

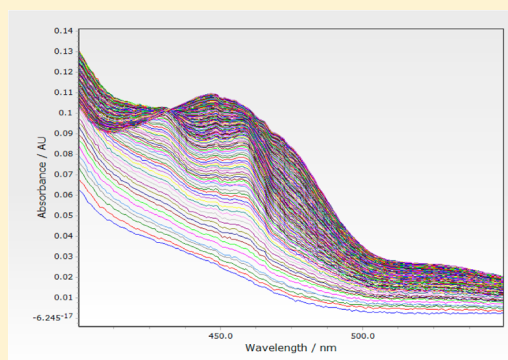
A Radical Intermediate in the Conversion of Pentachlorophenol to Tetrachlorohydroquinone by *Sphingobium chlorophenolicum*

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S Supporting Information

ABSTRACT: Pentachlorophenol (PCP) hydroxylase, the first enzyme in the pathway for degradation of PCP in *Sphingobium chlorophenolicum*, is an unusually slow flavin-dependent monooxygenase ($k_{\text{cat}} = 0.02 \text{ s}^{-1}$) that converts PCP to a highly reactive product, tetrachlorobenzoquinone (TCBQ). Using stopped-flow spectroscopy, we have shown that the steps up to and including formation of TCBQ are rapid ($5\text{--}30 \text{ s}^{-1}$). Before products can be released from the active site, the strongly oxidizing TCBQ abstracts an electron from a donor at the active site, possibly a cysteine residue, resulting in an off-pathway diradical state that only slowly reverts to an intermediate capable of completing the catalytic cycle. TCBQ reductase, the second enzyme in the PCP degradation pathway, rescues this nonproductive complex via two fast sequential one-electron transfers. These studies demonstrate how adoption of an ancestral catalytic strategy for conversion of a substrate with different steric and electronic properties can lead to subtle yet (literally) radical changes in enzymatic reaction mechanisms.



The industrial revolution has led to a significant expansion of the chemical repertoire on earth. The chemical industry has synthesized numerous compounds at commercial scale for use as novel materials, pharmaceuticals, textile dyes, pesticides, solvents, and explosives. These compounds can persist for years in the environment, often causing toxicity to many forms of life. Environmental microbes have evolved new metabolic pathways for degradation of some of these anthropogenic chemicals by patching together promiscuous activities of enzymes that normally serve other functions.¹

Promiscuous activities are often inefficient because new substrates with different steric and electronic properties do not bind optimally relative to catalytic groups in the active site, and/or because additional chemical steps not required for the original activity are needed. Even so, enzymes with promiscuous activities often accelerate such reactions by many orders of magnitude relative to the rates of uncatalyzed reactions.^{2–4} When a promiscuous activity becomes important for fitness, amplification of the gene encoding the promiscuous enzyme both provides more activity and sets the stage for divergence of the gene, ultimately leading to the emergence of an efficient new enzyme.

PCP hydroxylase exemplifies this situation. Most flavin-dependent phenol monooxygenases hydroxylate naturally occurring phenols at a carbon bearing a hydrogen substituent and generate hydroquinone or catechol intermediates. However, hydroxylation of PCP, obligatorily at a carbon bearing a chlorine substituent, generates a benzoquinone rather than a hydroquinone.⁵ The mechanism expected for PCP hydroxylase based upon the well-studied mechanism of *p*-hydroxybenzoate

hydroxylase^{6,7} is shown in Figure 1. The reductive half-reaction (blue) begins with substrate binding, which causes the flavin to move to the “out” position where it can be reduced by NADPH. The reduced flavin then moves back into the active site. During the oxidative half-reaction (yellow), the reduced flavin reacts with O₂ and then hydroxylates the substrate. Completion of the catalytic cycle requires expulsion of HCl from the unstable nonaromatic intermediate to form tetrachlorobenzoquinone (TCBQ), dehydration of the C_{4a}-hydroxyflavin, and release of products.

Here we describe studies that reveal a deviation in the canonical mechanism for this family of flavin-dependent monooxygenases, as well as the reason for the unusually inefficient activity of PCP hydroxylase (see Figure 2). The catalytic events that occur during turnover of PCP to TCBQ depart from those expected based upon the mechanisms of related flavin-dependent monooxygenases due to the unusual electronic characteristics endowed by the chlorine substituents on the aromatic ring. The presence of a chloride leaving group at the site of hydroxylation and the electron-withdrawing effect of the remaining four chlorine substituents result in formation of an unusually reactive product, TCBQ, which is both a potent electrophile⁸ and a strong oxidant.⁹ Presteady-state kinetic studies in conjunction with electron paramagnetic spectroscopy (EPR) show that TCBQ rapidly abstracts an electron from a

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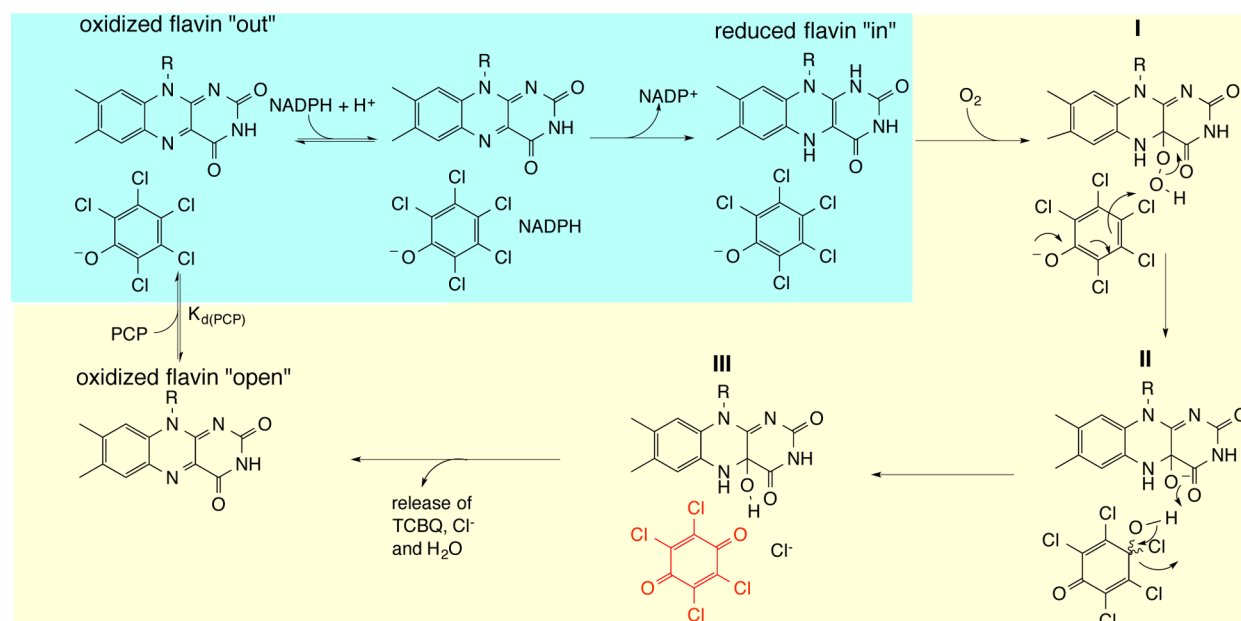


Figure 1. Expected mechanism of PCP hydroxylase. Blue box, reductive half-reaction; yellow box, oxidative half-reaction.

donor in the active site, generating an unreactive complex (IV) containing tetrachlorosemiquinone (TCSQ) and an enzyme-based radical that only slowly completes the catalytic cycle. We also describe how this dead-end complex is rescued by TCBQ reductase, the second enzyme in the PCP degradation pathway. TCBQ reductase is evolutionarily related to reductases that transfer electrons derived from NADH to Fe_2S_2 clusters in dioxygenases.¹⁰ Its role in PCP degradation is unusual because it transfers electrons to an organic acceptor (or acceptors) at the active site of PCP hydroxylase (pink box in Figure 2), rather than to an Fe_2S_2 cluster. Thus, the mechanisms of both PCP hydroxylase and TCBQ reductase are distinctly different from the typical mechanisms of their respective enzyme families.

