nonpolar environment of the CYP101 active site presumably lowered the pK_a of the imidazole nitrogen. Although the effect of pH was modest, the 101 min^{-1} activity of the Y96H–F87W–V247L triple mutant at pH 6.5 was the fastest observed in this work, and 3.2 times the wild-type activity.

SUMMARY AND CONCLUSIONS

HCE binding and reductive dehalogenation kinetics of CYP101 were investigated in a series of molecular variants designed to address the role of the polarity, size and topology of the substrate binding site. Individual mutations in the CYP101 active site appeared to act independently because there was good additivity of the effect of individual mutations in multiple mutants. Nevertheless, the protein engineering of CYP101 for HCE dehalogenation was more difficult than for the oxidation of many other substrates because of the conflicting demands of substrate binding and rate acceleration. HCE binding was strengthened only by increasing the active site hydrophobicity by the Y96A and Y96F mutations, but the polar phenol side-chain also played the crucial role of stabilizing the charge on the leaving group. It would appear that this circle could not be squared easily. It also seemed likely from our results that a positively charged side-chain, such as a protonated imidazole, in the active site might accelerate the rate but would also significantly weaken HCE binding.

Despite the modest increases in HCE reductive dehalogenation activity, with the most active mutant F87W–V247L being only 2.5 times as active as the wild-type at pH 7.4, molecular variants of CYP101 could have applications in bioremediation especially when the first, reductive dehalogenation step is slow. An interesting possibility is to introduce both the F87W–V247L and Y96F mutants into an organism so that the double mutant could reductively dehalogenate the contaminants at increased rates while the Y96F mutant, although less active, could lower the final concentration of the contaminant further by virtue of the much tighter binding.

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