Crystal Structure of the F87W/Y96F/V247L Mutant of Cytochrome P-450cam with 1,3,5-Trichlorobenzene Bound and Further Protein Engineering for the Oxidation of Pentachlorobenzene and Hexachlorobenzene*§

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We reported previously that the F87W/Y96F/V247L mutant of cytochrome P-450cam (CYP101) from Pseudomonas putida catalyzed the rapid oxidation of lightly chlorinated benzenes, but pentachlorobenzene oxidation was slow (Jones, J. P., O’Hare, E. J., and Wong, L. L. (2001) Eur. J. Biochem. 268, 1460–1467). In the present work, we determined the crystal structure of this mutant with bound 1,3,5-trichlorobenzene. The substrate was bound to crystallographically independent CYP101 molecules in at least three different orientations, which were distinguished by the angle between the benzene ring and the porphyrin, and one orientation contained an Fe-Cl interaction. In another orientation, the substrate was almost parallel to the heme, with a C–H bond closest to the iron. The enzyme/substrate contacts suggested that the L244A mutation should promote the binding of pentachlorobenzene and hexachlorobenzene by creating space to accommodate the extra chlorines. The F87W/Y96F/L244A/V247L mutant thus designed was found to oxidize pentachlorobenzene at a rate of 82.5 nmol (nmol CYP101)−1 min−1, 45 times faster than the F87W/Y96F/V247L parent mutant. The rate of hexachlorobenzene oxidation was increased 200-fold, to 2.0 min−1. Both substrates are oxidized to pentachlorophenol, which is degraded by micro-organisms. In principle, the F87W/Y96F/L244A/V247L mutant could have applications in the bioremediation of polychlorinated benzenes.

The cytochrome P-450 enzymes are mixed function oxidases that primarily catalyze the oxidation of C–H bonds in a great variety of endogenous and exogenous organic compounds, ranging from small alkanes to steroidal and polyaromatic compounds and very large molecules such as cyclosporin (1, 2). These reactions are important in biosynthesis, biochemistry, pharmacology, and clinical medicine. A great deal of research effort has been directed at understanding the relationship between structure, function, and reactivity (2) and to engineer these enzymes for biotechnological applications (3–5).

Polychlorinated aromatic compounds are hazardous and recalcitrant environmental contaminants and, as such, are classified as priority pollutants by the environment agencies of the United States and Europe (6, 7). The majority of the less chlorinated benzenes, biphenyls (polychlorinated biphenyls), and dioxins can be degraded by a consortium of micro-organisms (8–10). However, as the degree of chlorination increases, the compounds become more inert, and the heavily chlorinated homologues are partially degraded at slow rates or not at all. For example, pentachlorobenzene (PeCB)1 and hexachlorobenzene (HCB) are highly recalcitrant (11).

Aerobic micro-organisms degrade chlorinated aromatic compounds via initial attack by nonheme iron dioxygenases to form the cis-dihydriodiol. After re-aromatization to chlorocatechols, catechol 1,2-dioxygenases (another class of nonheme iron enzymes) cleave the aromatic ring (7, 10). Both of these dioxygenases appear to require certain ring positions to remain unsubstituted (12–14), and no strictly aerobic organism capable of degrading PeCB, HCB, and the heavily chlorinated polychlorinated biphenyls and dioxins has been isolated. Instead, these compounds are degraded by much slower reductive pathways (7).

Unlike the benzenes, polychlorinated phenols are readily mineralized by micro-organisms (15). The degradation pathways for heavily chlorinated phenols typically involve oxidation to chlorohydroquinones catalyzed by flavin-dependent monooxygenases (16–19). The reactive chlorohydroquinones are then dehalogenated by hydrolytic or reductive mechanisms. We have proposed that chlorophenol degrading micro-organisms could be genetically augmented with monooxygenase enzymes that can convert chlorinated benzenes to the phenol derivatives (20, 21). Provided that the introduced enzyme could oxidize the heavily chlorinated benzenes to the phenol derivatives at significant rates, the novel micro-organisms thus generated could degrade all chlorinated benzenes (Fig. 1).

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The atomic coordinates and structure factors (code 1J51) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ The on-line version of this article (available at http://www.jbc.org) contains a supplemental table and diagram.

