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Pentachlorophenol hydroxylase, a poorly functioning enzyme required for degradation of pentachlorophenol by *Sphingobium chlorophenolicum*

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Abstract

Several strains of *Sphingobium chlorophenolicum* have been isolated from soil that was heavily contaminated with pentachlorophenol (PCP), a toxic pesticide introduced in the 1930s. *S. chlorophenolicum* appears to have assembled a poorly functioning pathway for degradation of PCP by patching enzymes recruited via two independent horizontal gene transfer events into an existing metabolic pathway. Flux through the pathway is limited by PCP hydroxylase. PCP hydroxylase is a dimeric protein that belongs to the family of flavin-dependent phenol hydroxylases. In the presence of NADPH, PCP hydroxylase converts PCP to tetrachlorobenzoquinone (TCBQ). The k_{cat} for PCP (0.024 s^{-1}) is very low, suggesting that the enzyme is not well evolved for turnover of this substrate. Structure/activity studies reveal that substrate binding and activity are enhanced by a low pK_a for the phenolic proton, increased hydrophobicity, and the presence of a substituent ortho to the hydroxyl group of the phenol. PCP hydroxylase exhibits substantial uncoupling; the C4a-hydroxyflavin intermediate, instead of hydroxylating the substrate, can decompose to produce H_2O_2 in a futile cycle that consumes NADPH. The extent of uncoupling varies from 0 – 100% with different substrates. Uncoupling is increased by the presence of bulky substituents in the 3-, 4-, or 5-position, and lessened by the presence of a chlorine in the ortho position. The effectiveness of PCP hydroxylase is additionally hindered by its promiscuous activity with TCHQ, a downstream metabolite in the degradation pathway. The conversion of TCHQ to TCBQ reverses flux through the pathway. Substantial uncoupling also occurs during the reaction with TCHQ.

Pentachlorophenol (PCP) is designated as a priority pollutant by the U.S. Environmental Protection Agency. PCP was introduced as a pesticide in the 1930s and was once one of the most widely used biocides in the U.S., although current use is restricted to industrial wood

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Supporting information **available.** Supplementary materials and methods and supplementary figures 1-3 are available free of charge via the Internet at http://pubs.acs.org.

treatment applications (1). PCP and its metabolites tetrachlorobenzoquinone (TCBQ) and tetrachlorohydroquinone (TCHQ) are highly toxic chemicals that damage DNA by forming adducts (2) or causing strand breaks (3). PCP also uncouples oxidative phosphorylation (4, 5).

Several bacteria that mineralize PCP have been isolated from soil and ground water contaminated with PCP (6-10); the best studied of these is *Sphingobium chlorophenolicum* L-1[1]. Despite its ability to mineralize PCP, *S. chlorophenolicum* degrades PCP slowly and cannot tolerate high concentrations of PCP (11). Comparison of the whole genome sequence *of S. chlorophenolicum* with that of *S. japonicum*, a closely related Sphingomonad that degrades lindane (12), suggests that the PCP degradation pathway was assembled in a patchwork fashion using enzymes acquired via two different horizontal gene transfer events in addition to enzymes inherited from a progenitor of both species (13). The pathway (see Figure 1) begins with hydroxylation of PCP by PCP hydroxylase (PcpB) to form TCBQ (14). TCBQ reductase (PcpD) reduces TCBQ to TCHQ (14), which is converted to 2,6-dichlorohydroquinone (2,6-DCHQ) via two successive dehalogenation reactions catalyzed by TCHQ dehalogenase (PcpC) (15, 16). An Fe(II)-dependent extradiol dioxygenase (PcpA) then cleaves 2,6-DCHQ (17-19). Further steps leading to β-ketoadipate have been proposed (20). Flux through the pathway appears to be limited by the activity of PCP hydroxylase, as PCP accumulates to high levels in cells metabolizing PCP, while the concentration of downstream metabolites is much lower (21).

PCP hydroxylase is a member of a family of flavin-dependent phenol hydroxylases. Two members of this family, phenol hydroxylase and *p*-hydroxybenzoate hydroxylase, have been extensively characterized (22-32). The chemical events in the catalytic cycle are intimately coupled to conformational changes that control access of the substrate to the active site and interactions of the flavin with NADPH and the substrate, which occur at separate locations. The catalytic cycle begins with a conformational change that opens a solvent channel to the active site, allowing substrate to bind. Once substrate is bound, deprotonation of the hydroxyl group triggers movement of the flavin to the “out” conformation, where it can be reduced by NADPH. The reduced flavin reacts with O_2 to form C4a-hydroperoxyflavin, which moves to the “in” conformation and transfers a hydroxyl group to the substrate. The catalytic cycle is completed by tautomerization of the initial hydroxylation product and elimination of H_2O from C4a-hydroxyflavin, which returns the flavin to the oxidized state, and release of products, which requires a conformational change to the “open” form. Based on this body of work, we have proposed a comparable mechanism for PCP hydroxylase (14) (Scheme 1). Scheme 1 shows PCP binding in the phenolate form based on its predominant form in solution (pK_a = 4.5) and our studies of the binding of chlorinated phenols that suggest that deprotonation of the hydroxyl group increases affinity for the active site (see further below).

Here we characterize the poor catalytic abilities of PCP hydroxylase and address the ongoing debate over the identity of the product formed from PCP by PCP hydroxylase. PCP

[1]The *S. chlorophenolicum* L-1 strain originally deposited as ATCC 39723 has lost the ability to degrade PCP. It has been replaced by ATCC 53874.

is converted to TCBQ, as depicted in Scheme 1. The enzyme turns over substrate at a very low rate; k_{cat} is only 0.024 s^{-1}. Furthermore, it is unable to achieve complete coupling of the formation of C4a-hydroperoxyflavin and the hydroxylation of PCP. Consequently, excessive amounts of O_2 and NADPH are consumed during substrate turnover and H_2O_2 is produced due to elimination of H_2O_2 from C4a-hydroperoxyflavin (see Scheme 1). The spectrum of the enzyme-PCP complex indicates that the flavin is largely in the "out" conformation, which may account for the poor coupling efficiency. In addition to its poor ability to catalyze hydroxylation of PCP, the enzyme also does not exclude structurally similar molecules from the active site. Most detrimentally, TCHQ, a downstream metabolite, serves as a substrate for PCP hydroxylase, Hydroxylation of TCHQ regenerates TCBQ, an earlier metabolite in the pathway. The reaction is largely uncoupled in the presence of TCHQ, causing additional non-productive consumption of NADPH and generation of H_2O_2.

MATERIALS AND METHODS

Chemicals

PCP, TCP, 2,4,6-TriCP, 3,4,5-TriCP, 2,6-DCP, 2-CP, TCBQ, and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich. 2,3,4,5-TCP and 2,3,4,6-TCP were purchased from Supelco. TCHQ was purchased from Acros Organics. pQE30 was obtained from Qiagen. All other reagents were purchased from common commercial sources. Chlorinated phenols except for PCP were dissolved in DMSO prior to dilution into assay buffer. PCP was dissolved in 1 – 2 equivalents of 100 mM NaOH and then diluted in 50 mM potassium phosphate, pH 7.0. TCHQ solutions were prepared either in DMSO or in 1 – 2 equivalents of 100 mM NaOH, followed by dilution into 50 mM potassium phosphate, pH 7.0, containing a 1.5-fold molar excess of ascorbate to prevent oxidation to TCBQ. TCBQ was dissolved in acetone.

Purification of PCP Hydroxylase

The gene encoding N-terminal His_6-tagged PCP hydroxylase was cloned into pQE30 and introduced into *E. coli* M15 (pReP4). Cells were streaked onto a plate containing LB medium, kanamycin (25 μg/mL) and ampicillin (100 μg/mL). All subsequent growth media contained 25 μg/mL kanamycin and 100 μg/mL ampicillin. After overnight growth at 37 °C, 5 ml of LB was inoculated with a single colony from the plate. The culture was grown overnight with shaking at 37 °C and transferred into 1L of LB. The cells were grown with shaking at 37 °C until the OD_{600} reached approximately 0.6. The culture was then chilled on ice to 25 °C. Expression of PCP hydroxylase was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The cells were grown with shaking overnight at 25 °C and were harvested by centrifugation for 10 min at 5000 g at 4 °C. Cell pellets were stored at −20 °C.