EXPERIMENTAL PROCEDURES

Materials. PCP hydroxylase and TCBQ reductase were prepared and quantified as previously described.^{5,8} Concentrations for the enzymes are reported as flavin equivalents. PCP, TCBQ, protocatechuic acid and 3,4-dihydroxybenzoic acid were purchased from Sigma-Aldrich. Tetrachlorohydroquinone (TCHQ) was purchased from Acros Organic and dissolved in dimethyl sulfoxide at 50 mM prior to dilution into buffer. TCBQ was dissolved in N,N' -dimethylformamide at 50 mM prior to dilution into buffer.

Presteady State Kinetics. All enzymatic reactions were followed by stopped-flow spectrometry performed in 50 mM potassium phosphate, pH 7.0, at 22 °C by mixing equal volumes (30 μL) from two preloaded syringes. The stopped-flow instrument (Hi-Tech Scientific SF-61DX2) was used in diode-array mode to monitor wavelengths between 320 and 700 nm. Anoxic conditions were achieved by pre-equilibrating reagents in a glovebox flushed with nitrogen. Trace amounts of O_2 were removed by treating solutions with protocatechuic acid (0.05 U/ μL) and 3,4-dihydroxybenzoic acid (400 μM) as described by Patil and Ballou.¹¹ Air-tight syringes were used to transfer reagents from the glovebox to the stopped-flow instrument.

The reductive half-reaction of PCP hydroxylase was examined by mixing PCP hydroxylase (20 μM) that had been

preincubated with PCP (50 μM) with NAD(P)H (0.2–4 mM) under anoxic conditions in the stopped-flow instrument. The oxidative half-reaction was examined by preincubating PCP hydroxylase (20–50 μM) with PCP (0.9 equiv/PCP hydroxylase) and excess NADPH (100–400 μM) under anoxic conditions and subsequently mixing the anoxic reduced PCP hydroxylase with equal volumes of buffer containing O_2 (0–360 μM) in the stopped-flow instrument. For reactions in the presence of TCBQ reductase, TCBQ reductase (4–50 μM) and NADH (200–600 μM) were added to the anoxic mixture containing PCP hydroxylase, NADPH, and PCP.

The reduction of TCBQ reductase was studied by mixing the protein with saturating concentrations of NADH (100–200 μM) under anoxic conditions in the stopped-flow instrument. The reoxidation of TCBQ reductase was studied by mixing an anoxic preparation of the protein (27 μM) that had been incubated with NADH (200 μM) (to fully reduce the flavin and Fe_2S_2 cluster) with buffer containing atmospheric O_2 in the stopped-flow instrument.

Analysis of data collected by stopped-flow spectroscopy was performed using the software tools of Kinetic Studio (TgK Scientific) and ReactLab Kinetics Software (Jplus Consulting). Time-dependent absorbance changes at individual wavelengths were fit to single or double exponentials in Kinetic Studio. Wavelengths for this analysis were chosen based on favorable properties such as large differences in rate constants for two sequential kinetic steps or appropriate isosbestic points between intermediates, as described previously.¹² Goodness-of-fit was evaluated by observation of residuals and statistical values ($R^2 > 0.995$; residual standard error < 0.0005). Global fitting of diode-array data was performed in ReactLab using the rate constants derived from data collected at individual wavelengths as starting estimates. Goodness-of-fit was evaluated using observation of residuals and statistical values (standard deviation < 0.001; residual sum squares < 0.02). Good correlation between fitting of individual rate constants and global fitting was found. For example, the rate constant for formation of the C_{4a} -hydroperoxyflavin (I) obtained by global fitting ($2.6 \pm 0.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) was essentially identical to