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1 The abbreviations used are: PeCB, pentachlorobenzene; TCB, trichlorobenzene; HCB, hexachlorobenzene; PCP, pentachlorophenol; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid.
Structure-based CYP101 Engineering for PeCB Oxidation

We showed recently that the heme monoxygenase CYP101 from *Pseudomonas putida* (22, 23) could be engineered to oxidize all of the lightly chlorinated benzenes with high activity and coupling efficiency compared with the wild type enzyme (20, 21). The triple mutant F87W/Y96F/V247L oxidized 1,3,5-trichlorobenzene (1,3,5-TCB) with a catalytic turnover rate of 175 min⁻¹ and 57% coupling; this activity is more than sufficient for the application of this mutant in the bioremediation of the recalcitrant 1,3,5-TCB. This mutant also showed a fast NADH oxidation rate with PeCB as the substrate, but the PeCB oxidation rate was slow because of extensive uncoupling (21). Nevertheless, we were encouraged by the NADH turnover rate because this indicated that the PeCB was bound within the CYP101 active site, although not in the correct orientation to suppress uncoupling side reactions.

We report here our efforts to gain further insight into CYP101 substrate specificity and to improve the activity and coupling efficiency for the oxidation of PeCB and HCB by engineered CYP101 mutants. We determined the crystal structure of the F87W/Y96F/V247L mutant of CYP101 with 1,3,5-TCB bound within the active site. The structure provided new information on CYP101 substrate recognition and indicated an additional mutation that greatly increased the PeCB and HCB oxidation activity.

**Materials and Methods**

**General—**Enzymes for molecular biology were from New England Biolabs. Buffer components were from Anachem, and general reagents were from Sigma or Merck. NADH was from Roche Diagnostics. 1,3,5-TCB, PeCB, HCB, and PCP were from Sigma or Merck. UV-visible spectra were readily modeled as 1,3,5-TCB. Difference density in molecule C was recorded on a Varian CARY 1E spectrophotometer equipped with a Peltier cell temperature controller (±0.1 °C). The HPLC analyses were performed on a Gilson system.

**Enzymes and Molecular Biology—**General DNA and microbiological manipulations were carried out by standard methods (24). The CYP101 enzyme and the physiological electron transfer co-factor proteins putidaredoxin and putidaredoxin reductase were expressed in *Escherichia coli* and purified following literature methods (25–27). The purified proteins were stored at −20 °C in buffers containing 50% glycerol. Immediately before use in activity assays, glycerol was removed by gel filtration on a PD-10 column (Amersham Biosciences), eluting with 50 mM Tris, pH 7.4. Site-directed mutagenesis was carried out using the QuikChange kit from Stratagene. All of the CYP101 enzymes described in this work also contained the C334A mutation to prevent protein dimerization via disulfide bond formation (28). For convenience, the CYP101 active site, although not in the correct orientation to suppress uncoupling side reactions, was opened by a breakdown of symmetry may have occurred because soaking and entry of the substrate, we switched to the P1 space group where we were able to find two molecular replacement solutions corresponding to the two molecules/asymmetric unit. The P2₁ space group was also found in a similar study (29). However, when these solutions were used for the complex structure resulting from the soaking experiments, we were not able to refine the structure of the complex in the P2₁ space group. After realizing that a weakly bound substrate with very low occupancy, in the final structure it was more reasonable to model the density as a cluster of three water molecules. The final refinement parameters were Rwork = 18.6% and Rfree = 25.8%. The data collection and structure refinement statistics are summarized in Table I.

**NADH Turnover Rate Determinations—**All of the incubations were carried out at 30 °C. Incubation mixtures (1.7 ml) contained 50 mM Tris, pH 7.4, 200 mM KC1, 1 μM CYP101, 10 μM putidaredoxin, 1 μM putidaredoxin reductase, and 50 μM 1-bromobenzene (30). The mixtures were oxygenated and then equilibrated at 30 °C for 2 min. The PeCB and HCB substrates were added as 10 mM stocks in Me₃SO to a nominal final concentration in an incubation mixture of 100 μM. Precipitation of both substrates was observed. NADH was added to 116 μM (final A₅₄₀ = 1.00), and the absorbance at 340 nm was monitored. In the case of the F87W/Y96F/V247L mutant, the absorbance at 340 nm did not change because the product produced was the PCP product, which had an absorption maximum at 325 nm. However, this did not interfere with the determination of the rate of NADH consumption, which was calculated from the slope of the time-course plot using ε₅₄₀ = 6.22 mmol cm⁻². The maximum concentration of Me₃SO in an incubation reaction was 0.2% (v/v), and control experiments showed that Me₃SO at 2% (v/v) did not induce any changes in the...
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RESULTS AND DISCUSSION

