Pre-Steady-State Kinetic Studies of the Reductive Dehalogenation Catalyzed by Tetrachlorohydroquinone Dehalogenase†

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ABSTRACT: Tetrachlorohydroquinone dehalogenase catalyzes two successive reductive dehalogenation reactions in the pathway for degradation of pentachlorophenol in the soil bacterium *Sphingobium chlorophenolicum.* We have used pre-steady-state kinetic methods to probe both the mechanism and the rates of elementary steps in the initial stages of the reductive dehalogenation reaction. Binding of trichlorohydroquinone (TriCHQ) to the active site is followed by rapid deprotonation to form $TriCHQ^{-2}$ and subsequent formation of 3,5,6-trichloro-4-hydroxycyclohexa-2,4-dienone (TriCHQ*). Further conversion of TriCHQ* to 2,6-dichlorohydroquinone (DCHQ) proceeds only in the presence of glutathione. Conversion of TriCHQ to DCHQ during the first turnover is quite rapid, occurring at about 25 s^{-1} when the enzyme is saturated with TriCHQ and glutathione. The rate of subsequent turnovers is limited by the rate of the thiol−disulfide exchange reaction required to regenerate the free enzyme after turnover, a reaction that is intrinsically less difficult, but is hampered by premature binding of the aromatic substrate to the active site before the catalytic cycle is completed.

Tetrachlorohydroquinone (TCHQ)[1] dehalogenase catalyzes the reductive dehalogenation of TCHQ to trichlorohydroquinone (TriCHQ) and then to 2,6-dichlorohydroquinone (DCHQ) in the pathway for degradation of pentachlorophenol (PCP) by the soil bacterium *Sphingobium chlorophenolicum* (*1*). The poor function of TCHQ dehalogenase, as well as other enzymes in this pathway, suggests that this pathway has been assembled by recruiting existing enzymes from at least two other pathways to serve new functions. Studies of the reasons for the poor function of evolving enzymes can provide insights into the contingencies and consequences involved in evolution of a new activity using an existing scaffold that may not be ideal, but nevertheless provides an activity that enhances the fitness of the bacterium.

TCHQ dehalogenase is a member of the zeta class of the glutathione *S*-transferase (GST) superfamily (*2*). Most GSTs catalyze a simple reaction consisting of a nucleophilic attack of glutathione upon an electrophilic substrate to form a glutathione conjugate. However, a few members of the superfamily catalyze more complex reactions. The enzymes most closely related to TCHQ dehalogenase, maleylacetoacetate and maleylpyruvate isomerases, catalyze the glutathione-dependent isomerization of a double bond from the cis to the trans configuration, a reaction that requires glutathione, but regenerates it, an interesting contrast to the reductive dehalogenation of TCHQ and TriCHQ, which requires 2 equiv of glutathione and results in their oxidation to glutathione disulfide.

Previous studies have led to the mechanism proposed in Figure 1. An early finding that the enzyme produced aberrant products when oxidized led to the identification of Cys13 as a critical residue for catalysis (*3*). The failure of the enzyme to turn over pentachlorophenol and tetrachlorophenol indicated that both hydroxyl groups are required for reactivity and was interpreted at the time as evidence for the intermediacy of **III**, formation of which requires the presence of two hydroxyl groups (*4*). The intermediacy of **IV** was suggested by studies of the C13S enzyme, which catalyzes only part of the reaction and produces a mixture of DCHQ and GS-DCHQ from TriCHQ, depending upon the pH (*4*). (Both products can be formed from intermediate **IV**, and the distribution of products would be expected to be dependent upon pH.) The formation and turnover of a covalent intermediate between Cys13 and glutathione (ESSG) was demonstrated by rapid-quench flow studies (*5*). The conversion of ESSG to GSSG and free enzyme was characterized using chemically synthesized ESSG and shown to occur with a k_{cat} of 103 ± 15 s^{-1} and a $K_{M,GSH}$ of 18.6 ± 5 mM at pH 7.7 (*6*).