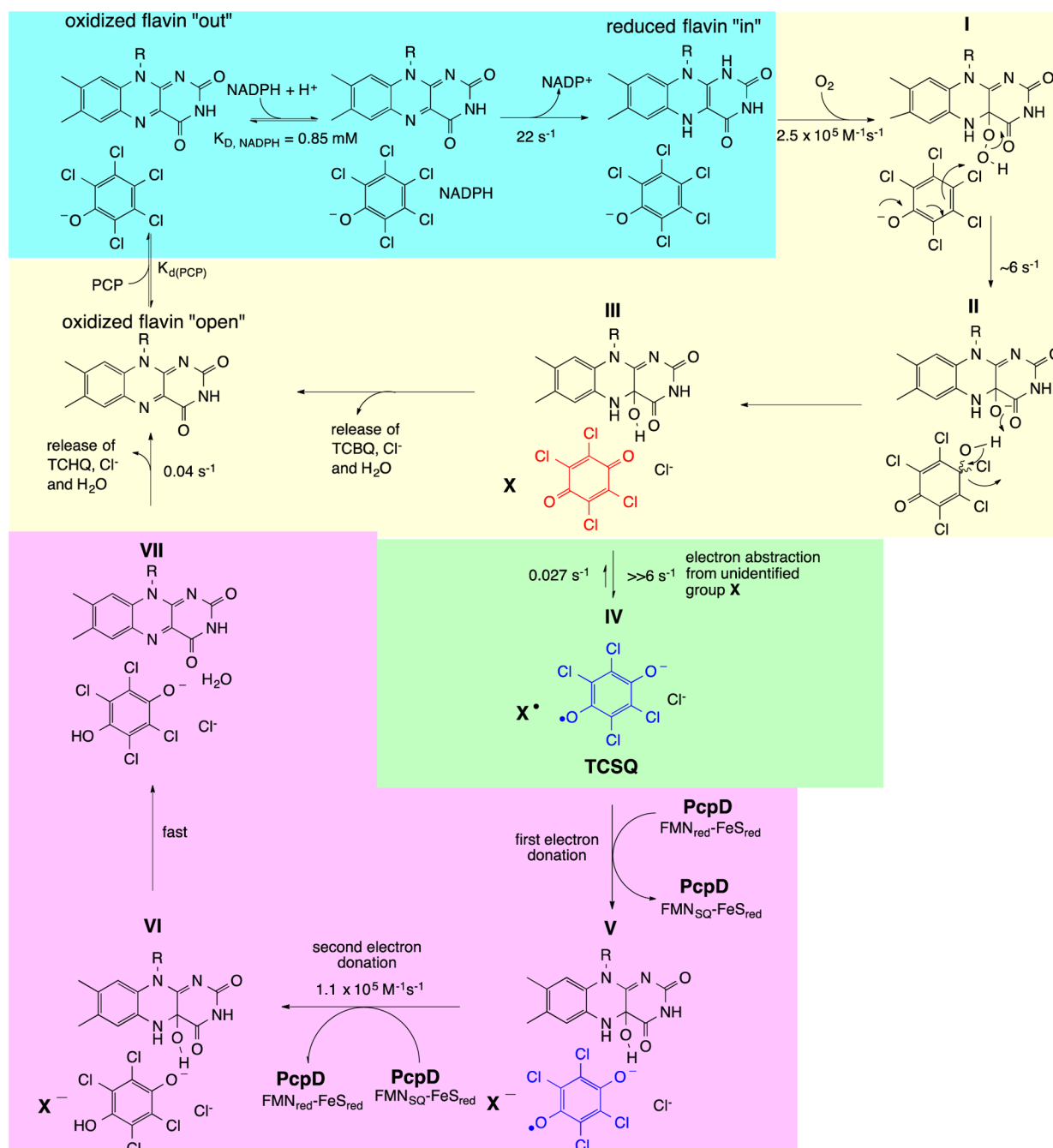


Figure 2. Mechanism of PCP hydroxylase alone and in combination with TCBQ reductase. TCBQ is shown in red and TCSQ is shown in blue. Blue box, reductive half-reaction; yellow box, oxidative half-reaction; green box, off-pathway detour; pink box, proposed role of TCBQ reductase in rescuing the off-pathway intermediate.

that derived by fitting the data at 402 nm to a single exponential $(2.5 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Baseline-corrected spectra of intermediates and products in the PCP hydroxylase-catalyzed reaction in the presence of TCBQ reductase were computed as follows. The initial spectrum of reduced PCP hydroxylase in the absence of TCBQ reductase was subtracted from the corresponding spectrum in the presence of a particular concentration of TCBQ reductase to provide a spectrum of TCBQ reductase at the beginning of the experiment. This spectrum was then subtracted from the spectra of intermediates at that TCBQ reductase concentration to provide difference spectra that reflect the absorption properties of PCP hydroxylase. (This procedure is justified by the experiments

described in the text that show that the spectrum of TCBQ reductase does not change over the range of wavelengths needed to analyze the PCP hydroxylation reaction.) For reactions containing both PCP hydroxylase and TCBQ reductase, kinetic events after formation of oxidized PCP hydroxylase (>10 s) were not analyzed further as they displayed complex multiphasic spectral changes that may be due to depletion of NAD(P)H, potential substrate cycling, and reoxidation of the flavin of TCBQ reductase.

Electron Paramagnetic Resonance (EPR) Spectroscopy. Samples for EPR were prepared as follows. TCSQ was generated by mixing equimolar concentrations (50 μM) of TCBQ and TCHQ in 50 mM potassium phosphate, pH 7.0.

TCSQ is formed rapidly by disproportionation. EPR spectra of TCSQ were then acquired either at 140 K (frozen) or 298 K (in solution). (Measurements in solution were possible because TCSQ is reasonably stable in the presence of O₂; $t_{1/2}$ for oxidation of TCSQ is 22 min at room temperature in potassium phosphate, pH 7.) Samples for analysis of potential radicals generated during turnover of PCP hydroxylase were generated by prereducing the enzyme (170 μ M) with NADPH (300 μ M) in the presence of limiting PCP (150 μ M) under anoxic conditions, followed by mixing with an equal volume of buffer containing ambient O₂. Samples were quenched by immersion in liquid nitrogen at intervals between 20 and 180 s and spectra were acquired at 140 K.

EPR spectroscopy was performed using a Bruker Elexsys 500 spectrometer equipped with a Bruker Super High QE (SHQE) cavity resonator and a liquid nitrogen temperature control system. Spectra were recorded at 140 or 293 K at 9.45 GHz. Typical parameters for low temperature spectra were as follows: microwave power of 0.0796 mW, modulation amplitude of 2 G, modulation frequency of 100 kHz, conversion time and time constant both set to 20.48 ms, and a sweep width of 100 G. Typical parameters for spectra recorded at 293 K were as follows: microwave power of 2 mW, modulation amplitude of 1 G, modulation frequency of 100 kHz, conversion time and time constant both set to 20.48 ms, and a sweep width of 50G. Quantitation of the EPR signal was performed by double integration. Five different concentrations of 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) in water were used as standards. The concentration of the TEMPOL stock solution was determined using $\epsilon_{492} = 13.4 \text{ M}^{-1} \text{ cm}^{-1}$ for TEMPOL in water.¹³ Magnetic field calibration was performed using 1,3-bisdiphenylene-2 phenylallyl (BDPA) as a standard (peak-to-peak line width, 0.45–0.8 G; g -value, $2.00254 \pm 3.2 \times 10^{-5}$; Bruker, Manual for Xenon software).

RESULTS

Presteady State Kinetic Studies Reveal That the Reductive Half-Reaction of PCP Hydroxylase Is Efficient.

Presteady state kinetic experiments were carried out to measure the rates of the elementary steps during turnover of PCP by PCP hydroxylase and to identify the step that limits turnover to an unusually slow rate of 0.02 s^{-1} . Reduction of the active site flavin by NADPH was monitored by stopped-flow spectroscopy under anoxic conditions. The observed rate constant at each concentration of NADPH can be derived from a fit of the kinetic data to a single exponential function [$A_{460} = A_{0,460} + A_{f,460} (\exp(-k_{\text{red}}t))$] (Figure 3). Assuming that flavin reduction is irreversible, values for k_{red} over a range of NADPH concentrations can be used to derive the $K_{\text{D,NADPH}}$ ($850 \pm 82 \text{ }\mu\text{M}$) and the maximal rate of hydride transfer from NADPH to the flavin ($22 \pm 1.1 \text{ s}^{-1}$) (inset, Figure 3). In the presence of physiological concentrations of NADPH ($\sim 100 \text{ }\mu\text{M}$), the rate of flavin reduction is 2 s^{-1} , substantially faster than k_{cat} (0.02 s^{-1}). For comparison, *p*-hydroxybenzoate hydroxylase catalyzes reduction of the flavin in the presence of 2,4-dihydroxybenzoate with a k_{red} of 2.5 s^{-1} and a $K_{\text{D,NADPH}}$ of $\sim 4 \text{ mM}$.¹⁴ Phenol hydroxylase catalyzes reduction of the flavin in the presence of resorcinol with a k_{red} of 16 s^{-1} and a $K_{\text{D,NADPH}}$ of $67 \text{ }\mu\text{M}$.¹⁵ PCP hydroxylase prefers NADPH over NADH; when NADH was used as the cosubstrate, saturation was not observed and $k_{\text{red}}/K_{\text{D}}$ was ~ 10 -fold lower than that for NADPH (Table 1, Supporting Information Figure 1).