All protein purification steps were performed at 4 °C. The harvested cells were resuspended in lysis buffer (4 mL/g of cells) consisting of 50 mM HEPES, pH 8.0, containing 300 mM sodium chloride, 10 mM imidazole and 1 mM TCEP supplemented with Sigma protease inhibitor cocktail for use in purification of His-tagged proteins. The cells were lysed by two passages through a French pressure cell at 12,000 psi. PCP hydroxylase was purified on a

Ni-NTA column according to the QIAexpressionist protocol (Qiagen). The pooled PCP hydroxylase fractions were dialyzed against 20 mM HEPES, pH 7.0, containing 100 mM NaCl, 1 mM TCEP, and one molar excess of FAD (with respect to the concentration of PCP hydroxylase). Following concentration to 2 mL using Amicon centrifugal filters (Millipore, Ireland), the protein preparation was injected onto a Superdex 200 column (GE Healthcare). Fractions containing dimeric PCP hydroxylase were pooled and dialyzed as described above. Finally, PCP hydroxylase was dialyzed against 50 mM potassium phosphate, pH 7.0, for 4 h. The final protein sample was concentrated to approximately 20 mg/ml (300 μM based upon monomer concentration), flash frozen in liquid nitrogen, and stored at −70 °C. Analysis by SDS-PAGE indicated that the enzyme was homogeneous. All concentrations for the enzyme are given in terms of monomer concentration.

The concentration of flavin in purified PCP hydroxylase was determined in 8 M urea from the absorbance at 446 nm (ε_{446} = 10,600 $M^{-1}cm^{-1}$ (33)). The protein concentration was determined in 8 M urea by subtracting the contribution of the flavin to the absorbance at 280 nm, which was determined experimentally to be 1.94 × (A_{446}), from the total absorbance at 280 nm. A value of ε_{280} = 68,500 $M^{-1}cm^{-1}$ for the apoprotein was determined from the absorbance of a solution of protein whose concentration was known based upon amino acid analysis.

Dependence of PCP Hydroxylase Activity on Flavin Content

Samples of PCP hydroxylase with varying FAD content were prepared by incubation of the enzyme (300 μM) at room temperature for 1 h with varying concentrations of FAD (from 0 – 300 μM) in 20 mM HEPES, pH 7.0, supplemented with 1 mM TCEP. The protein was then recovered by gel filtration using Illustra NAP-5 columns filled with Sephadex G-25 (GE Healthcare). Further removal of flavin could be accomplished by repeating the gel filtration step.

HPLC Analysis of the Products Formed by PCP Hydroxylase

PCP hydroxylase was incubated with substrate (PCP, TCP, or TCHQ) and NADPH in 300 μL 50 mM potassium phosphate, pH 7.0, at 25 °C. Ascorbate (25 μM) was typically added to prevent non-enzymatic oxidation of TCHQ. Control reactions were carried out in the absence of enzyme and/or NADPH. When measuring formation of TCBQ, β-ME (6 mM) was included in the reaction mixture to trap TCBQ as 2,3,5,6-tetrakis[(2-hydroxyethyl)thio]-1,4-hydroquinone (THTH; ε_{350} = 2175 $M^{-1}cm^{-1}$). The reactions were quenched, typically after 1 min, by addition of 300 μL ethyl acetate containing 50 μM *p*-nitrophenol (internal standard) while vortexing. Following centrifugation at 16,000 × g for 1 min, the ethyl acetate layer (150 μL) was recovered. The solvent was removed by evaporation and the residue re-dissolved in 80% aqueous TFA (0.1%)/20% acetonitrile for analysis of THTH or 40% aqueous TFA (0.1%)/60% acetonitrile for analysis of TCBQ, TCHQ, PCP and TCP. The reaction components were resolved on a Zorbax SB-C18 (4.6 × 150 mm) HPLC column (Agilent Technologies) using a 10-80% gradient of acetonitrile containing 0.1% aqueous TFA over 23 min. The detector was set at 295 nm for detection of TCBQ, PCP and TCP, 301 nm for detection of TCHQ and *p*-nitrophenol, and 350 nm for

detection of THTH. Quadruplicate aliquots were analyzed for each time point and each reaction was repeated at least three times.

Determination of Dissociation Constants by Absorbance Spectroscopy

PCP hydroxylase (7.1 μM) in 50 mM potassium phosphate, pH 7.0, was titrated with various chlorinated phenols (1 μM – 5 mM) dissolved in exactly the same solution of protein. Absorbance spectra (350 – 550 nm) were collected at room temperature using an HP8453 Diode Array Spectrophotometer at more than 20 concentrations of each ligand and the spectrum of PCP hydroxylase alone was subtracted from each. Dissociation constants were calculated using Equation 1 from the absorbance differences at 388 nm for PCP, TCP, and 2,6-DCP and the absorbance differences at 444 nm for 2,4,6-TriCP, 3,4,5-TriCP, and 3,5-DCP.

$$\Delta Abs = \frac{K_D + [E]_{tot} + [S] - \sqrt{(K_D + [E]_{tot} + [S])^2 - 4[E]_{tot}[S]}}{2[E]_{tot}} \quad \text{Equation 1}$$

Spectroscopic Assays of Enzyme Activity

Formation of the hydroxylated product and depletion of NADPH were measured following ethyl acetate extraction of the reaction mixture. PCP hydroxylase (0.15 – 5 μM) was incubated with substrate (1 – 1000 μM) and NADPH (160 μM) in 1 mL 50 mM potassium phosphate, pH 7.0, at room temperature. Reactions with PCP and TCHQ were performed in the presence of 6 mM β-ME to trap the TCBQ product as THTH. Reactions with other phenols carrying a chlorine at the 4-position were performed in the presence of ascorbate (5 mM) to trap the benzoquinone product as a hydroquinone. Aliquots (0.2 mL) were removed at varying time points up to 10 min (corresponding to less than 20% depletion of substrate) and the reactions were quenched by vortexing with ethyl acetate (0.2 mL). Following centrifugation at 16,000 × g for 1 min, the ethyl acetate layer was removed and product concentration was measured by UV-Vis spectroscopy. For each substrate listed, the extinction coefficients for the corresponding product were determined by allowing a known amount of substrate to react to completion in the presence of excess enzyme and NADPH: TCP, ε_{310} = 7700 $M^{-1}cm^{-1}$; 2,4,6-TriCP, ε_{305} = 4502 $M^{-1}cm^{-1}$; 2,6-DCP, ε_{303} = 4384 $M^{-1}cm^{-1}$. (At these wavelengths, the corresponding substrate had no significant absorption.) The extinction coefficient of THTH was determined to be ε_{350} = 2175 $M^{-1}cm^{-1}$ based on the absorbance of product formed by reacting a known amount of TCBQ with β-ME. The extinction coefficient of TCHQ was confirmed to be 7700 $M^{-1}cm^{-1}$ based on the absorbance of a solution prepared by weighing a known amount of substrate. NADPH remaining in the aqueous layer was quantified by UV-Vis spectroscopy (ε_{340} = 6,200 $M^{-1}cm^{-1}$). All reactions were performed more than three times with time points taken in triplicate. For K_m determinations, the assay volume was increased to 5 mL, and 1 mL aliquots were extracted with 0.22 mL ethyl acetate to allow concentration of product in the ethyl acetate layer. The concentration of each substrate or uncoupler was varied over a range equal to or greater than 0.2 – 5× the apparent K_m. For effectors, depletion of NADPH can be monitored at 340 nm because no complicating changes in the spectrum due to transformations of the phenol and the hydroxylated products occur. Determinations of the

K_M for effectors were performed by monitoring depletion of NADPH in a reaction volume of 200 μL in a 96-well Costar plate (#3635). The decrease in absorption at 340 nm was monitored every 5 – 10 s for 5 – 10 min in a Varioskan plate reader (Thermo Electron Corporation). The linear part of each reaction rate was corrected for the rate of NADPH consumption observed in the absence of the effector. The Michaelis-Menten (Equation 2), an equation including substrate inhibition (Equation 3), or an equation describing cooperative substrate kinetics (Equation 4) were used to perform a weighted least-squares fit of the data.

$$v_{obs}=\frac{V_{max}[S]}{K_m+[S]} \quad \text{Equation 2}$$

$$v_{obs}=\frac{V_{max}[S]}{K_m+[S]+\frac{[S]^2}{K_i}} \quad \text{Equation 3}$$

$$v_{obs}=\frac{V_{max}[S]^n}{K_{50}^n+[S]^n} \quad \text{Equation 4}$$

Hydrogen Peroxide Assays

H_2O_2 was quantified using a colorimetric assay based upon oxidation of Fe(II) in complex with xylenol orange (34). The assay mixture contained 250 μM $(NH_4)_2Fe(II)(SO_4)_2$, 25 mM H_2SO_4, 100 μM xylenol orange, and 100 mM sorbitol. The mixture was prepared immediately before use by combining one volume of 25 mM $(NH_4)_2Fe(II)(SO_4)_2$ in 2.5 M H_2SO_4 with 100 volumes of 100 mM sorbitol containing 100 μM xylenol orange. A 50 μL aliquot of sample or H_2O_2 standard was added to 1 mL of assay mixture and incubated for 30 – 45 minutes at room temperature. The increase in absorbance at 560 nm was measured with a Hewlett-Packard 8453 Diode Array Spectrophotometer. A standard curve was determined prior to the measurements. None of the components in standard reaction mixtures (PCP, TCBQ, TCHQ, NADPH, NADP) except for PCP hydroxylase at high concentrations (>3 μM) interfered with the assay. All reactions in the presence of substrate or effector (100 μM) and NADPH (160 μM) were performed in triplicate and the average rates were determined from at least three separate experiments. Depletion of H_2O_2 by reaction with PCP hydroxylase was determined in reactions that contained 0 or 5 μM PCP hydroxylase and 50 μM H_2O_2 in 50 mM potassium phosphate, pH 7.0. Samples were removed at 1 min intervals and analyzed for H_2O_2 as described above.