Here we report pre-steady-state kinetic studies that provide more detailed information about the initial steps in the reaction and justify a revision of the previously proposed mechanism. These studies indicate that conversion of TriCHQ to DCHQ during the initial turnover is remarkably fast, given the need to disrupt the aromaticity of the ring to accomplish substitution of a chlorine by a hydrogen. The catalytic efficiency in the initial dehalogenation steps contrasts with

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[1] Abbreviations: DCBQ, 2,6-dichlorobenzoquinone; DCHQ, 2,6-dichlorohydroquinone; DTT, dithiothreitol; ESSG, tetrachlorohydroquinone dehalogenase Cys13-glutathionyl disulfide; GS-DCHQ, 3,5-dichloro-2-*S*-glutathionylhydroquinone; GSH, glutathione; GS-Me, *S*-methylglutathione; GSSG, glutathione disulfide; GST, glutathione transferase; TCHQ, tetrachlorohydroquinone; TriCHQ, T, trichlorohydroquinone; TriCHQ*, 3,5,6-trichloro-4-hydroxycyclohexa-2,4-dienone.

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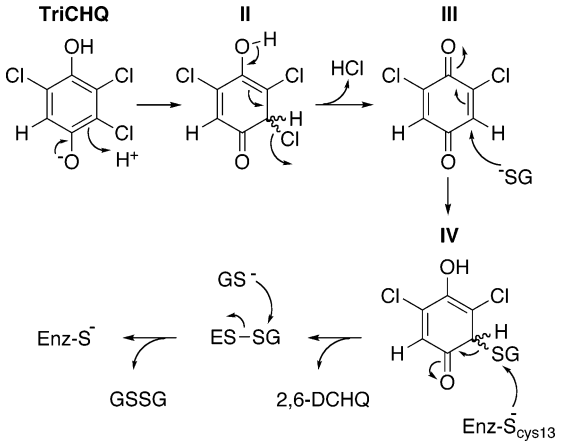


FIGURE 1: Previously proposed mechanism for TCHQ dehalogenase.

the poor performance of the enzyme in the final stages of the reaction, in which binding of the aromatic substrate to the active site prior to completion of the thiol−disulfide exchange reaction required to regenerate the free enzyme brings catalysis to a halt.

EXPERIMENTAL PROCEDURES

Reagents. All reagents were purchased from common commercial sources.

Enzyme Preparation. TCHQ dehalogenase has two cysteine residues, one in the active site (Cys13) and one on the surface (Cys156). Cys156 is not involved in catalysis. As its presence interferes with some of our experimental procedures, we routinely use the C156S mutant enzyme for kinetic studies. Substitution of Ser for Cys at position 156 does not appear to affect any properties of the enzyme (*3*). C156S TCHQ dehalogenase was expressed and purified using an *N*-linked glutathione−Sepharose affinity matrix as previously described (*7*). TCHQ dehalogenase obtained in this manner is fully active as judged by active site titration and contains no enzyme in which the active site Cys13 has been oxidized. The enzyme was treated for 30 min with 5 mM DTT and exchanged into various buffers prior to experiments by dialysis or by size exclusion chromatography using a Sephadex G25 column (Amersham Biosciences).

The gene encoding K16A C156S TCHQ dehalogenase was generated by mutagenesis of the gene for C156S TCHQ dehalogenase in pET-21b (Novagen) using the QuikChange II mutagenesis kit (Stratagene). The following primers were used to introduce the mutation: forward, 5′-CCATGTCGATCTGTTCGATGGCGACCCGTCTGGCGATGGAGG-3′; reverse, 5′-CCTCCATCGCCAGACGGGTCGCCATCGAACAGATCGACATGG-3′. The K16A C156S mutant protein was expressed as previously described (*7*). The cells were harvested by centrifugation at 10000*g* at 4 °C for 10 min. After lysis and removal of cell debris as previously described (*7*), the lysate was dialyzed for 3 h against 25 mM Tris−HCl, pH 8.0, containing 1 mM DTT, 1 mM EDTA, and 0.1 mM glutathione. The lysate was diluted with an equal volume of 25 mM Tris−HCl, pH 8.0, containing 5 mM DTT, 1 mM EDTA, and 0.5 mM glutathione and loaded onto a 30 mL Q Sepharose HP column (Amersham Biosciences) equilibrated with the same buffer. Proteins were eluted with a gradient of 0−30% 1 M KCl in the same buffer. Fractions containing TCHQ dehalogenase were concentrated with an Amicon Ultra 5000 molecular weight cutoff spin filter (Millipore). The sample was then diluted 10-fold with 25 mM Tris−HCl, pH 8.0, containing 5 mM DTT and 0.5 mM glutathione, and again concentrated to a volume of less than 2 mL. The sample was loaded onto a Mono Q HR 10/10 column (Amersham Biosciences) equilibrated with 25 mM Tris−HCl, pH 8.0, containing 5 mM DTT and 0.5 mM glutathione. Proteins were eluted with a gradient of 0−30% 1 M KCl in the same buffer also containing 1 mM EDTA. Fractions were analyzed by SDS−PAGE. Fractions containing pure K16A C156S TCHQ dehalogenase were combined and concentrated. Samples of protein were stored at 4 °C for no longer than one week.