The Structure of the F87W/Y96F/V247L Mutant with 1,3,5-TCB Bound—The CYP101 mutant F87W/Y96F/V247L was crystallized from cacodylate buffer and PEG8000. Soaking the crystals in the well solution with 100 μl 1,3,5-TCB added did not give good quality crystals. The conditions were varied, and we found that soaking in MES buffer for 5 days gave the best results. Longer soak times resulted in deterioration of the crystals and lower data resolution. The crystals of the F87W/Y96F/V247L mutant with 1,3,5-TCB bound in the active site diffracted to beyond 2.0 Å resolution, and the structure at 2.2 Å resolution was solved by molecular replacement methods. There were four protein molecules (A, B, C, and D) in the unit cell, three of which (A, B, and C) contained the 1,3,5-TCB substrate within the active site, whereas only water molecules were modeled in the substrate pocket in molecule D. The 1,3,5-TCB substrate was bound in different orientations in molecules A, B, and C.

We also obtained another crystal form under similar crystallization conditions. These crystals belonged to the space group P2₁, with two enzyme molecules in each unit cell. One molecule was the substrate-free form, whereas the other had a 1,3,5-TCB substrate bound in a very similar orientation to that found in molecule A of the monoclinic crystals. However, this P2₁ form diffracted only to 2.5 Å resolution, and further refinement was not pursued.

General Structural Features—The electron density of the first 9 amino acid residues was not observed in any of the four molecules in the unit cell, otherwise the mutant retained all of the structural features of the wild type (31). Some general structural parameters for the mutant molecules are compared with those for the wild type in Table I. The Cα backbones of all four molecules were almost superimposable on that of the wild type, with the exception of a small rotation (89o compared with the orthorhombic crystal form of the wild type (31)).

The electron density of the heme spin state equilibration nor increases in the rate of NADH consumption.

| Table I | X-ray data collection, refinement, and common structural parameters |
|---------|-------------------------------------------------------------------|
| Unit cell | 62.47, 66.90, 95.54 Å |
| Space group | P1 |
| Number of molecules in unit cell | 4 (A, B, C, D) |
| Refinement | 50 – 2.2 Å |
| Total number of reflections | 285817 |
| Unique reflections | 74970 |
| Completeness (highest resolution shell) | 95.8% (90.1%) |
| Rmerge | 7.9% |
| R因子 | 18.6% |
| Rfree | 25.8% |
| Residues not modeled | A1–A9, B1–B9, C1–C9, D1–D9 |
| Number of molecules with 1,3,5-TCB bound | 3 (A,B,C) |
| rmsd from restraint values | bond lengths: 0.0013 Å |
| | bond angles: 1.74° |
| rmsd between Cα backbone of molecules | WT vs. A: 0.40 Å; WT vs. B: 0.46 Å; WT vs. C: 0.38 Å; WT vs. D: 0.46 Å; |
| | A vs. B: 0.39 Å; A vs. C: 0.16 Å; A vs. D: 0.39 Å; B vs. C: 0.38 Å; B vs. D: 0.18 Å; C vs. D: 0.37 Å |
| Fe-S (cysteine 357) distance | A: 2.23 Å; B: 2.07 Å; C: 2.20 Å; D: 2.09 Å (WT, 2.20 Å) |
| Fe-porphyrin plane distance | A: 0.32 Å; B: 0.36 Å; C: 0.36 Å; D: 0.35 Å (WT, 0.37 Å) |
| Ramachandran analysis: most favored (additionally allowed) | A: 88.2% (11.8%); B: 85.9% (13.8%); C: 87.9% (12.1%); D: 84.5% (15.5%) |

The planes of the aromatic side chains at the 87 and 96
positions in the wild type and all four molecules of the mutant were virtually superimposable, although the main chain and side chain of the 87 residue were shifted by \( \sim 0.4 \) Å. Similar slight shifts were also observed for the 247 residue. The Phe 98 phenyl ring was rotated by \( \sim 20^\circ \) in all four molecules, apparently to avoid steric hindrance between Phe 98 CE and Leu 247 CD. The indole NH group of Trp 87 pointed toward the heme, whereas the benzene ring was oriented toward the Leu 247 side chain. The orientation of the geminal methyl groups of Leu 247 varied slightly in the four protein molecules, but in all cases the closest distance between the Trp 87 and Leu 247 side chains was \( 4.0 - 4.3 \) Å, compared with \( 6.5 \) Å between Phe 87 and Val 247 in the wild type. It is evident that the three mutations had only minor effects on the structure of CYP101.