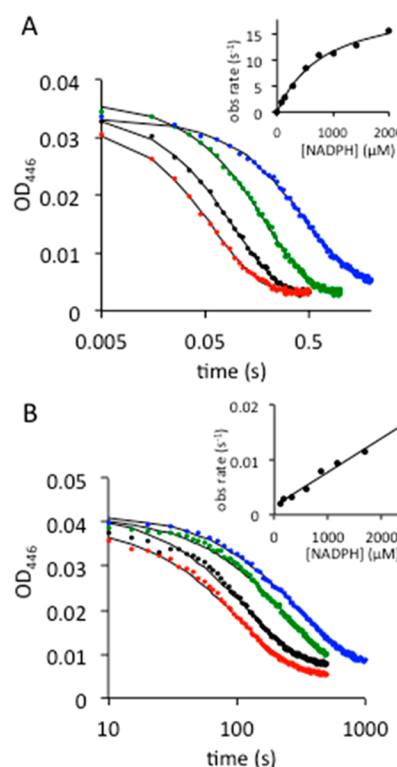


Figure 3. Kinetics of the reductive half-reaction of PCP hydroxylase. Oxidized PCP hydroxylase (20 μ M) in (A) the presence or (B) the absence of 50 μ M PCP was mixed with varying concentrations of NADPH (0.2–8 mM) under anoxic conditions in a stopped-flow instrument. Representative data sets collected at 446 nm are shown. Symbols represent observed data points, and lines indicate the fit to a single exponential. NADPH concentrations after mixing are (A) blue, 0.17 mM; green, 0.57 mM; black, 1.48 mM; and red, 4.0 mM; (B) blue, 0.16 mM; green, 0.54 mM; black, 1.4 mM; red, 3.8 mM. Replots derived from more extensive data sets, including replicates, are shown in the inset.

Table 1. Kinetic Parameters for the Reduction of PCP Hydroxylase by NAD(P)H under Anoxic Conditions^a

substrates	K_{D} for NAD(P)H (μ M)	k_{red} (s^{-1})	$k_{\text{red}}/K_{\text{D}}$ ($\text{M}^{-1} \text{ s}^{-1}$)
PCP/NADPH	850 ± 82	22 ± 1.1	$(2.6 \pm 0.2) \times 10^4$
PCP/NADH	n.a.	n.a.	$(2.4 \pm 0.3) \times 10^3$
none/NADPH	n.a.	n.a.	6.0 ± 0.6

^an.a.: not applicable.

Reduction of the flavin in flavin-dependent monooxygenases is typically very slow in the absence of substrate.⁷ This characteristic prevents formation of the C_{4a}-hydroperoxyflavin (I) when there is no substrate in the active site; elimination of H₂O₂ from the C_{4a}-hydroperoxyflavin generates a reactive oxygen species and wastes a molecule of NADPH.¹⁶ Reduction of the flavin in PCP hydroxylase is also slow in the absence of PCP, and the enzyme is not saturated by NADPH concentrations up to 2.5 mM; $k_{\text{red}}/K_{\text{D}}$ is ~ 4000 times lower in the absence than in the presence of PCP (Table 1, Figure 3B). We conclude that PCP effectively promotes movement of the flavin to the “out” position, which allows reduction by NADPH, and that this process occurs at a rate comparable to those of other enzymes and substantially higher than k_{cat} .

An Unexpected Off-Pathway Radical Intermediate Is Formed during the Oxidative Half-Reaction of PCP

Hydroxylase. The oxidative half-reaction of PCP hydroxylase was examined by prereducing PCP hydroxylase in the presence of a substoichiometric concentration of PCP under anoxic conditions and mixing this solution with oxygenated buffer in a stopped-flow instrument. We expected that all of the steps included in the yellow box in Figure 1 would occur, although some might not be detected. In general, an intermediate is detectable if it accumulates due to a large difference between the rate constant for its formation and the rate constant for its conversion to the next intermediate in the catalytic cycle.

Three kinetic processes were observed during the oxidative half-reaction in the presence of 90 μM O_2 (Figure 4A). The

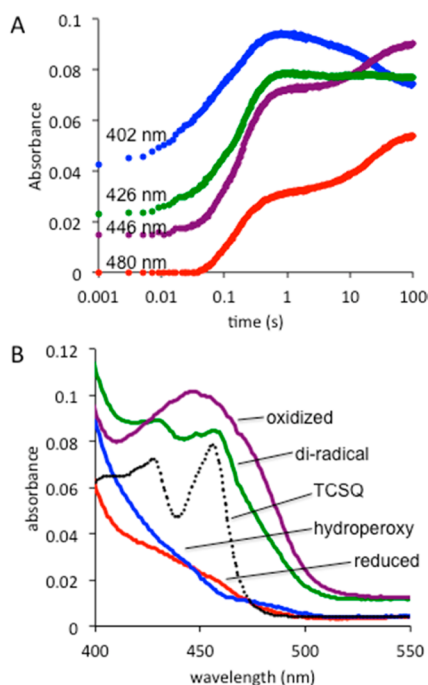


Figure 4. Spectroscopic changes and intermediates observed during the oxidative half-reaction of PCP hydroxylase. Reduced PCP hydroxylase (28 μM , anoxic) in the presence of PCP (22 μM) was mixed with an equal volume of oxygenated buffer and the reaction was monitored by diode array spectroscopy in a stopped-flow apparatus. (A) Time course of changes at 402 nm (blue), 426 nm (green), 446 nm (black), and 480 nm (red). (B) Calculated spectra for intermediates detected during the oxidative half-reaction. Reduced flavin (red), C_{4a} -hydroperoxyflavin (blue), diradical intermediate (green), and oxidized flavin (purple). Also shown is the spectrum of TCSQ (dotted black).

observed rate constant for the first process can be obtained by fitting the change in absorbance at 402 nm over the first second to the following equation: $k_{\text{obs}} = A_{0,402} + A_{f,402}(1 - \exp(-k_{\text{obs}}t))$. This process can be assigned to formation of the C_{4a} -hydroperoxyflavin (I) because k_{obs} is linearly dependent on the concentration of O_2 (Figure 5). The second-order rate constant of $(2.5 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (at 22 $^\circ\text{C}$) is comparable to those for formation of the C_{4a} -hydroperoxyflavin intermediate by phenol hydroxylase¹⁵ and *p*-hydroxybenzoate hydroxylase^{12,17,18} ($1\text{--}3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at 4 $^\circ\text{C}$). Like those enzymes, PCP hydroxylase is not saturated at concentrations of O_2 up to half the atmospheric concentration.^{15,19}

Further analysis of the oxidative half-reaction of PCP hydroxylase by global fitting of the kinetic data enabled measurement of the rate constants for two additional steps as

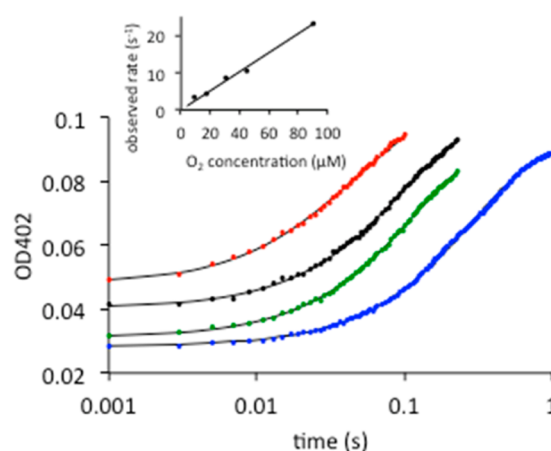


Figure 5. Dependence of the change in absorbance at 402 nm upon the concentration of O_2 . An anoxic solution of reduced PCP hydroxylase (40 μM) and PCP (36 μM) was mixed with buffer equilibrated with varying concentrations of O_2 in a stopped-flow apparatus. Representative data sets acquired after mixing with various concentrations of O_2 [18 μM (blue), 60 μM (green), 90 μM (black), and 180 μM (red)] O_2 are shown. Symbols represent observed data points. Lines are derived from the best fit of the data to a single exponential. The observed rate constants from these and additional data sets, including replicates, are plotted against the final concentration of O_2 in the reaction mixture in the inset.