C156S TCHQ dehalogenase Cys13-glutathionyl disulfide (ESSG) was prepared as previously described (*7*).

Preparation of TriCHQ, DCHQ, and GS-DCHQ. TriCHQ was prepared from trichlorobenzoquinone as previously described (*2*). TriCHQ was dissolved in ethanol, and the concentration was determined by the absorbance at 306 nm ($\epsilon_{306} = 4.5\ mM^{-1}\ cm^{-1}$). Solutions of TriCHQ in ethanol were prepared the day of the experiment. In experiments with TriCHQ, the ethanol concentration was less than 1% by volume. Control experiments were carried out to ensure that the addition of ethanol did not affect the results. DCHQ was prepared from dichlorobenzoquinone (DCBQ) using the procedure developed for preparation of TriCHQ. Purity was assessed by ^1H and ^{13}C NMR spectroscopy and HPLC analysis. GS-DCHQ was prepared from the reaction of TriCHQ and glutathione catalyzed by C13S TCHQ dehalogenase (as described for preparation of GS-TriCHQ) (*8*).

Kinetic Analysis of Glutathione Binding. The rate of binding of glutathione to TCHQ dehalogenase was measured by monitoring the change in the intrinsic fluorescence of the protein on an Applied Photophysics SX17.MV stopped-flow instrument at 20 °C using a cell with a 1 cm path length. TCHQ dehalogenase (1−10 μM) in 200 mM potassium phosphate, pH 7.0 or 8.0, was mixed with an equal volume of glutathione (20−1600 μM) in 200 mM potassium phosphate, pH 7.0 or 8.0. The concentration of glutathione was always at least 10-fold greater than that of protein so that pseudo-first-order conditions were met. Fluorescence was excited at 282 nm, and emission was detected using a 310 or 320 nm high-pass filter. At least 10 transients were collected and averaged. The data were fit with a single exponential.

Kinetic Analysis of TriCHQ Binding. Absorbance and fluorescence changes due to binding of TriCHQ to TCHQ dehalogenase were measured on an Applied Photophysics SX17.MV stopped-flow instrument at 20 °C using a cell with a 1 cm path length. TCHQ dehalogenase (0.4−20 μM) in 200 mM potassium phosphate, pH 8.0, containing 0.005% ascorbate, was mixed with an equal volume of TriCHQ (4−200 μM) in 200 mM potassium phosphate, pH 8.0, containing 0.005% ascorbate. The concentration of TriCHQ was always at least 10-fold greater than that of protein so that pseudo-first-order conditions were met. In some experiments, each syringe also contained *S*-methylglutathione (GS-Me) (500 μM). Changes in absorbance were monitored at 320, 345, 370, and 385 nm. Fluorescence was excited at 292 nm and emission detected using a 310 nm high-pass filter. At least

five transients were collected and averaged, and the data were fit with a double exponential.