Poulos et al. (31) noted previously that the structure of CYP101 does not show an obvious substrate access channel, and dynamic fluctuations are required to allow entry of a potential substrate. The close position of the Trp 87 and Leu 247 side chains at the top of the CYP101 active site indicates that the structural fluctuations are sufficient to move both of these sterically demanding side chains out of the way, such that an incoming substrate, e.g. 1,3,5-TCB, can enter and bind in the active site.

1,3,5-TCB Binding in Molecule A—The active site structure of molecule A with 1,3,5-TCB bound, together with the electron density for the substrate, is shown in Fig. 2. (The contact distances between 1,3,5-TCB and the heme and protein side chains are detailed in the supplemental table. There were no water molecules in the active site.) The substrate was located mainly over pyrroles A and D and the meso carbons CHA and CHB. The C-4 atom of 1,3,5-TCB was at a distance of 4.01 Å above the heme iron with a C/Fe/S (Cys 357) angle of 170°, compared with 4.21 Å and 164° for camphor C-5 in the wild type structure. The angle between the 1,3,5-TCB plane and the porphyrin was 28°, and these two aromatic systems were in van der Waals’ contact.

The heme and 1,3,5-TCB chlorine atoms played very important roles in the enzyme-substrate interactions. There was
strong van der Waals’ interaction between Cl-3 and pyrrole D. Cl-5 was close to the meso carbon CHB, and it also contacted the Val295 side chain (Fig. 3). Both Cl-3 and C-2 contacted the Leu244 side chain, and Cl-3 also contacted the backbone of Gly248. The Cl-1 atom was 3.00 Å away from the indole nitrogen of Trp87, indicating a hydrogen bonding interaction. This chlorine atom was also within hydrogen bonding distance (3.40 Å) of a carboxylate oxygen of Asp297. The hydrogen bond to the indole NH of Trp87 appeared to pull the Cl-1/C-1 end of the 1,3,5-TCB molecule slightly upwards and tilt the plane of the ring away from being parallel to the porphyrin.

The substrate binding orientation in molecule A, with the benzene ring almost parallel to and in van der Waals’ contact with the porphyrin ring might be considered to be very favorable for both 1,3,5-TCB binding and oxidation. The substrate is close to the heme, preventing access of water to the iron atom during the catalytic cycle. The substrate C-4 atom is poised over the iron atom, at a comparable distance to camphor C-5 in the wild type, and so efficient attack by the ferryl intermediate is expected. The predicted product would be 2,4,6-trichlorophenol, as observed experimentally.

1,3,5-TCB Binding in Molecule B—The 1,3,5-TCB binding orientation in molecule B was related to that in molecule A by a tilt of the plane of the benzene about the C-4 atom, increasing the angle between the ring and the porphyrin from 28 to 66°. The Cl-3, C-4, and Cl-5 atoms remained over pyrrole D and the CHB meso carbon, but all three atoms moved closer to the heme, resulting in more substrate/heme contacts. The Cl-1 atom moved away from the pyrrole NH (3.80 Å versus 3.04 Å) and toward the benzene CZ2 and CH2 atoms of the Trp87 indole side chain and into contact with CG of the Leu247 side chain. The ring tilt also brought C-2 away from Leu244 and into contact with Leu247, whereas C-6 moved away from Val295 and came into contact with the Val396 side chain.

The C-4 atom of 1,3,5-TCB was 3.40 Å from the heme iron, with a C-4/Fe/S (Cys357) angle of 163°. The predicted substrate oxidation product for the binding orientation in molecule B would be 2,4,6-trichlorophenol, in agreement with the experiment.