RESULTS AND DISCUSSION

Purification of the Dimeric Form of PCP Hydroxylase

His_6-tagged PCP hydroxylase was purified to apparent homogeneity (based on SDS-PAGE) on a Ni-NTA column. However, gel filtration analysis showed three prominent species with apparent molecular weights of 129 kDa, 295 kDa, and >500 kDa. When TCEP or DTT was included throughout the purification, the 129 kDa species predominated, suggesting that the

higher molecular weight species arose by covalent cross-linking between surface-exposed cysteine residues. The molecular weight of His_6-tagged PcpB is 60 kDa. Thus, the 129 kDa species is a dimer, as observed for other flavin monooxygenases such as phenol hydroxylase and *p*-hydroxybenzoate hydroxylase (25, 35, 36). The dimeric species is yellow (i.e. loaded with flavin) upon elution from the gel filtration column, while the other species are nearly colorless and have no observable activity.

Determination of the Level of Flavin in Purified PCP Hydroxylase

The amount of flavin in purified samples of PCP hydroxylase was determined after denaturation with 8 M urea to release the flavin into solution. The absorbance of the sample was measured at 446 nm and an extinction coefficient of 10.6 $mM^{-1}cm^{-1}$ was used to calculate the flavin concentration. The sample was then diluted 5-fold and the protein concentration was determined as described in Materials and Methods. The flavin content of the purified enzyme was generally 60-80%. Flavin loading could be increased to 90-100% by incubation of the enzyme with excess flavin at room temperature in the presence of 1 mM TCEP (see Materials and Methods). As shown in Figure 2, activity is proportional to the level of flavin, with a linear increase in activity up to one equivalent of flavin/monomer. All kinetic parameters reported in this paper were obtained with enzyme that had been fully reconstituted with flavin.

Development of a New Assay for PCP Hydroxylase Activity

Measurement of PCP hydroxylase activity is difficult because the enzyme is very inefficient. Furthermore, the product formed from PCP (TCBQ) is unstable, reacting with NADPH and various nucleophiles in the solution. Reduction of TCBQ by NADPH forms TCHQ, which is itself a substrate for PCP hydroxylase and is converted back to TCBQ (see further below). To overcome these difficulties, we developed an assay procedure that rapidly and quantitatively traps TCBQ as 2,3,5,6-tetrakis[(2-hydroxyethyl)thio]-1,4-hydroquinone (THTH, Scheme 2). The structure of THTH was assigned by NMR (Supplementary Figure 1). The adduct is stable to further manipulation and can be separated from other substrates and products by HPLC (Figure 3). Using this procedure with *p*-nitrophenol as an internal standard, we can precisely quantify both substrates and products by HPLC and achieve a mass balance accounting for all of the substrate initially added to the reaction mixture.

Identification of the Products formed by Hydroxylation of PCP, TCP and TCHQ

The initial description of PCP hydroxylase reported that both PCP and TCP were converted to TCHQ in the presence of the enzyme and NADPH (37-39). The reaction with PCP was reported to use two NADPH/PCP, whereas the reaction with TCP used only one NADPH/TCP. However, based upon the mechanism of flavin monooxygenases, (40, 41), we would predict that PCP would be converted to TCBQ, rather than TCHQ (see Scheme 1). In agreement with this proposed mechanism, we previously demonstrated the formation of TCBQ from PCP and identified an enzyme (PcpD) that catalyzes the reduction of TCBQ to TCHQ (14). These results have been called into question by Su et al., who maintain that the TCBQ we detected was an artifact resulting from oxidation of TCHQ and that PCP hydroxylase actually converts PCP to TCHQ (42). Consequently, we have reinvestigated

this issue by comparing the products formed using both PCP and TCP as substrates. As shown in Scheme 2, hydroxylation of PCP should produce TCBQ, and hydroxylation of TCP should produce TCHQ.

Reaction mixtures containing PCP hydroxylase (10 μM), PCP or TCP (100 μM), and NADPH (300 μM) were incubated for one minute to minimize the effects of oxidation of TCHQ and/or non-enzymatic reduction of TCBQ by NADPH. Reaction mixtures included β-ME (6 mM) to trap any TCBQ formed as THTH. We also added 25 μM ascorbate to protect any TCHQ formed from oxidation. Under these conditions, reaction of TCBQ with β-ME is faster than reduction by ascorbate or NADPH, so formation of THTH is fast and essentially quantitative. Importantly, TCHQ is stable under these conditions (Figure 3, Table 1). Control reactions carried out in the absence of enzyme demonstrated that TCBQ was quantitatively converted to THTH and that TCHQ was quantitatively recovered as TCHQ under these conditions (see Table 1).

Incubation of PCP with PCP hydroxylase and NADPH results in formation of THTH; no TCHQ is detected (see Figure 3 and Table 1). If β-ME is omitted from the reaction mixture, some of the PCP that disappears is recovered as TCHQ, presumably due to non-enzymatic reduction of the initial TCBQ product by excess NADPH in the reaction mixture. However, 28% of the expected product is missing. Control experiments show that PCP hydroxylase reacts rapidly with TCBQ, depleting up to a five-fold molar excess of TCBQ within one minute in the absence of NADPH (see Supplementary Figure 2). Thus, we expect that TCBQ partitions between rapid reactions with PCP hydroxylase and NADPH in the absence of β-ME.

Incubation of TCP with PCP hydroxylase and NADPH results in formation of TCHQ as the major product (see Figure 3 and Table 1), demonstrating that TCHQ can be detected if it is formed during the reaction. However, a small amount of THTH is also detected. The THTH might have been formed from TCBQ generated by oxidation of TCHQ during the incubation. However, control experiments demonstrated that TCHQ was entirely stable when the reaction was carried out in the absence of the enzyme. Consequently, we considered the possibility that TCHQ, which resembles PCP, might serve as a substrate for PCP hydroxylase. Hydroxylation of TCHQ would be predicted to form TCBQ (see Scheme 2). Indeed, incubation of TCHQ with the enzyme in the presence of NADPH and β-ME for one minute resulted in 23% conversion to THTH.

These results conclusively demonstrate that PCP is converted to TCBQ by PCP hydroxylase. However, we must address the reasons for the discrepancies between our results and those of Su et al. (42) Su et al. did not report a mass balance for their experiments and did not compare the amount of TCHQ formed to the amount of PCP consumed. We suspect that the TCHQ detected in their experiments was due to a small amount of non-enzymatic reduction of TCBQ to TCHQ by NADPH in the reaction mixture. Neither did they demonstrate that TCBQ could be recovered after control reactions carried out in the absence of either the enzyme or of NADPH. Thus, we suspect that TCBQ was not detected due to reaction with the enzyme and NADPH. Su et al. argue that our observation of TCBQ as the reaction product was due to oxidation of TCHQ upon ethyl acetate extraction. This argument is based

upon inspection of the UV spectra of TCHQ and TCBQ in phosphate buffer and ethyl acetate. Supplementary Figure 3 shows that TCHQ is not affected by extraction into ethyl acetate based upon its chromatographic behavior. The shift in the UV spectrum of TCHQ observed in ethyl acetate is caused by solvatochromism (i.e. the change in the absorption band accompanying the change of the solvent polarity), rather than by oxidation of TCHQ to TCBQ.

Turnover of PCP by PCP Hydroxylase is Extremely Slow

We have assayed PCP hydroxylase activity by following both consumption of PCP and formation of TCBQ (monitored by capturing TCBQ as THTH). All experiments were carried out at ambient O_2 concentrations, and thus the derived kinetic parameters are apparent parameters. The apparent $k_{cat,PCP}$ determined by measuring the initial rate of THTH formation is 0.024 s^{-1} (see Figure 4a). The K_m for PCP is below 1 μM and could not be determined accurately due to lack of sensitivity in the assay procedure (ε_{350} = 2175 $M^{-1}cm^{-1}$ for THTH) and the high concentration of protein needed to detect activity (~1 μM).