Equilibrium Binding Experiments. Binding of glutathione was monitored by observation of the change in fluorescence of TCHQ dehalogenase (1.5 μM) upon titration with glutathione in 200 mM potassium phosphate, pH 7.0 or 8.0, at 20 °C using a Photon Technology International QM-2000-6SE fluorescence spectrometer. Fluorescence was excited at 282 nm (4 nm band-pass) and emission detected at 343 nm (8 nm band-pass). The solution was stirred for 60 s after the addition of each aliquot of glutathione and the fluorescence intensity determined as the average intensity of an 8 s reading. The observed fluorescence was then corrected for dilution, which was minimal. Each titration experiment was carried out three or four times. The fluorescence signal was plotted versus glutathione concentration and fit to eq 1, in which $[S]_0$ refers to the total concentration of glutathione. The total change in fluorescence (ΔF), initial fluorescence (F_0), and dissociation constant ($K_{D,GSH}$) were obtained from each fit. In the figure shown, the fluorescence intensity was normalized by dividing the observed fluorescence by F_0.

$$F = F_0 + \Delta F[S]_0/([S]_0 + K_D) \quad (1)$$

Binding of TriCHQ was monitored by observation of the change in fluorescence (290 nm excitation, 2 nm band-pass; 330 nm emission, 8 nm band-pass) upon addition of TriCHQ to TCHQ dehalogenase (2 μM) in 200 mM potassium phosphate, pH 7.0 or 8.0, containing 0.005% ascorbate, in the absence or presence of GS-Me (1 mM). The data were corrected for dilution. The small inner-filter effect caused by TriCHQ was corrected for using the method of Gauthier et al. (*9*). The titration experiment was carried out three times. The magnitude of the fluorescence signal was plotted versus the concentration of TriCHQ and fit to either eq 1 (a single hyperbolic function) or eq 2 (the sum of two hyperbolic functions). A quadratic binding equation (eq 3) was used to fit the data when the enzyme concentration was more than one-fifth of the K_D (eq 3). ($[S]_0$ refers to the total concentration of TriCHQ and $[E]_0$ to the total concentration of enzyme.) The total change in fluorescence (ΔF), initial fluorescence (F_0), and dissociation constant (K_D) were obtained from each fit. (The dissociation constant for TriCHQ is an apparent dissociation constant because, as will be shown below, binding of TriCHQ to the enzyme is a two-step process, and the apparent dissociation constant is a function of the equilibrium constants for both steps.) In the figures shown, the fluorescence intensity was normalized by dividing the observed fluorescence by F_0.

$$F = F_0 + \Delta F_\alpha[S]_0/([S]_0 + K_{D\alpha}) + \Delta F_\beta[S]_0/([S]_0 + K_{D\beta}) \quad (2)$$

$$F = F_0 + \Delta F \times \left(\frac{(K_D + [E]_0 + [S]_0) - \sqrt{(K_D + [E]_0 + [S]_0)^2 - 4[E]_0[S]_0}}{2[E]_0}\right) \quad (3)$$

UV/Vis Spectra of TriCHQ in Solution. The absorbance of TriCHQ (150 μM) in the presence of ascorbate (0.003%) and 200 mM potassium phosphate, pH 5.0 or 8.0, was measured in a 1 cm cuvette. Small aliquots of sodium hydroxide (5 N) were added to adjust the pH between 5.0 and 12.0. Figure 3a shows the change in absorbance of TriCHQ (150 μM) upon formation of $TriCHQ^-$ as the pH increases. Formation of $TriCHQ^-$ results in an increase in absorbance at 325 nm ($\Delta\epsilon_{325} = 4.6$ mM^{-1} cm^{-1}) and a decrease in absorbance at 298 nm ($\Delta\epsilon_{298} = -2.0$ mM^{-1} cm^{-1}). Formation of $TriCHQ^{-2}$ from $TriCHQ^-$ (Figure 3b) results in an increase in absorbance at 347 nm ($\Delta\epsilon_{347} = 3.7$ mM^{-1} cm^{-1}) and a decrease in absorbance at 318 nm ($\Delta\epsilon_{318} = -2.0$ mM^{-1} cm^{-1}).

UV/Vis Spectra of TriCHQ Bound to TCHQ Dehalogenase. The spectrum of TriCHQ (170 μM) in 200 mM potassium phosphate, pH 8.0, containing 0.1% ascorbate was measured in a 1 cm cuvette. The spectrum of TriCHQ was also measured under the same conditions in the presence of TCHQ dehalogenase (100 μM) with and without GS-Me (800 μM). When present, the absorbance due to TCHQ dehalogenase and ascorbate was subtracted from the spectrum of TriCHQ.