well as calculation of the spectra of the C_{4a} -hydroperoxyflavin and two additional intermediates. The first of these steps occurs with a rate constant of $5.5 \pm 0.6 \text{ s}^{-1}$ and leads to formation of an intermediate with a spectrum that has a double peak in the flavin region (Figure 4B; λ_{max} at 429 and 457 nm). This spectrum does not resemble that of any previously described flavin intermediate seen during turnover by phenol hydroxylase²⁰ or *p*-hydroxybenzoate hydroxylase.^{12,21–24}

The first hint to the identity of this intermediate came from the observation that its calculated spectrum shows peaks at 429 and 457 nm (Figure 4B), wavelengths at which the spectrum of tetrachlorosemiquinone (TCSQ) also has distinctive peaks. EPR analysis at 140 K of the PCP hydroxylase reaction mixture after manual mixing of the reaction components and freezing at various times after mixing revealed a radical signal that is essentially identical to that of a sample of TCSQ at the same temperature (Figure 6). This radical decayed during the hydroxylation reaction with an apparent rate constant of 0.022 s^{-1} (Figure 6, inset). The EPR spectrum of TCSQ at 140 K has not previously been reported. However, the radical spectrum of our TCSQ standard at 298 K had $g = 2.00535$ and $\Delta H = 0.845 \text{ G}$ (Supporting Information Figure 2), consistent with a previous literature report of $g = 2.00535$, $\Delta H = 0.8 \text{ G}$.²⁵

Taken together, the presteady state kinetic data and the EPR spectrum suggest that TCSQ is formed during the catalytic cycle of PCP hydroxylase. We postulate that formation of TCSQ is an off-pathway detour resulting from transfer of an electron from a donor at the active site to the strongly oxidizing TCBQ (Figure 2). [$E(Q/Q^{\bullet-})$ for TCBQ is +650 mV.⁹] Comparison of the amplitude of the EPR signal obtained by freezing the reaction 45 s after addition of O_2 with the amplitude of the signals from known concentrations of TEMPO indicated the presence of 0.10 spin equivalents per flavin of PCP hydroxylase.

The spectrum of intermediate IV (green line in Figure 4B) is noticeably broader than that of TCSQ alone. The absorbance at

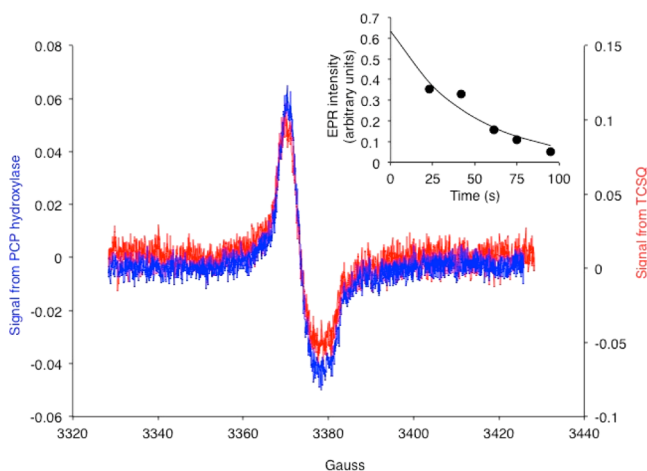


Figure 6. EPR spectrum of the enzyme-bound radical and time-course of radical decay. PCP hydroxylase (170 μM) was incubated with NADPH (300 μM) in the presence of limiting PCP (150 μM) under anoxic conditions, followed by mixing with an equal volume of buffer containing ambient O_2 . The reaction was quenched at varying times by freezing in liquid N_2 . The radical signal from PCP hydroxylase has $g = 2.0063$ and line-width $\Delta H = 7.5$ G (blue). For comparison, a sample of TCSQ under the same conditions gave a signal at $g = 2.0061$ and line width $\Delta H = 7.2$ G (red). The inset shows the time-dependent decrease in the radical signal on PCP hydroxylase. Symbols represent observed data points and the line is derived from a best-fit using a single exponential.

wavelengths less than 450 nm can be attributed to the C_{4a} -hydroxyflavin. However, the absorbance at wavelengths greater than 470 nm cannot be accounted for by either TCSQ or the C_{4a} -hydroxyflavin. (Spectra of complexes of the C_{4a} -hydroxyflavin with various intermediates and products have little absorbance above 460 nm ($<1 \text{ mM}^{-1} \text{ cm}^{-1}$).²⁶) The absorbance at 480 nm increases in a biphasic manner (Figure 4A). The first phase of this change correlates with the appearance of IV. We propose that this signal is due to a charge-transfer complex between TCBQ and an electron donor on the enzyme that subsequently converts to a diradical complex containing TCSQ and an enzyme-based radical. The apparently concurrent observation of both a charge-transfer complex and TCSQ is likely due to an inability to resolve the signals resulting from the formation of the charge-transfer complex and its rapid conversion to the diradical species.

The final step observed by stopped-flow analysis corresponds to the decay of the diradical to oxidized flavin (Figure 4). The rate constant of 0.027 s^{-1} for this step correlates well with k_{cat} (0.02 s^{-1}) and with the rate constant for the disappearance of the radical signal as measured by EPR (0.022 s^{-1}), suggesting that the diradical species is a kinetically competent intermediate. These data suggest that the rate-limiting step for PCP hydroxylase is the slow reversion of IV to III, which

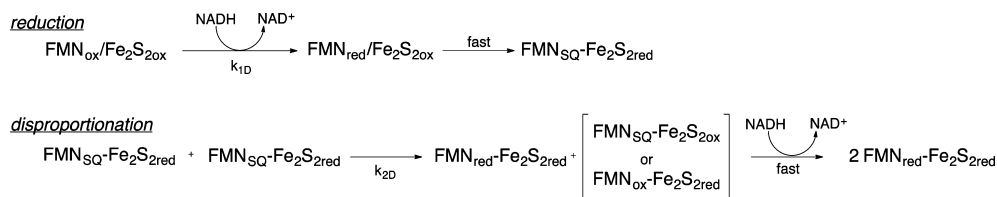
requires transfer of an electron from TCSQ back to the active site radical. Once III is reformed, the rate constant for dehydration to form the oxidized flavin must be faster than 0.027 s^{-1} .

Intermediates II and III do not accumulate during turnover of PCP. We can estimate the rate constants for formation of these intermediates according to the following logic. The rate of formation of I is 30 s^{-1} when the anoxic solution of PCP hydroxylase and PCP is mixed with buffer containing atmospheric O_2 . The rate of formation of TCSQ is 5.5 s^{-1} . Thus, one of the steps between I and IV must occur at a rate of 5.5 s^{-1} . Since intermediates II and III do not accumulate, conversion of I to II must be rate-limiting for formation of TCSQ, and the subsequent steps must be much faster. These suppositions agree with our chemical intuition; hydroxylation of PCP is likely to be more difficult than elimination of HCl from the unstable hydroxylation product and electron transfer to TCBQ from an electron donor at the active site.

Spectroscopic Signals Due to Reduction and Oxidation of TCBQ Reductase Do Not Interfere with Analysis of Single Turnovers by PCP Hydroxylase. We previously found that a transient physical interaction between PCP hydroxylase and TCBQ reductase prevents release of TCBQ to solvent.⁸ This interaction protects cells from damage by the highly reactive TCBQ. This finding raises the possibility that interaction with TCBQ reductase might alter the mechanism and/or kinetics of the PCP hydroxylase reaction.