The low values of $K_{M,PCP}$ suggest that the enzyme is likely to be saturated during degradation of PCP. Previous studies (21) have shown that cells harvested from medium containing 670 μM PCP contained 4.6 mM PCP, most of which would have been concentrated in the membrane due to the hydrophobicity of PCP. If conversion of PCP to TCBQ is much slower than its release from the membrane, then PCP would equilibrate between the membrane and cytoplasm, and its concentration should be about 120 μM. Thus, the enzyme would have been saturated and flux through the pathway would have been limited by $k_{cat,PCP}$.

Previous studies of the kinetic parameters of PCP hydroxylase have reported widely varying values for PCP reaction. Xun and Orser reported a $k_{cat,PCP}$ of 17 ± 5 s^{-1} and a K_M of 30 ± 7 μM for the native enzyme purified from *S. chlorophenolicum* by measuring the initial rate of formation of TCHQ after two minutes of reaction (37). Given these kinetic parameters, the substrate would have been depleted within two seconds under the reported experimental conditions (PCP hydroxylase, 5 μM, PCP, 20-50 μM and NADPH, 100 μM). Thus, these kinetic parameters may be erroneous.

Wang et al. measured a $k_{cat,PCP}$ of 0.033 s^{-1} and a $K_{M,PCP}$ of 50 μM by following consumption of PCP using initial PCP concentrations between 20 and 80 μM (43). This is not a sufficient range of substrate concentrations to allow an accurate determination of K_M. Furthermore, the GST-tagged enzyme was isolated as an insoluble apoenzyme and reconstituted with flavin after solubilization with detergent, purification by glutathione affinity chromatography, and cleavage of the fusion protein. Given the uncertainties as to the proper refolding of the protein and the flavin content achieved by this procedure, we cannot be sure that these parameters accurately reflect those for the native enzyme.

Nakamura et al. measured kinetic parameters for PCP hydroxylase using a GC/MS assay for TCHQ and PCP concentrations ranging between 0.4 and 20 μM (44). They reported a $K_{M,PCP}$ of 0.5 μM and a k_{cat} of 0.009 s^{-1}. The relatively small 3-fold difference in $k_{cat,PCP}$

between this work and ours could be due to the fact that activity was assessed by following TCHQ formation; as demonstrated above, TCHQ is actually a substrate for the enzyme. Since some of the TCBQ formed by hydroxylation of TCHQ would have reacted with the enzyme, the rate of appearance of TCHQ would have been lower than the actual rate of depletion of PCP.

The $k_{cat,PCP}$ we have measured for PCP hydroxylase is much lower than those reported for most other flavin monooxygenases. The k_{cat} for phenol hydroxylase with phenol as substrate is 9 s^{-1} (22). The k_{cat} for *p*-hydroxybenzoate hydroxylase with *p*-hydroxybenzoate as substrate is 57 s^{-1} (45). However, many phenol hydroxylases are promiscuous, and hydroxylate substrate analogues with lower efficiency. For example, *p*-hydroxybenzoate hydroxylase has a k_{cat} = 1.4 s^{-1} for turnover of 2,3,5,6-tetrafluoro-*p*-hydroxybenzoate (22). The slow rate of substrate turnover by PCP hydroxylase suggests that PCP may not be the optimal substrate for the enzyme. This is not unexpected, as PCP has only been present in the environment since the 1930s, and thus PCP hydroxylase likely evolved to hydroxylate some other substrate. In addition, the reaction might be slow due to the presence of five electron-withdrawing substituents on the aromatic ring. The presence of five fluorine substituents slows the k_{cat} for phenol hydroxylase by 10-fold from 7.8 s^{-1} for phenol to 0.7 s^{-1} for pentafluorophenol (46). Chlorine substituents are more electron-withdrawing than fluorine substituents (for Cl, σ_{meta} = 0.37 and σ_{para} = 0.23; for F, σ_{meta} = 0.34 and σ_{para} = 0.06), so chlorine substituents would be expected to further diminish the reactivity of a phenolic substrate.

Formation of the C4a-hydroperoxyflavin Intermediate and Hydroxylation of PCP are Uncoupled

The rates of formation of TCBQ and disappearance of NADPH during turnover of PCP should be equal. However, in the presence of 100 μM PCP and 160 μM NADPH, the rate of disappearance of NADPH significantly exceeds the rate of formation of TCBQ (see Table 3). These data suggest that formation of the C4a-hydroperoxyflavin is not tightly coupled to hydroxylation of PCP (see Scheme 1). Such uncoupling is common in flavin monooxygenases that hydroxylate phenolic compounds, particularly when non-physiological substrates are used. Uncoupling has been observed with phenol hydroxylase when *p*-fluorophenol, *p*-chlorophenol, resorcinol, *p*-aminophenol, *o*β-MEthylphenol and even phenol itself are used as substrates (22). In extreme cases, compounds that cannot be hydroxylated but nevertheless bind to the active site act as “effectors”, facilitating reduction of the flavin by NADPH, reaction with O_2 and stoichiometric production of H_2O_2. For example, 5-hydroxypicolinate, 6-hydroxynicotinate, and 3,4-dihydroxybenzoate serve as effectors for *p*-hydroxybenzoate hydroxylase (26).

To confirm the uncoupling of the PCP hydroxylase reaction, we measured the rate of H_2O_2 formation. In the presence of PCP, the rate of H_2O_2 formation accounts for the difference between the rates of NADPH consumption and formation of TCBQ (see Table 3). Notably, one in five catalytic cycles fails to hydroxylate PCP and generates H_2O_2 instead. Even in the absence of PCP, PCP hydroxylase slowly consumes NADPH and forms a small amount of H_2O_2 (see Table 3). The discrepancy between the rates of NADPH consumption (0.0034

s^{-1}) and H_2O_2 formation (0.0014 s^{-1}) in the absence of substrate or effector can be attributed to depletion of H_2O_2 by reaction with components of the reaction such as PCP hydroxylase. (When PCP hydroxylase (5 μM) was incubated with 50 μM H_2O_2, more than 40% of the H_2O_2 disappeared within 5 minutes (data not shown).)

Uncoupling at the active site of PCP hydroxylase has not been previously recognized, although it is consistent with previously reported findings. Xun et al. (37) reported that 1.4 equivalents of O_2 and 2 equivalents of NADPH were consumed for each PCP consumed. These data were interpreted as evidence for the conversion of PCP to TCHQ, which was predicted to require two equivalents of NADPH. Since we know now that PCP hydroxylase converts PCP to TCBQ, consuming 1 NADPH, and that there is significant uncoupling during the reaction, these data can now be explained in a different way. The excess consumption of O_2 was due to the uncoupled reaction, and the excess consumption of NADPH was due to both the uncoupled reaction and the non-enzymatic reaction of NADPH with TCBQ.

Spectroscopic Analysis Suggests that the Flavin is Preferentially in the "Out" Conformation in the Presence of PCP

Extensive structural and spectroscopic characterization of phenol hydroxylase and *p*-hydroxybenzoate hydroxylase have shown that the flavin can occupy three different conformations in the active site (30). When the flavin is in the "open" conformation, substrate can access the active site. In the "out" conformation, the flavin can interact with NADPH. In the "in" conformation, the flavin is positioned near the substrate and, when the flavin-C4a-hydroperoxide is formed, is optimally aligned to transfer a hydroxyl group to the substrate. The differences in solvent exposure in these three positions result in changes in the absorbance spectrum of the flavin. Typically, movement of the flavin to the "out" position is correlated with an increase in absorbance at 444 nm.

PCP hydroxylase, like other flavin monooxygenases, undergoes ligand-dependent conformational changes as detected by changes in the spectrum of the enzyme-bound flavin (see Figure 5). The spectral changes are similar to those observed previously for phenol hydroxylase (36) and *p*-hydroxybenzoate hydroxylase (27), particularly for substrate analogs for which the reaction is partially or fully uncoupled. The tendency for the flavin to prefer the "out" conformation may be responsible for the uncoupling that occurs during turnover in the presence of PCP. After reduction of the flavin and reaction with O_2, the C4a-hydroperoxyflavin must adopt the "in" conformation in order to hydroxylate the substrate. If the C4a-hydroperoxyflavin lingers in the "out" position in the presence of PCP, elimination of H_2O_2 will occur more often.

The changes in the flavin spectrum can be used to measure the dissociation constants for substrates and substrate analogs. PCP binds extremely tightly, with a dissociation constant of 36 nM (see Figure 6). Such tight binding of substrate is unusual for metabolic enzymes in general and for this class of enzymes in particular. For *p*-hydroxybenzoate hydroxylase, the K_D for *p*-hydroxybenzoate is 43 μM (27). Phenol hydroxylase shows cooperative substrate binding, with K_D values ranging between 1 and 19 μM (36).