Rapid Quench-Flow Experiments. For measurement of the pre-steady-state formation of DCHQ, C156S TCHQ dehalogenase (20 μM) in 200 mM potassium phosphate, pH 7.0 or 8.0, containing 1 mM glutathione and 0.1% ascorbate was mixed with an equal volume of TriCHQ (400 μM) in the same buffer containing 0.1% ascorbate at 20 °C in an Applied Photophysics rapid quench-flow instrument. For attempts to detect intermediates during the first turnover, C156S TCHQ dehalogenase (360 μM) in 200 mM potassium phosphate, pH 7.0, containing 2 mM glutathione and 0.1% ascorbate was mixed with an equal volume of TriCHQ (420 μM) in the same buffer containing 0.1% ascorbate at 5 °C in the rapid quench-flow instrument. After various times, reactions were quenched by addition of an equal volume of 1 N HCl and collected. Products were analyzed by HPLC. Samples were injected on a Microsorb-MV 300-5 C18 column (250 × 4.6 mm, Varian) at a flow rate of 1 mL/min in 0.1% trifluoroacetic acid for 4 min, after which a gradient to 60% acetonitrile was developed over 13 min. Under these conditions, glutathione eluted at 5.3 min, GSSG at 10.0 min, GS-DCHQ at 12.8 min, DCHQ at 14.0 min, and TriCHQ at 16.0 min. The concentrations of substrates and products were plotted versus time. The data were fit to a single exponential.

Single-Turnover Stopped-Flow Experiments. Changes in absorbance were monitored at 345, 370, and 385 nm after TCHQ dehalogenase (180 μM) in 200 mM potassium phosphate buffer, pH 8.0, containing 0.005% ascorbate and either 1 mM GS-Me or 1 mM glutathione, was mixed with an equal volume of TriCHQ (40 μM) in 200 mM potassium phosphate buffer, pH 8.0, containing 0.005% ascorbate and either 1 mM GS-Me or 1 mM glutathione, on the stopped-flow instrument. At least five transients were collected and averaged.

RESULTS

Binding of Glutathione to TCHQ Dehalogenase. Binding of glutathione to TCHQ dehalogenase causes a decrease in the intrinsic fluorescence of the protein. The change in fluorescence was monitored during titration of TCHQ dehalogenase with glutathione at pH 7.0 (see Figure 2a) and pH 8.0 (data not shown). The data were fit to eq 1 to obtain the dissociation constant, $K_{D,GSH}$, of 90 ± 10 μM at pH 7.0 and 17 ± 1.5 μM at pH 8.0.

The rate of binding of glutathione to TCHQ dehalogenase was measured by following the decrease in enzyme fluo-