Since both PCP hydroxylase and TCBQ reductase contain flavins, we first performed a minimal kinetic characterization of TCBQ reductase to identify potentially conflicting absorption and/or kinetic properties. TCBQ reductase is homologous to phthalate dioxygenase reductase, and mechanistic studies of this enzyme²⁷ provide a model for the reduction of TCBQ (Scheme 1). TCBQ reductase is a homotrimer containing a flavin and an Fe_2S_2 cluster in each subunit. Reaction of NADH with oxidized TCBQ reductase ($\text{FMN}_{\text{ox}}\text{-Fe}_2\text{S}_{2\text{ox}}$) (Supporting Information Figures 3 and 4) results in initial reduction of the flavin, followed by rapid transfer of an electron to the Fe_2S_2 cluster to give an $\text{FMN}_{\text{SQ}}\text{-Fe}_2\text{S}_{2\text{red}}$ monomer. Disproportionation between two such monomers generates a fully reduced monomer ($\text{FMN}_{\text{red}}\text{-Fe}_2\text{S}_{2\text{red}}$) and a one-electron reduced monomer ($\text{FMN}_{\text{ox}}\text{-Fe}_2\text{S}_{2\text{red}}$ or $\text{FMN}_{\text{SQ}}\text{-Fe}_2\text{S}_{2\text{ox}}$) within 2 s. In the presence of excess NADH, the spectroscopic signature of the FMN_{SQ} (a peak at 525 nm) forms and disappears rapidly, indicating that the one-electron reduced monomer is rapidly reduced to the fully reduced $\text{FMN}_{\text{red}}\text{-Fe}_2\text{S}_{2\text{red}}$. The fully reduced enzyme can transfer an electron to O_2 , but this reaction is quite slow; at $90 \mu\text{M}$ O_2 , electron transfer to O_2 occurs at only 0.025 s^{-1} (Supporting Information Figure 5). The presence of the semiquinone form of TCBQ reductase produced by electron transfer to O_2 was confirmed by EPR (Supporting Information Figure 6).

Scheme 1



This investigation revealed three fortunate characteristics of TCBQ reductase that enabled presteady state kinetic analysis of PCP hydroxylase in the presence of TCBQ reductase. Fully reduced TCBQ reductase ($\text{FMN}_{\text{red}}\text{--Fe}_2\text{S}_2_{\text{red}}$) has little absorbance in the region where TCSQ and the oxidized flavin formed at the active site of PCP hydroxylase absorb (Supporting Information Figure 4). Further, oxidized TCBQ reductase, which we expect to be generated during turnover in the presence of PCP hydroxylase, is rapidly reduced by NADH ($79 \pm 2 \text{ s}^{-1}$), preventing accumulation of oxidized flavin on TCBQ reductase that would interfere with our ability to detect oxidized flavin at the active site of PCP hydroxylase. Thus, TCBQ reductase is spectroscopically silent at the wavelengths needed to analyze the intermediates formed during the oxidative half-reaction of PCP hydroxylase. However, the absorbance of the $\text{FMN}_{\text{SQ}}\text{--Fe}_2\text{S}_2_{\text{red}}$ form of TCBQ reductase at a longer wavelength (525 nm) (Supporting Information Figure 4) allows us to detect transfer of a single electron from the fully reduced $\text{FMN}_{\text{red}}\text{--Fe}_2\text{S}_2_{\text{red}}$ form to a substrate.

TCBQ Reductase Rescues the Unreactive Diradical Formed during Turnover of PCP. Kinetic analysis of events during the oxidative half-reaction of PCP hydroxylase in the presence of TCBQ reductase revealed three observable processes, as for the reaction with PCP hydroxylase alone. The calculated spectra of the TCSQ intermediate and the final product (Figure 7) are essentially identical to those seen during

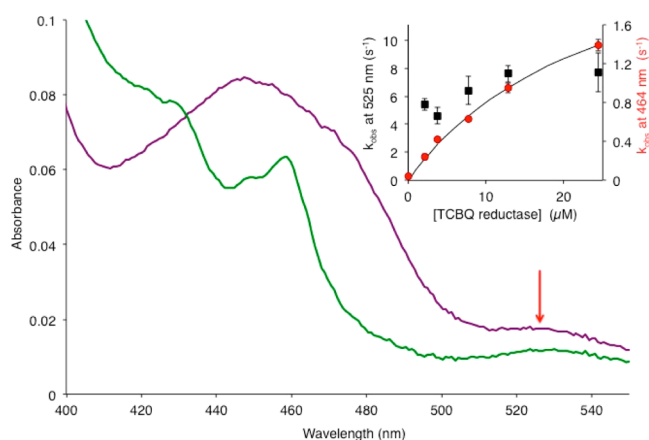


Figure 7. Calculated spectra of the diradical intermediate (green) and oxidized PCP hydroxylase (purple) formed during the oxidative half-reaction of PCP hydroxylase in the presence of TCBQ reductase. Reduced PCP hydroxylase ($28 \mu\text{M}$) in the presence of PCP ($22 \mu\text{M}$), NADPH ($90 \mu\text{M}$), and NADH ($90 \mu\text{M}$) and various concentrations of TCBQ reductase ($4.2\text{--}48.8 \mu\text{M}$) was mixed with an equal volume of oxygenated buffer and monitored by diode array spectroscopy in a stopped-flow apparatus. The calculated spectra shown in the figure were obtained from the reaction carried out in the presence of $24.4 \mu\text{M}$ TCBQ reductase. The inset shows k_{obs} for the appearance of the peak at 525 nm (left axis, squares) and k_{obs} for the disappearance of the signal at 464 nm due to TCSQ (right axis, circles). Observed rate constants were obtained by fitting the Michaelis–Menten equation to the data.

the reaction of PCP hydroxylase alone (Figure 4B), except for the presence of a peak at 525 nm that can be attributed to the $\text{FMN}_{\text{SQ}}\text{--Fe}_2\text{S}_2_{\text{red}}$ form of TCBQ reductase. The rate constants for formation of C_{4a} -hydroperoxyflavin (I) and TCSQ remained essentially unchanged in the presence of TCBQ reductase (Table 2). However, TCBQ reductase is directly involved in the following steps (Figure 2, highlighted in pink).

The intermediate state depicted by the green line in Figure 7 includes both TCSQ (indicated by the double peak in the region between 420 and 470 nm) and the $\text{FMN}_{\text{SQ}}\text{--Fe}_2\text{S}_2_{\text{red}}$ form of TCBQ reductase (indicated by the peak at 525 nm). The concomitant appearance of both species suggests that they form at the same time (unlikely since TCSQ is formed in the absence of TCBQ reductase), or that formation of TCSQ is followed immediately by a single electron transfer from TCBQ reductase (Figure 2, pink box). This electron must reduce the unidentified active-site radical in PCP hydroxylase to form V because the absorbance spectrum indicates that TCSQ is still present. The rate of this electron transfer is independent of the concentration of TCBQ reductase (black data points in the inset to Figure 7) for reasons that are not understood, yet not unprecedented.²⁸

The final observed process is reduction of TCSQ by TCBQ reductase concomitant with dehydration of VI to form the oxidized flavin (VII), which absorbs maximally at 460 nm (Figure 7). Since this process is accelerated by TCBQ reductase in a concentration-dependent manner (red data points in the inset to Figure 7), we conclude that the electron transfer that results in reduction of TCSQ limits the rate of conversion of VI to VII, and that dehydration of the flavin occurs rapidly after the electron is transferred. Alternatively, dehydration of the flavin might occur first, and be followed rapidly by electron transfer. However, the observation that the rate constant is proportional to the concentration of TCBQ reductase makes this scenario less likely.