1,3,5-TCB Binding in Molecule C—We had great difficulty in fitting a unique substrate binding orientation to the electron density above the heme in molecule C. Interestingly, all of the orientations that fitted the density reasonably well contained a close distance (≤3.2 Å) between a substrate chlorine atom and the heme iron, suggesting a bonding interaction. However, all of these orientations also contained one or more very short (≤2.5 Å) substrate-heme contacts. See text for further discussion.
ble interpretations of the data are that (a) there is direct bonding interaction between a chlorine lone pair of electrons and the heme iron and (b) there are a number of closely related and perhaps interconverting 1,3,5-TCB binding orientations such that the electron density is smeared out and the fit to individual orientations is poor. Direct interaction between the heme iron and a lone pair on an exogenous organic molecule has been reported in the crystal structure of wild type CYP101 with 5-exo-hydroxycamphor bound, in which the hydroxyl group is directly bonded to the iron center (32).

There are a number of common features in the different potential 1,3,5-TCB binding orientations in molecule C. All of the orientations could be considered to be derived from that in molecule B by further tilting about C-4 and rotation about the Cl-1/C-1/C-4 vector. There are additional movements so that the Cl-3 atom is at different distances from the heme iron and the Cl-3/Fe/S (Cys357) angles also varied. One orientation is shown in Fig. 5. The distance between Cl-3 and the heme iron was 3.2 Å, which was the longest Fe-Cl distance among all of the orientations. There was one particularly short contact (2.6 Å) between Cl-5 and the porphyrin atom ND. The Cl-1 atom moved further away from the indole NH compared with the substrate in molecule B, so that it pointed into the gap between the Trp37 and Leu247 side chains. The angle between the substrate benzene ring and the porphyrin was increased to 115° from 66° in molecule B.

Because the Fe–Cl bond must be broken for dioxygen binding to occur, the modeled substrate binding orientations in molecule C did not allow the direct prediction of the product formed. However, it might be presumed that once the bond is broken the substrate might assume orientation(s) similar to that in molecule B, and 2,4,6-trichlorophenol would be the product.

**Molecule D**—The electron density above the heme in molecule D could not be fitted to 1,3,5-TCB at full occupancy, and the fit was only marginally better at lower occupancy values. We therefore modeled the density with a cluster of three hydrogen-bonded water molecules. However, there are too many uncertainties, even in this model, for any detailed inferences to be drawn from the structure.

**Implications in CYP101 Activity and Further Protein Engineering**—The present crystal structure offered a framework for rationalizing the role of different active site residues in the binding and oxidation of 1,3,5-TCB by CYP101 (Table II). We suggested previously that the Y96F mutation most likely promoted the binding of hydrophobic organic compounds by increasing the active site hydrophobicity (20, 33–37).

We found that introducing the F87W mutation increased both the NADH turnover rate and coupling for 1,3,5-TCB oxidation by more than 10-fold. Adding the V247L mutation increased the NADH turnover rate further, but there was little effect on the coupling. Close examination of the structure of the wild type and the F87W/Y96F/V247L mutant complex suggested a plausible explanation. In the wild type structure there was a “pocket” near the top of the active site. This pocket, 6–6.5 Å across, was defined by the side chains of the residues at positions 87, 96, 98, 185, and 247 and capped on the top by Met184 and Phe193 (Fig. 6). Although too small to accommodate the entire 1,3,5-TCB molecule, it was sufficiently large to bind part of it (e.g. one of the chlorine atoms and one or more ring carbons) so that on average the 1,3,5-TCB substrate could be located further away from the heme. Consequently 1,3,5-TCB oxidation by the wild type would be slow and extensively uncoupled.

In the structure of the F87W/Y96F/V247L mutant complex, the Trp37 indole side chain pointed toward the Leu247 butyl group, and these two side chains approached each other to within 4.3 Å, compared with 6.5 Å between Phe97 and Val247 in the wild type structure. The pocket in the mutant was therefore much smaller and, in a static picture at least, effectively closed to substrate binding. This should constrain the 1,3,5-TCB to bind closer to the heme, thus increasing the NADH turnover rate and coupling (21, 23). The F87W mutation on its own would be less effective in closing down this pocket, hence the slightly lower activity of the F87W/Y96F mutant. The increase in high spin heme content (see Table II), in particular from the Y96F mutant to the F87W/Y96F double mutant, is consistent with this argument. We note that the presence of a direct Fe-Cl interaction in molecule C would explain the incomplete conversion of the heme in the F87W/Y96F/V247L mutant to the high spin form upon 1,3,5-TCB binding at 30 °C, even though the
The data are the means of at least three experiments, with all data for each parameter being within 15% of the mean. % HS (± 5%) is the high spin heme content in the presence of excess substrate. N is the NADH turnover rate, $k_b$ is the product formation rate (2,4,6-TCP for 1,3,5-TCB; PCP for PeCB and HCB), and both rates are given in nmol (nmol CYP101)$^{-1}$ min$^{-1}$. ND, no product observed; —, not determined.