Insights into Substrate Binding from Structure/Activity Relationships

We examined the binding specificity of PCP hydroxylase by titrating the enzyme with various chlorinated phenols. Binding of all of the tested compounds results in increased absorption at 444 nm, consistent with a shift of the flavin to the “out” position. (See Figure 5 for examples of difference spectra). However, the difference spectra vary depending upon the ligand. In particular, a change in absorbance at 388 nm is only observed for some ligands. The different spectroscopic signatures do not correlate with any obvious property of the ligand such as the number or pattern of substituents, pK_a, logP, or solvent accessible surface area. The observed differences may reflect not only differences in the equilibrium between the three positions of the flavin, but also smaller differences in the positioning of the flavin that influence hydrogen bonding and/or hydrophobic interactions between the flavin and the protein.

The affinities of the various chlorinated phenols for PCP hydroxylase were determined by titration of the ligands in the presence of a constant concentration of enzyme (see Figures 6 and 7). Binding affinity appears to depend on three factors: 1) pK_a; 2) hydrophobicity; and 3) the presence of a substituent ortho to the hydroxyl group. PCP, which has the lowest pK_a and the highest logP, was by far the tightest binding ligand. Dissociation constants for the tetra-chloro compounds TCP and TCHQ were in the low micromolar range; these compounds have pK_as similar to that of PCP, but they are less hydrophobic. Trichloro- and dichlorophenols, which have higher pK_as and are less hydrophobic, bind even less tightly. Notably, tri- and dichlorophenols with an ortho chlorine substituent bound more tightly than isomers with an ortho hydrogen. These results suggest that the substrate binding pocket of PCP hydroxylase prefers a hydrophobic compound with a deprotonated hydroxyl group and a bulky substituent at the ortho position. Further, since PCP is the tightest binding substrate, we suspect that the pocket is very hydrophobic and that substrates that are smaller than PCP cannot interact optimally with surrounding protein.

The preference for binding the phenolate form of the substrate is interesting from a mechanistic standpoint. A proton must be removed from the hydroxyl group in order for the aromatic ring to attack the C4a-hydroperoxyflavin intermediate (see Scheme 1). In phenol hydroxylase and *p*-hydroxybenzoate hydroxylase, the substrates have relatively high pK_as, and the proton is removed by an internal proton transport network (47). In contrast, PCP is nearly completely ionized at neutral pH; thus, there is no need for deprotonation to occur at the active site.

Insights into Substrate Reactivity from Structure/Activity Relationships

We measured steady state kinetic parameters for turnover of PCP, TCP, 2,6-DCP and TCHQ (see Table 2 and Figure 4), as well as consumption of NADPH in the presence of two effectors (3,5-DCP and 3,4,5-TriCP, see further below). Surprisingly, the plots for various substrates are different; the plot for 2,6-DCP is hyperbolic, the plot for TCP is sigmoidal, and the plot for TCHQ shows substrate inhibition above a concentration of 40 μM. The physiological concentration of TCHQ is on the order of 5 μM (21), so the substrate inhibition observed at high conentrations is likely not physiologically relevant. However, the substrate inhibition does complicate the determination of the catalytic constants. The line

shown in Figure 4d applies when the K_M is set to be equal to the K_i, and is shown for illustrative purposes only. Reliable values for K_M, K_i and k_{cat} cannot be determined from these data. We can conclude only that the value of k_{cat} must be higher than the highest observed rate constant (0.015 s^{-1}) and the K_M must be greater than 7 μM.

The results of these kinetic studies suggest that TCP is more reactive than PCP, in agreement with previous studies (38). The presence of a halogen substituent at C-4 should decrease the electron density at the 4-position, which would correspondingly decrease the HOMO-LUMO interaction required for transfer of the hydroxyl group. The increased steric bulk of a chlorine substituent at C-4 should further decrease the HOMO-LUMO interaction by impeding close approach of the terminal oxygen of the peroxyl group to the substrate. These results also suggest that the reason for the poor activity of PCP hydroxylase with PCP cannot be attributed to the electron-withdrawing effects of the chlorine substituents, as the k_{cat} for TCP is higher than that for 2,6-DCP. Indeed, the chlorine substituents may actually increase the reactivity of the ring by decreasing the pK_a of the hydroxyl group and obviating the need for proton removal.

We examined the extent of uncoupling in the presence of a larger set of chlorinated phenols using a fixed concentration of substrate (100 μM) and NADPH (160 μM) (see Table 3). (Note that only some of the substrates are at saturating concentrations, so these rate constants are not necessarily equivalent to k_{cat}.) More complete kinetic analyses to obtain values for k_{cat} and K_M were determined for depletion of NADPH in the presence of the effectors 3,4,5-TriCP and 3,5-DCP (see Table 2). Values for k_{cat} are the same for both effectors and may reflect the intrinsic rate of elimination of H_2O_2 when the C4a-hydroperoxyflavin is unable to approach the phenolic substrate.

Two features of the substrate strongly affect the extent of uncoupling: 1) a chlorine atom at the ortho position decreases the extent of uncoupling; and 2) steric bulk at positions 3, 4 and 5 increases the extent of uncoupling. These factors can be understood based upon the orientation of the substrate and flavin that must be achieved for hydroxyl transfer. Figure 8 shows the orientation of the flavin, with the peroxy group modeled in, with respect to the substrate in *p*-hydroxybenzoate hydroxylase. (Note that the substrate is hydroxylated at the position *ortho* to the hydroxyl group by *p*-hydroxybenzoate hydroxylase, rather than at the *para* position, as occurs at the active site of PCP hydroxylase.) The substrate is fixed firmly in the active site by interactions between the carboxylate and hydroxyl substituents and the protein. The peroxy substituent on the flavin approaches the substrate from above the ring. The data presented above for PCP hydroxylase demonstrate that a chlorine substituent at the ortho position increases binding affinity, suggesting that there is a pocket that accommodates the chlorine at this position. A two-point attachment of a planar substrate in the active site mediated by interactions with the negatively charged oxygen and the ortho chlorine substituent would suffice to fix the orientation of the planar substrate in the active site. In the absence of a chlorine substituent at the ortho position, the substrate might twist in the active site, impeding movement of the C4a-hydroperoxyflavin over the face of the ring and thus leading to uncoupling. Similarly, bulky substituents around the 4-position, to which the hydroxyl group is transferred, increase the extent of uncoupling, presumably by impeding the approach of the C4a-hydroperoxyflavin. The predicted effects of substituents

are consistent throughout the set of chlorinated phenols. 2,6-DCP shows no uncoupling; it has a chlorine at the ortho position to enforce orientation and no bulky substituents to hinder hydroxylation. At the other extreme, 3,5-DCP, 3,4-DCP and 3,4,5-TriCP are all effectors, causing complete uncoupling. These compounds have no chlorine at the ortho position to fix the orientation of the ring and have steric bulk around the 4-position. Interestingly, the turnover of TCHQ is more highly uncoupled than that of PCP itself, suggesting that the presence of a hydroxyl group is more problematic than the presence of a chlorine at the 4-position.

CONCLUSIONS

The findings reported here suggest that PCP hydroxylase has not yet evolved to be an optimal catalyst for hydroxylation of PCP. The enzyme is rather non-specific, which is consistent with its poor catalytic efficiency, as precise orientation of substrates in proximity to catalytic groups is critical for catalytic efficiency. The enzyme is unable to couple formation of C4a-hydroperoxyflavin and hydroxylation of the substrate efficiently, leading to excess consumption of NADPH and production of H_2O_2. Finally, the enzyme is unable to discriminate between PCP and TCHQ, a downstream metabolite. When TCHQ is bound, the uncoupled reaction causes substantial production of H_2O_2 at the active site. Furthermore, hydroxylation of TCHQ forms TCBQ. This reaction generates a futile cycle in which NADPH is consumed unproductively. Since the K_M for PCP is lower than that for TCHQ, and PCP is present at a higher concentration *in vivo* (21), the active site of PCP hydroxylase will be primarily engaged in converting PCP to TCBQ. The uncoupling that occurs with PCP, and to a lesser extent with TCHQ, will deplete NADPH and generate substantial quantities of H_2O_2, causing oxidative stress. TCBQ generated by PCP hydroxylase may damage cytoplasmic proteins if it is not efficiently captured and converted to TCHQ by TCBQ reductase. The multiple shortcomings of PCP hydroxylase and the intrinsic toxicity of TCBQ may explain the inability of *S. chlorophenolicum* to degrade high levels of PCP (11).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

β-ME	beta-mercaptoethanol
IPTG	isopropyl-β-D-thiogalactopyranoside
PCP	pentachlorophenol
TCBQ	tetrachlorobenzoquinone