These data indicate that TCBQ reductase rescues the unreactive off-pathway state of PCP hydroxylase by two consecutive electron transfers to different acceptors. This intervention accelerates the formation of the oxidized flavin by up to 50-fold. However, k_{cat} in the presence of TCBQ reductase is still remarkably slow (0.04 s^{-1} , ref 8). We conclude that a conformational change occurring after reoxidation of PCP hydroxylase or release of product(s) limits the rate of overall turnover in the presence of TCBQ reductase.

DISCUSSION

Given the toxicity of PCP and its relatively recent introduction into the environment, the assembly of a new pathway for degradation of PCP is an impressive feat. The PCP degradation pathway in *S. chlorophenolicum* begins with conversion of PCP to an extremely toxic intermediate, TCBQ.⁵ This initial step may seem surprising from a teleological standpoint; why would an organism “choose” to generate such a reactive intermediate? The answer to this question is likely that formation of TCBQ is an inevitable result of the enzymatic repertoire from which *S. chlorophenolicum* could have drawn an enzyme capable of initiating degradation of PCP. Aerobic microbes typically initiate degradation of phenols by hydroxylating a position ortho or para to the existing hydroxyl substituent using a flavin-dependent monooxygenase. Such enzymes are abundant in environmental microbes. Thus, the most likely source of an enzyme that can do anything with PCP is a flavin-dependent monooxygenase whose normal function is to hydroxylate a naturally occurring phenol. Most commonly, phenols are hydroxylated at a position carrying a hydrogen, a reaction that leads to formation of a hydroquinone after elimination of H_2O from the hydroxylated intermediate. In contrast, hydroxylation at a position carrying a leaving group leads to an intermediate that undergoes *gem* elimination to form a benzoquinone. Phenol hydroxylase,²⁹ *p*-hydroxybenzoate hy-

Table 2. Kinetic Parameters for the Oxidative Half-Reaction of PCP Hydroxylase in the Presence of Varying Concentrations of TCBQ Reductase^a

[TCBQ reductase] (μM)	formation of I ($\times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$)	I \rightarrow IV (s^{-1})	IV \rightarrow V (at 525 nm; s^{-1})	V \rightarrow VI (s^{-1})
0	2.8 ± 0.19	6.0 ± 0.2	n.a.	n.a.
2.1	3.0 ± 0.21	5.8 ± 0.3	5.4 ± 0.4	0.24 ± 0.029
3.8	3.3 ± 0.28	6.7 ± 0.5	4.6 ± 0.6	0.42 ± 0.010
7.7	3.6 ± 0.51	6.7 ± 0.4	6.4 ± 1.0	0.63 ± 0.010
12.8	3.4 ± 0.32	8.3 ± 1.5	7.6 ± 0.6	0.95 ± 0.052
24.4	3.2 ± 0.24	10.8 ± 3.4	7.7 ± 1.4	1.39 ± 0.063

^aDerived from global kinetic fitting (unless indicated otherwise). n.a.: not applicable.

droxylase,³⁰ and anthranilate hydroxylase³¹ have been shown to produce benzoquinone products from fluorinated substrate analogs by this mechanism. Since PCP contains five chlorines, formation of a benzoquinone is an inevitable consequence of hydroxylation as catalyzed by a flavin-dependent monooxygenase.

PCP hydroxylase is a remarkably slow enzyme; k_{cat} is only 0.02 s^{-1} . Our initial expectation was that turnover was slow because hydroxylation of a phenol with so many electron-withdrawing chlorine substituents was difficult. Surprisingly, we found that PCP hydroxylase acts as efficiently and via the same mechanism as other flavin-dependent monooxygenases even through the hydroxylation step. The mechanism only departs from the canonical phenol hydroxylase mechanism when the strongly oxidizing TCBQ is formed in the active site. Electron transfer to TCBQ results in a diradical intermediate (IV) that only slowly completes the catalytic cycle. This unprecedented diradical intermediate is not dictated by a change in the fundamental mechanism of typical flavin-dependent monooxygenases, but rather by the unique characteristics of the polychlorinated substrate.

Formation of TCSQ at the active site appears to be preceded by formation of a charge-transfer complex that absorbs at wavelengths longer than 470 nm. A transient charge-transfer complex with similar properties has been observed between ubiquinone and an active site cysteine during oxidation of DsbA by DsbB.^{32,33} This charge-transfer complex shows a broad absorbance peak with a maximal absorbance at about 525 nm and forms at rate comparable to that of the increase in absorbance at 480 nm that we attribute to formation of a charge-transfer complex at the active site of PCP hydroxylase. Further, the increase in absorbance due to the charge-transfer complex is comparable in the two systems. The OD₄₈₀ increases by ~ 0.03 in a solution containing $10 \mu\text{M}$ PCP hydroxylase. For the DsbB/DsbA reaction, the OD₅₀₀ increases by ~ 0.05 in a solution containing $40 \mu\text{M}$ of each protein.

An estimate of the amount of TCSQ present at the active site can be obtained by subtracting the estimated contributions of the C_{4a}-hydroxyflavin and the charge-transfer complex from the absorbance at 460 nm of the intermediate shown in the green line in Figure 4B. The absorbance of the C_{4a}-hydroxyflavin alone is not known, but in complexes of flavin-dependent monooxygenases containing the C_{4a}-hydroxyflavin and either an intermediate or product, its absorbance is comparable to that of the C_{4a}-peroxyflavin. If the absorbance of the charge-transfer complex is symmetric around 480 nm, then the absorbance of 460 nm would be approximately equal to that at 500 nm. Based upon these approximations and the extinction coefficient of TSCQ ($\epsilon_{461} = 6600 \text{ M}^{-1} \text{ cm}^{-1}$, ref 34), complex IV contains about 0.7 enzyme equivalents of TCSQ. While this is only an

estimate, it suggests that a substantial amount of TCSQ can be accounted for by the spectroscopic evidence.

Unexpectedly, the amplitude of the EPR signal we measure during turnover is half that expected for a complex containing TCSQ and a protein-based radical. Quantitation of the EPR signal 45 s after initiation of the reaction indicates the presence of 0.10 spin equivalents per active site. Based upon the stopped-flow data, we would expect 0.11 equiv of TCSQ at this point, as well as 0.11 equiv of the radical formed from the electron donor. Clearly there must be a second radical that was not observable via the methods we employed in these experiments. Our attempts to trap the active site radical with the spin traps α -phenyl-*N*-tert-butyl nitron (PBN) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*n*-oxide (DEPMPO) were unsuccessful, probably because the active site is inaccessible during turnover.