| CYP101 enzyme | 1,3,5-TCB % HS | 1,3,5-TCB $k_b$ | Coupling | PeCB % HS | PeCB $k_b$ | Coupling | HCB % HS | HCB $k_b$ | Coupling |
|---------------|---------------|---------------|----------|-----------|-----------|----------|---------|-----------|----------|
| Wild type     | < 5           | 6.4 ± 0.07    | 11.1%    | < 5       | 3         | ND       | 4       | ND        | ND       |
| Y96F          | 20 ± 108      | 12 ± 11%      |          | < 5       | 10 ± 0.02 | 0.24%    | 3 ± 0.001| 0.04%     |
| F87W/Y96F     | 65 ± 224      | 115 ± 11%     | 51%      | < 5       | 90 ± 1.4  | 1.6%     | 11 ± 0.01| 0.08%     |
| F87W/Y96F/V247A | 70 ± 305     | 117 ± 5.7%    | 57%      | < 5       | 91 ± 1.8  | 1.9%     | 9 ± 0.01 | 0.15%     |
| F87W/Y96F/L244A | — — —        | — — —          | 55 ± 311 | 82.5 ± 24%| 20 ± 1.1 | 4.2%     | 101 ± 2.1| 4.2%      |

* The data for 1,3,5-TCB is from Ref. 21.

structure showed that there were no water molecules in the active site.

The observed substrate binding orientation in molecules A and B correctly predicted the product of 1,3,5-TCB oxidation. As discussed earlier the potential binding orientations in molecule C could also lead to 2,4,6-trichlorophenol as the only product. The effective exclusion of water and the close approach of a C–H bond to the heme iron in molecules A and B were consistent with the high coupling efficiency (57%) for the F87W/Y96F/V247L mutant. It was notable, however, that the coupling was not the >95% observed for camphor oxidation by the wild type, even though there was no water in the active site, and the 1,3,5-TCB substrate was poised over the heme for attack by the ferryl intermediate. The crystal structure revealed a number of possible factors. First, multiple substrate binding orientations were observed, even at 100 K. It is likely that at ambient temperature the 1,3,5-TCB substrate has some mobility. As noted by Raag and Poulos (38), mobility of a substrate in the active site, for example in the form of interconversion between the orientations in molecule B and C by substrate rotation, could interfere with the iron-dioxygen interaction and lead to uncoupling.

The 1,3,5-TCB binding orientation in molecule B may also be significant. The crystal structure, which is a static picture, showed that the Trp87 and Leu247 side chains appeared to act as a set of "double lids" to close off the top of the active site. However, these side chains also had to move out of the way, probably as a result of the normal dynamic structural fluctuations of the protein, to allow the substrate to enter the active site. These fluctuations could also allow 1,3,5-TCB in molecule B to move into the pocket at the top of the active site and away from the heme for some of the time. Consequently, the coupling efficiency would be reduced. In this context we note that all of the potential substrate binding orientations in molecule C had the substrate Cl-1 atom just beneath the Trp87 and Leu247 side chains. Once the Fe–Cl bond is broken, the same uncoupling mechanism could also come into operation. We therefore believe that the orientation in molecule A is probably the most "productive," whereas those in B and C could lead to uncoupling.

The 1,3,5-TCB binding orientation and enzyme-substrate contacts suggested a strategy and other mutations that could enhance the activity of CYP101 for the oxidation of the highly recalcitrant, heavily chlorinated benzenes. Our earlier work showed that the F87W/Y96F/V247L mutant had a fast NADH turnover rate but poor coupling for PeCB oxidation. We therefore sought further mutations that could promote PeCB binding in the orientation observed for 1,3,5-TCB in molecule A.

Viewing the structure from above the active site, the Leu244 side chain fitted neatly between Cl-1 and Cl-3 of 1,3,5-TCB (Fig. 3). There was no space to accommodate the extra chlorine atom in PeCB or HCB. These two substrates could not be bound in the near-parallel orientation and consequently the coupling efficiencies were low. One solution would be to introduce the L244A mutation (39–41) to create space to promote PeCB and HCB binding in this orientation. We therefore prepared the new mutants F87W/Y96F/L244A, F87W/Y96F/V247A, and F87W/Y96F/L244A/V247L to examine the effect of mutations at the 244 and 247 positions on the PeCB and HCB oxidation activity of CYP101.