TCHQ	tetrachlorohydroquinone
TCP	2,3,5,6-tetrachlorophenol
THTH	2,3,5,6-tetrakis[(2-hydroxyethyl)thio]-1,4-hydroquinone
2,4,6-TriCP	2,4,6-trichlorophenol
3,4,5-TriCP	3,4,5-trichlorophenol
2,6-DCP	2,6-dichlorophenol
3,5-DCP	3,5-dichlorophenol
TCEP	tris(2-carboxyethyl)phosphine

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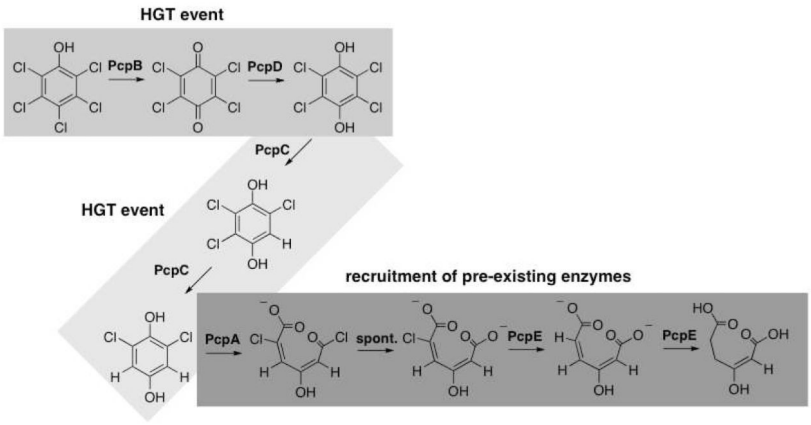


Figure 1.

Pathway for degradation of PCP in *Sphingobium chlorophenolicum*. PcpB, PCP hydroxylase; PcpD, TCBQ reductase; PcpC, TCHQ dehalogenase; PcpA, 2,6-dichlorohydroquinone dioxygenase; GSH, glutathione; HGT, horizontal gene transfer.

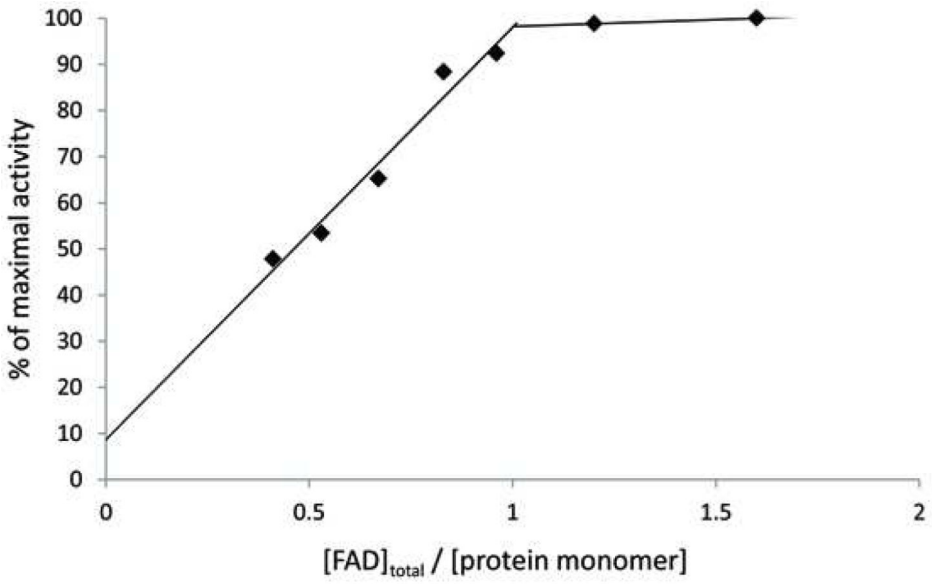


Figure 2.

Dependence of PCP hydroxylase activity on FAD. Comparison of activities of PCP hydroxylase samples differentially loaded with FAD. Reaction mixtures containing 5 μM PCP hydroxylase, 100 μM PCP, 6 mM β-ME, and 300 μM NADPH were incubated for 3 min at room temperature and THTH was quantified by HPLC.

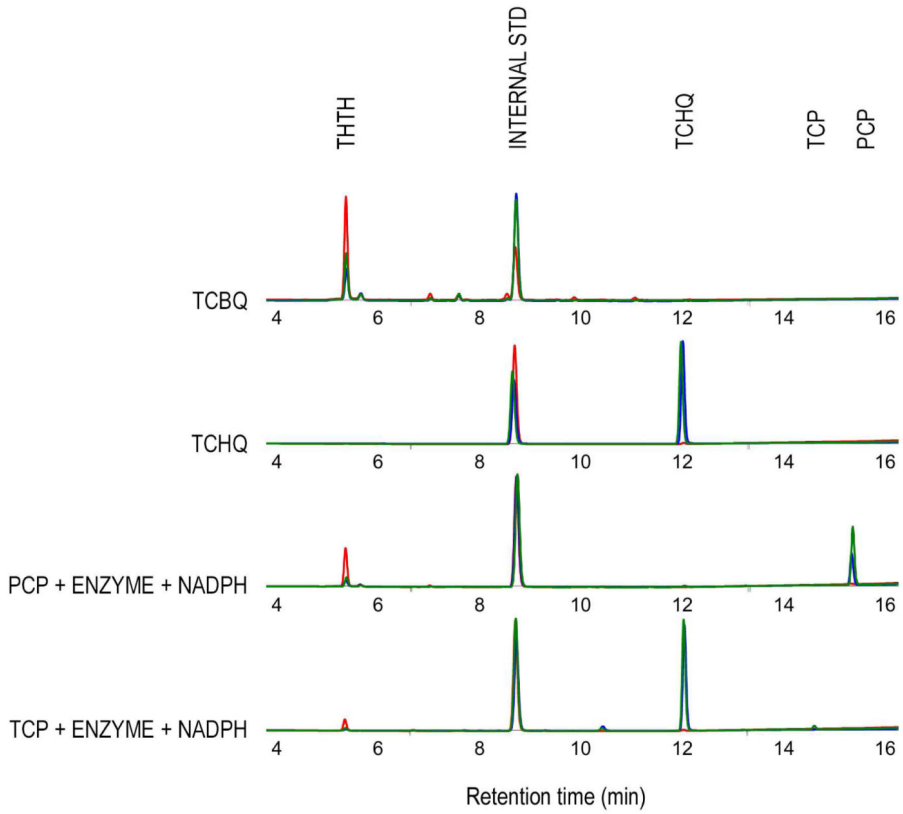


Figure 3.

Representative HPLC traces showing the products formed by PCP hydroxylase from PCP, TCP, and TCHQ. Each substrate (100 μM) was incubated in 50 mM potassium phosphate, pH 7.5, containing 6 mM β-ME and 25 μM ascorbate, for 1 min at room temperature. PCP hydroxylase (10 μM) and NADPH (300 μM) were added as indicated. Since the various compounds absorb optimally at different wavelengths, signals detected at different wavelengths are overlaid in the chromatograms. Green, 295 nm; blue, 310 nm; red, 350 nm. *p*-Nitrophenol was used as an internal standard.