Possible sources of the electron that reduces TCBQ to TCSQ include the deprotonated forms of cysteine, tyrosine, or the C_{4a}-hydroxyflavin. Formation of radicals from any of these species is unprecedented in flavin-dependent monooxygenases. Although there is no literature precedent for the spectrum of a deprotonated C_{4a}-hydroxyflavin, such a radical should give a detectable EPR signal, as the common neutral and anionic flavin radicals are easily detectable by X-band EPR.^{35,36} Tyrosine radicals are easily detectable by EPR and typically display hyperfine interactions with protons on the aromatic ring and the methylene group.^{37,38} Our EPR signal shows no hyperfine coupling (as expected for TCSQ, which lacks protons on the aromatic ring). In contrast, the electron spin relaxation of thiyl radicals is so fast that they can only be detected at temperatures below 80 K.³⁹ Thus, if the diradical complex contains a thiyl radical, its amplitude would be half of that expected and the signal should look like that of TCSQ alone. Further, cysteine radicals do not absorb above 400 nm,⁴⁰ and therefore would not be detectable in the region of the spectrum probed during our stopped-flow kinetic studies.

PCP hydroxylase is only 29% identical to *p*-hydroxybenzoate hydroxylase (PDB 1DOC) and phenol hydroxylase (1FOH), for which structures are available. Although multiple sequence alignments of PCP hydroxylase with pfam 01494, to which all three of these enzymes belong, suggest that one or possibly two cysteines may be near the active site, the high level of sequence divergence, the different structural and electronic properties of the substrates, and the differences in the regiochemistry of hydroxylation make extrapolation from the available structural information tenuous. Future structural and kinetic experiments will probe the roles of cysteine residues in catalysis and electron transfer between TCBQ reductase and the active site of PCP hydroxylase.

Our data suggest that elimination of H₂O to regenerate the oxidized flavin cofactor cannot occur in complex IV. If the active-site radical is indeed a thiyl radical, perhaps the

dehydration of the C_{4a}-hydroxyflavin requires Cys to act as a general acid or general base. A radical would be unable to perform either function. Alternatively, formation of TCSQ and a side-chain-based radical might perturb the structural and/or electronic environment at the active site such that dehydration becomes very slow. We plan to solve the crystal structure of PCP hydroxylase to reveal whether a cysteine residue occupies a position close enough to donate an electron to TCBQ, but not close enough to permit nucleophilic attack on the electron-deficient ring. More detailed information about the structure of the active site will help us interpret the results of site-directed mutagenesis of residues that may be involved in electron transfer to TCBQ and/or dehydration of the C_{4a}-hydroxyflavin, as well as in transfer of electrons from TCBQ reductase into the active site of PCP hydroxylase.

Formation of the off-pathway diradical intermediate **IV** is thermodynamically favored but not irreversible; occasionally the enzyme returns to a state in which the C_{4a}-hydroxyflavin can undergo dehydration. Because dehydration is fast and irreversible, it eventually pulls the enzyme out of the unreactive diradical state. Thus, we now understand why PCP hydroxylase has such a slow turnover number in vitro.

Based upon our previous observation that TCBQ reductase can reduce TCBQ in solution,⁸ we anticipated that it would transfer two electrons or a hydride to TCBQ at its own active site. However, we now have clear evidence that the electrons are donated one at a time to the PCP hydroxylase:TCSQ complex. We propose that the first electron reduces the enzyme-based radical and the second electron reduces TCSQ to TCHQ. Thus, in the case of both PCP hydroxylase and TCBQ reductase, mechanistic studies of the individual enzymes in solution give an only partially accurate picture of the mechanism by which PCP is converted to TCHQ in the presence of both enzymes, and by extension, in vivo.

It is particularly intriguing that the dramatic mechanistic intervention by TCBQ reductase has a large effect on the rate at which dehydration of the C_{4a}-hydroxyflavin can occur, but only a 2-fold effect on k_{cat} . We previously speculated that the low turnover number of PCP hydroxylase might be a result of selective pressure to minimize the possibility of TCBQ escaping to the cytoplasm before the enzyme can interact with TCBQ reductase.⁸ However, the interaction between the proteins is very efficient; delivery of electrons to the diradical intermediate **IV** is at least 50-fold faster than k_{cat} . Our presteady state kinetic studies show that the rate-limiting step in the coupled PCP hydroxylase:TCBQ reductase system is either the conformational change needed to open the active site to allow products to depart, or the release of TCHQ and/or chloride. Turnover is clearly not limited by any of the multiple chemical steps required for conversion of PCP to TCHQ. Whether the low turnover is a consequence of selection to minimize the rate of formation of TCHQ or an indication that PCP hydroxylase has not yet evolved to be a highly efficient catalyst remains an open question.

In other cases in which benzoquinone intermediates are known to be formed during degradation of a phenol, the gene encoding the hydroxylase is clustered with a gene encoding a reductase.^{41,42} This is the case in *S. chlorophenolicum* as well,⁴³ which suggests that this two-enzyme system had already evolved the ability to convert a naturally occurring phenol with a leaving group at the position of hydroxylation to a hydroquinone before it was recruited to deal with the more problematic substrate PCP.

The results reported here beg the question of how the reductases in other hydroxylase:reductase systems deal with benzoquinone intermediates. Interestingly, there are different pairings in hydroxylase:reductase systems, as if the system can be made up of two interchangeable parts. In *Alcaligenes* sp. strain NyZ215,⁴¹ which can metabolize *o*-nitrophenol, the reductase is homologous to TCBQ reductase (38% identity), but the hydroxylase is unrelated to PCP hydroxylase, containing an unusual cytochrome *b*₅ domain on the C-terminal end that could serve to accept electrons from the reductase. In *Pseudomonas* WBC-3, which can mineralize *p*-nitrophenol,⁴² the hydroxylase is distantly related to PCP hydroxylase (25% identity), but the reductase (PnpB) is not related to TCBQ reductase. The hydroxylase involved in degradation of 2,4,6-trichlorophenol by *Cupriavidus necator*⁴⁴ is a two-component monooxygenase that is unrelated to PCP hydroxylase. A benzoquinone reductase has not been identified. The benzoquinone intermediates formed in these systems are considerably less reactive than TCBQ because they carry fewer electron-withdrawing substituents. We do not know whether these systems sequester benzoquinones like the PCP hydroxylase/TCBQ reductase system. We also do not know whether diradical intermediates are generated in the presence of benzoquinone intermediates that are weaker oxidants than TCBQ.

Our studies have revealed interesting insights into the consequences of adopting an ancestral catalytic strategy to carry out a reaction on a novel substrate. Typically, flavin-dependent monooxygenases that hydroxylate phenols generate hydroquinone or catechol intermediates that are neither electrophilic or strong oxidants. However, adoption of the typical mechanistic strategy for this enzyme family for hydroxylation of PCP leads to formation of a strong oxidant, TCBQ. The abstraction of an electron from an unidentified electron donor at the active site by TCBQ is responsible for the slow turnover of PCP hydroxylase in vitro. This situation is remedied in the presence of TCBQ reductase. We do not know whether the ability of TCBQ reductase to transfer electrons to two different acceptors in the active site was part of its pre-existing function, or is a new feature due to the presentation of two radicals in the active site of PCP hydroxylase. Similarly, is not clear whether the success of the two enzymes working in concert is an elegant mechanism resulting from natural selection or just a fortuitous outcome of the inherent catalytic capabilities of TCBQ reductase that manages to correct a “fumble” on the part of PCP hydroxylase. In either case, the net effect is conversion of PCP to TCHQ without damage to either enzyme or other cellular molecules.

■ ASSOCIATED CONTENT

§ Supporting Information

Supplementary Figures 1–6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS

PCP, pentachlorophenol; TCBQ, tetrachlorobenzoquinone; TCHQ, tetrachlorohydroquinone; TCSQ, tetrachlorosemiquinone; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl

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