**Polychlorinated Benzene Oxidation Activity of CYP101 Mutants**—The rates of NADH turnover and product formation and the derived coupling efficiencies for PeCB and HCB oxidation by the CYP101 enzymes are given in Table II. Wild type CYP101 showed slow but detectable NADH turnover activity with these two substrates, but no products were observed, whereas all of the mutants oxidized both PeCB and HCB to PCP.

Considering the PeCB data first, the results showed that the crystal structure-based protein engineering approach was successful. The F87W/Y96F/L244A/V247L mutant oxidized PeCB at a rate of 82.5 min$^{-1}$ and with 24% coupling efficiency. This substrate oxidation activity was 45 times higher than the parent F87W/Y96F/V247L mutant, and the most significant factor was the 10-fold increase in the coupling (Table II). The importance of both the L244A and V247L mutations was clearly demonstrated by comparison between the activities of the F87W/Y96F/V247L, F87W/Y96F/V247A, and F87W/Y96F/L244A/V247L mutants. The V247A mutation was expected to open up the pocket at the top of the active site (Fig. 6). PeCB could bind away from the heme, hence both the activity and coupling of the F87W/Y96F/V247A mutant were very low. The L244A mutation, on the other hand, could create the space necessary for PeCB to adopt the apparently favorable binding orientation observed for 1,3,5-TCB in molecule A. The high spin heme content was increased, and the coupling of the F87W/Y96F/L244A mutant was 10-fold higher than those for the F87W/Y96F and F87W/Y96F/V247L mutants. However, the L244A mutation on its own was not sufficient to induce a very high proportion of PeCB molecules to bind closer to the heme, hence the small effect on the NADH turnover rate. It is interesting that combining the "pull" of the L244A mutation with the "push" of the V247L increased the high spin heme content and enabled the enzyme to have both fast NADH turnover activity and reasonable coupling efficiency for PeCB oxidation.

In general terms the activity trends for HCB oxidation mirrored those for PeCB. The F87W/Y96F/L244A/V247L mutant was again the most active (200 times faster than the F87W/Y96F/V247L mutant), but both the activity and coupling were much lower than for PeCB. The lower solubility and chemical reactivity of HCB compared with PeCB might be significant factors. It should be noted, however, that the PeCB and HCB
oxidation rates cannot be compared directly, because the conversion of HCB to PCP by oxidative dechlorination required four electrons from two molecules of NADH. The likely mechanism of this reaction proceeds via an arene oxide or a ketone intermediate generated by an NIH shift step involving chlorine (Scheme 1). Such a shift has been observed recently in the flavin monooxygenase-mediated oxidation of benzene compounds (42). Reduction by two electrons, loss of chloride, and protonation gives the PCP product.

The extra complication with HCB was that the reduction steps in Scheme 1 might be slow but tightly coupled, whereas the initial oxidative attack could have a faster NADH oxidation rate but low coupling. We have no evidence to determine whether the rate-limiting step was the first electron transfer to initiate the CYP101 catalytic cycle for the oxidation of HCB or the electron transfer from the ferrous heme to the proposed arene oxide/ketone intermediate to expel the chloride ion. Nevertheless, both of these reactions were expected to be faster if the substrate was bound close to the heme, hence the higher activity of the F87W/Y96F/L244A/V247L mutant.

Summary and Conclusions—The crystal structure of the CYP101 active site mutant F87W/Y96F/L244A/V247L with 1,3,5-TCB binding orientations were observed. One such orientation, with the benzene ring being almost parallel to and in van der Waals’ contact with the heme is likely to be the most favorable for faster and more tightly coupled substrate oxidation. By examining the enzyme-substrate contacts, we designed and prepared the F87W/Y96F/L244A/V247L mutant and showed that it had significantly increased activity for the oxidation of the most heavily chlorinated benzenes PeCB and HCB. The observed activities are sufficient for the application of this quadruple mutant in the effective bioremediation of PeCB and HCB. The success of the combined approach of crystal structure determination and further rational redesign suggests that the oxidation of other environmental contaminants by engineered CYP101 and other cytochrome P-450 enzymes and the subsequent degradation of their metabolites, may also be possible.

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