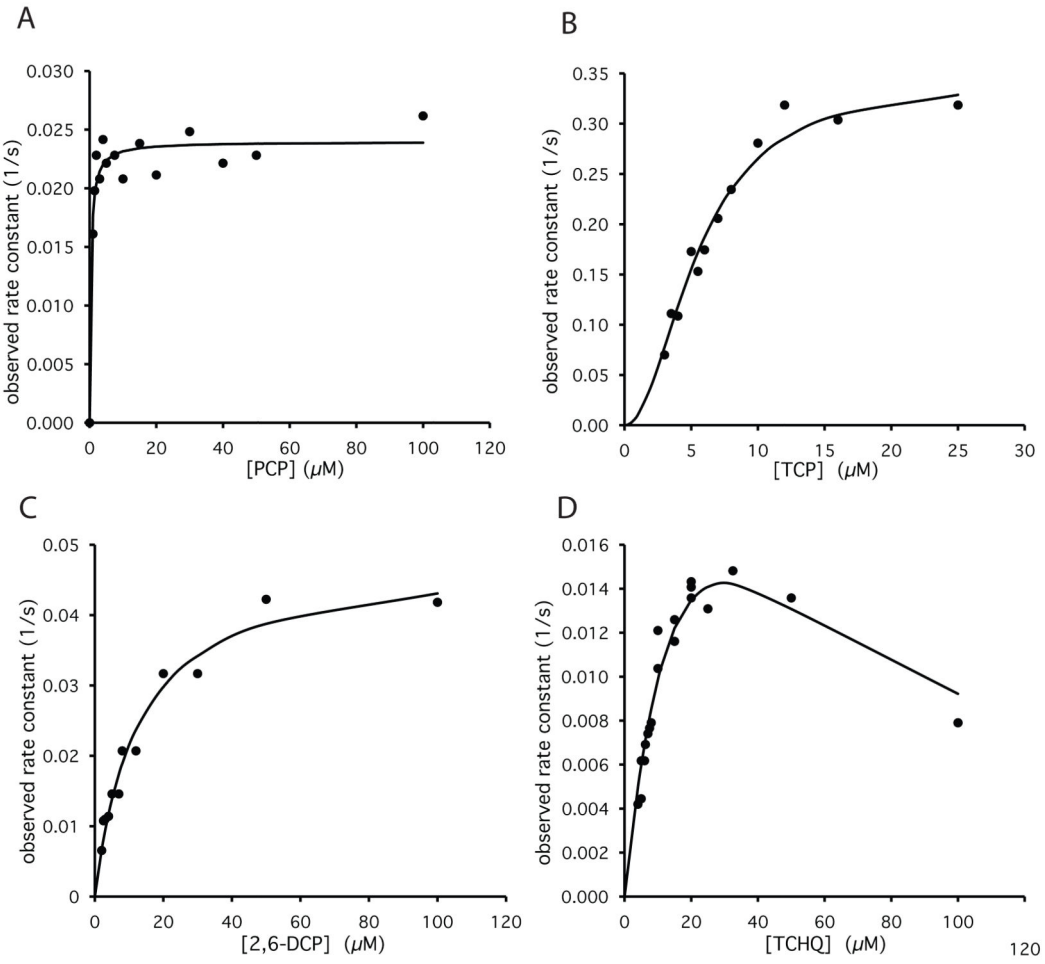


Figure 4.

Relationship between reaction velocity and substrate concentration for A) PCP; B) TCP; C) 2,6-DCP; and D) TCHQ. Reactions were carried out in 50 mM potassium phosphate, pH 7.0, containing 160 μM NADPH, 6 mM β-ME, 25 μM ascorbate, and PCP hydroxylase.

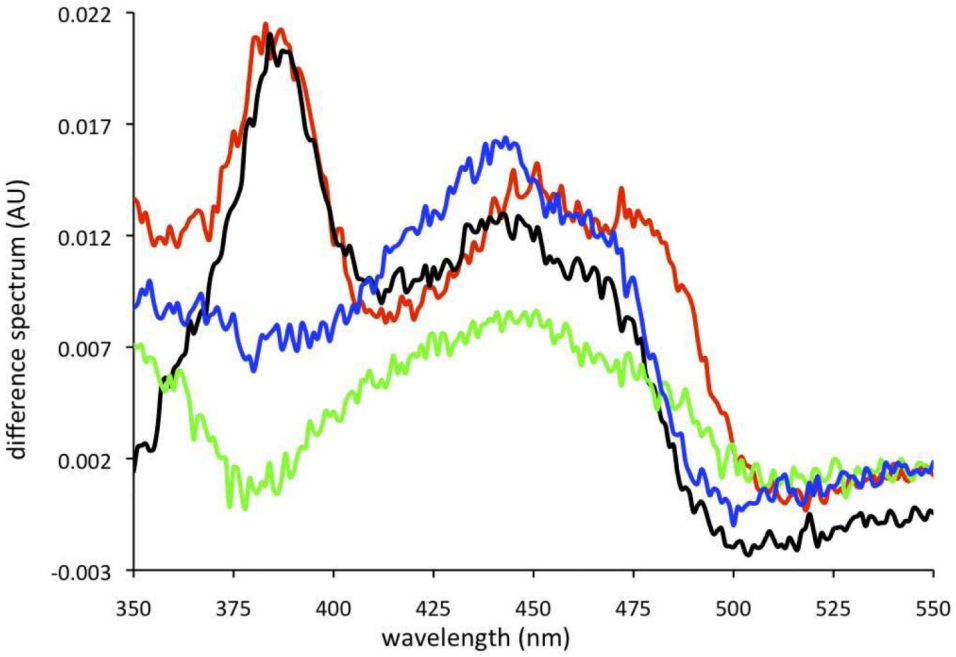


Figure 5.

Overlay of representative difference spectra for the flavin of PCP hydroxylase (7.1 μM) in the presence vs. absence of various ligands: PCP (red, 15 μM), TCP (black, 18 μM), 2,4,6-TriCP (blue, 80 μM) and 3,5-DCP (green, 2500 μM).

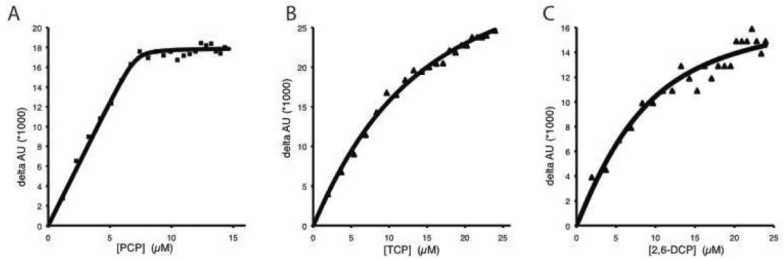


Figure 6.

Perturbation of the flavin spectrum at 388 nm by binding of PCP (A), TCP (B) and 2,6-DCP (C).

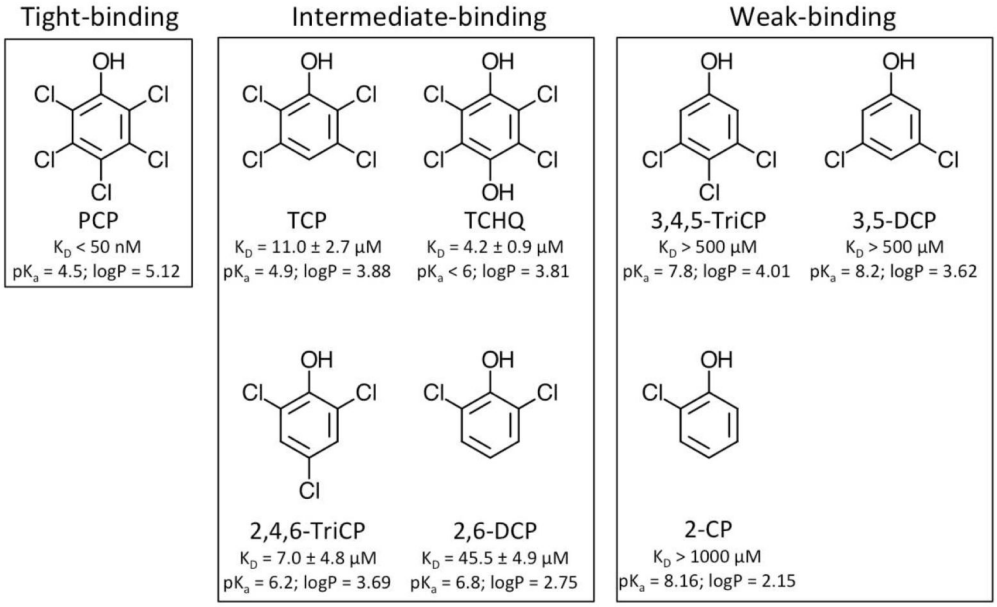


Figure 7.

Summary of binding constants, pK_as and logP values for various chlorinated phenols.

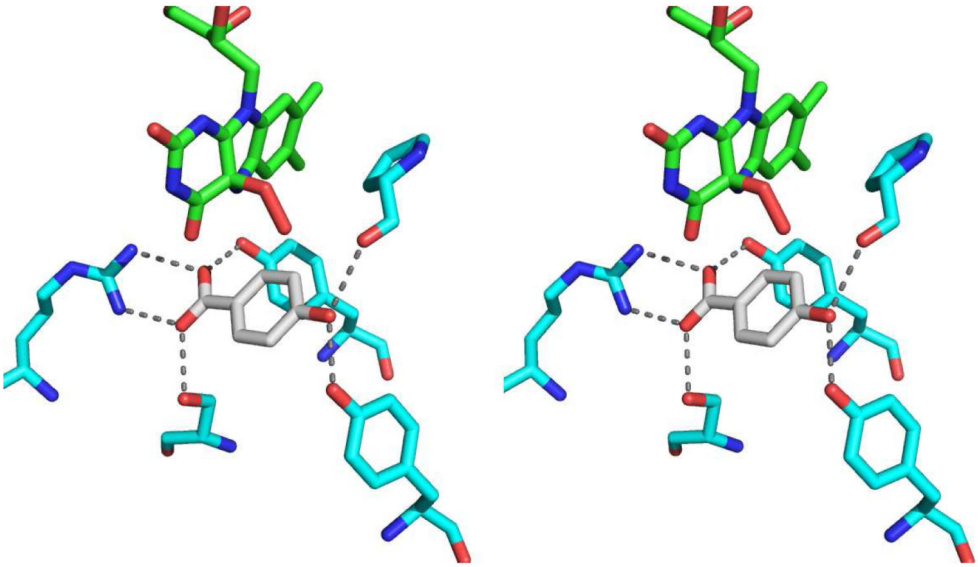
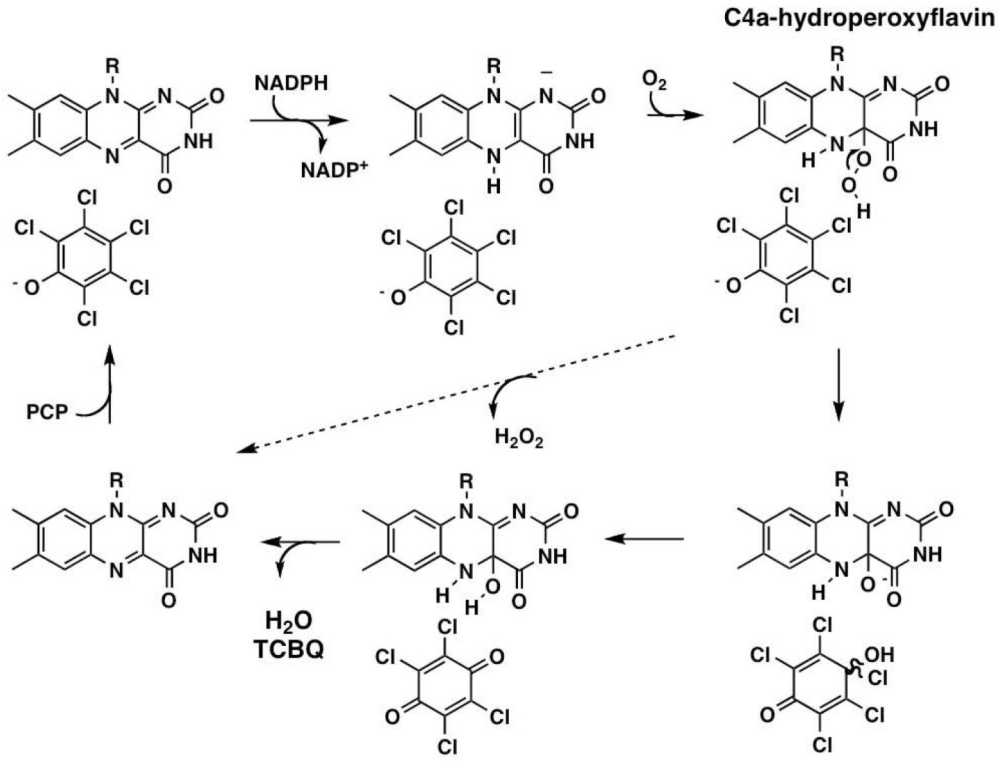
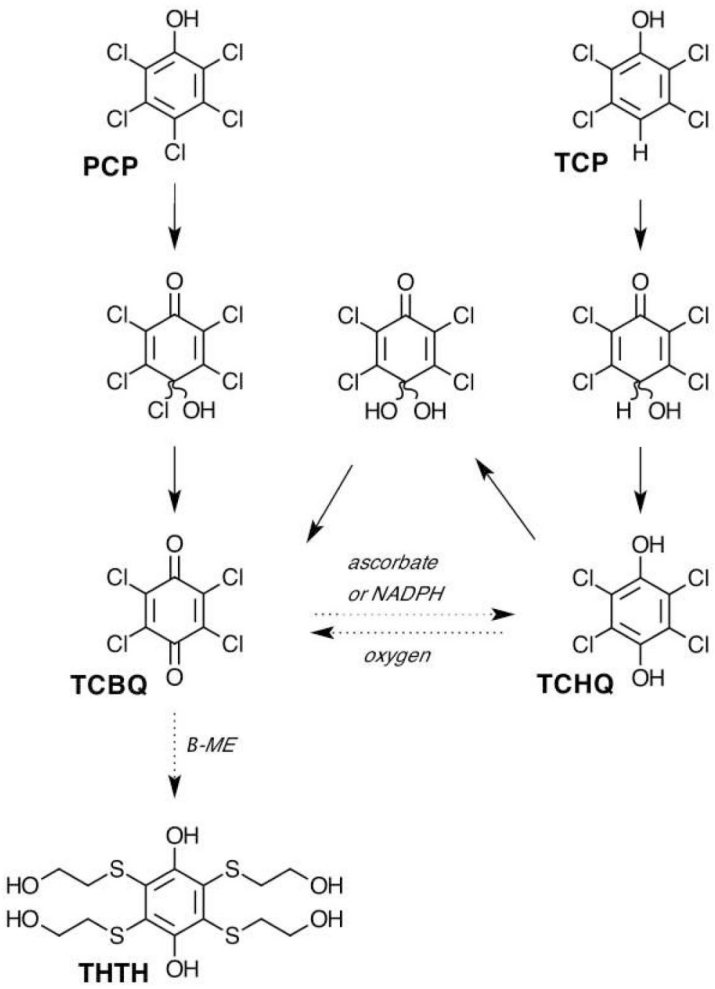


Figure 8.

Stereo view of the active site of *p*-hydroxybenzoate hydroxylase showing the substrate (*p*-hydroxybenzoate) in grey and a C4a-hydroperoxyflavin modeled in place of the FAD in the "in" position (PDB 1PBE).



Scheme 1.



Scheme 2.

Table 1

Analysis of the products formed by PCP hydroxylase from PCP, TCP and TCHQ.[a]

Reactant	Conc (μM)	PCP hydroxylase (μM)	NADPH (μM)	(β-ME (μM)		Reaction components after 1 min incubation at room temperature			
						THTH	TCHQ	PCP	TCP
Enzymatic reactions									
PCP	100	10	300	6		28 ± 2		70 ± 2	
	100	10	300				18 ± 1	75 ± 1	
TCP	100	10	300	6		9 ± 1	75 ± 8		10 ± 1
	100	10	300				78 ± 11		10 ± 1
TCHQ	100	10	300	6		23 ± 2	77 ± 2		
Control reactions									
TCBQ	20			6		21 ± 4			
	20	10		6		17 ± 3			
	20	10	300	6		21 ± 3			
TCHQ	20			6			20 ± 1		
	20		300	6			21 ± 1		

[a] All reactions were performed in the presence of 25 μM ascorbate.

Table 2

Steady-state kinetic parameters for PCP hydroxylase in the presence of 160 μM NADPH

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
PCP	< 1[a]	0.024 ± 0.002[a,b]	n.a.
TCP	5.6 ± 3.6[c] (nH=2)	0.32 ± 0.03[b]	(5.7 ± 1.2) × 10^4
2,6-DCP	12.6 ± 3.1	0.046 ± 0.006[b]	3809 ± 970
TCHQ	n.d.	> 0.015[a,b]	n.a.
3,4,5-triCP	59 ± 12	0.010 ± 0.001[d]	169 ± 37
3,5-DCP	98 ± 13	0.010 ± 0.0012[d]	102 ± 14

n.a. = not applicable; n.d. = not determined; nH – Hill coefficient

[a] In the presence of 6 mM β-ME

[b] k_{cat} for hydroxvlation reaction

[c] This is actually a K_{50} (see Equation 4).

[d] k_{cat} for disappearance of NADPH during completely uncoupled reaction

Table 3

Rates of the uncoupled reaction in the presence of substrates and effectors of PCP hydroxylase.[a]

Substrate	Product appearance (s^{-1})	NADPH disappearance (s^{-1})	NADPH /product	H_2O_2 appearance (s^{-1})
PCP	0.020 ± 0.003[b]	0.025 ± 0.001[b]	1.3[b]	0.0070 ± 0.0009
TCP	0.076 ± 0.008	0.111 ± 0.018	1.5	0.0132 ± 0.008
TCHQ	0.0048 ± 0.0004[c]	0.030 ± 0.003[c]	6.3[c]	0.035 ± 0.005
2,4,6-triCP	0.0033 ± 0.0002[b]	0.0048 ± 0.003[b]	1.5[b]	0.0012 ± 0.0002
3,4,5-triCP	none	0.012 ± 0.001	n.a.	n.d.
2,6-DCP	0.060 ± 0.002	0.051 ± 0.004	0.9	0.0011 ± 0.0002
3,5-DCP	none	0.0079 ± 0.0016	n.a.	0.0029 ± 0.0006
3,4-DCP	none	0.0045 ± 0.0010	n.a.	n.d.
none	none	0.0034 ± 0.0008	n.a.	0.0014 ± 0.0002

n.a. = not applicable; n.d. = not determined

[a] All reactions were performed in the presence of 100 μM substrate and 160 μM NADPH.

[b] In the presence of 5 mM ascorbate.

[c] In the presence of 6 mM β-ME