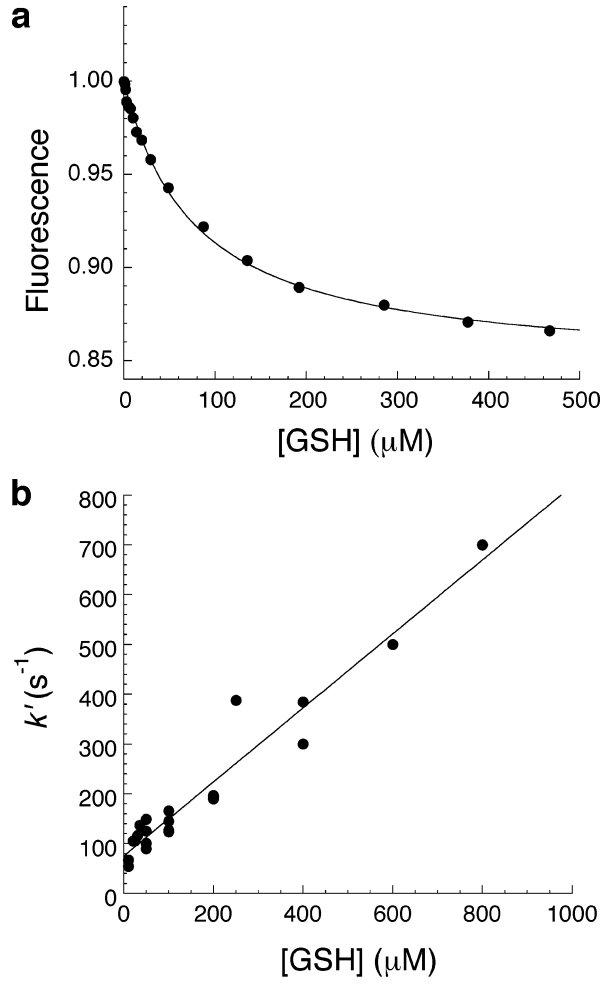


FIGURE 2: (a) Fluorescence change due to binding of glutathione to TCHQ dehalogenase. The decrease in fluorescence was monitored as glutathione was added in small increments (≤1 vol %) to TCHQ dehalogenase (1.5 μM) in 200 mM potassium phosphate, pH 7.0. The data were fit to eq 1 to give $K_{D,GSH} = 90 \pm 10$ μM. (b) Concentration dependence of the pseudo-first-order rate constant for binding of glutathione to TCHQ dehalogenase (0.5−5 μM) in 200 mM potassium phosphate, pH 7.0. The data were fit to eq 4.

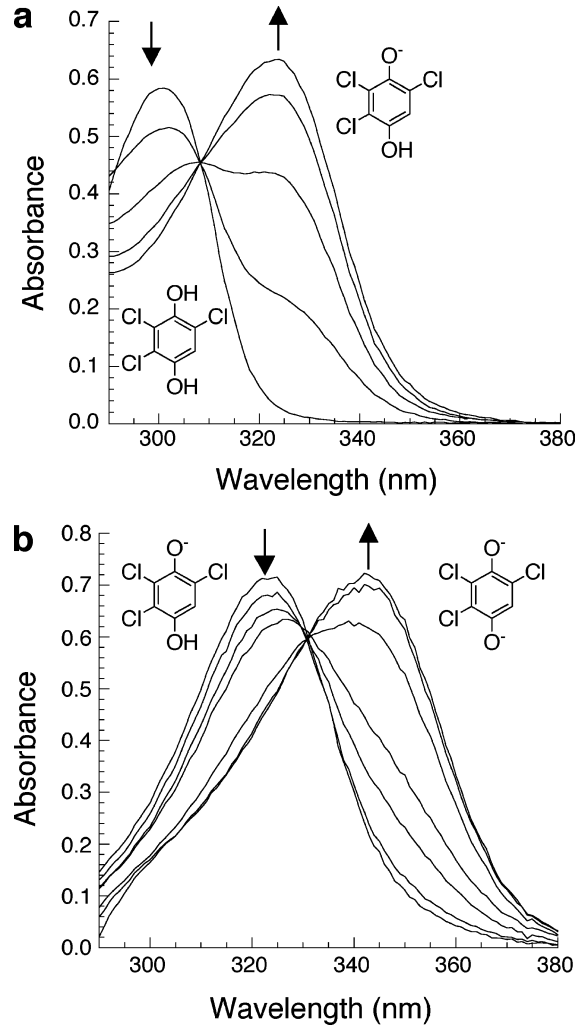


FIGURE 3: (a) Change in absorbance of TriCHQ (150 μM) in 200 mM potassium phosphate, pH 5.0 (800 μL), containing 0.003% ascorbate upon addition of 6 μL aliquots of sodium hydroxide (5 N) to a final pH of 8.0. (b) Change in absorbance of TriCHQ (150 μM) in 200 mM potassium phosphate, pH 8.0 (800 μL), containing 0.003% ascorbate upon addition of 1 μL aliquots of sodium hydroxide (5 N) to a final pH of 11.

rescence after mixing of the enzyme with glutathione in a stopped-flow instrument. The time dependence of the fluorescence signal followed a single exponential. At pH 7.0, the rate constant k' obtained by fitting the data to a single exponential increased linearly with glutathione concentration and showed no signs of saturation (see Figure 2b). Similar data were obtained at pH 8.0 (not shown). These data are consistent with a simple binding equilibrium as shown in Scheme 1. (The designation GS(H) indicates that the protonation state of the thiol is uncertain.) The observed rate constant for glutathione binding under pseudo-first-order conditions is given by eq 4. Values for k_1 and k_{-1} obtained by fitting the data in Figure 2b to eq 4 are given in Table 1.

$$k' = k_1[\mathrm{GSH}] + k_{-1} \quad (4)$$

Glutathione is deprotonated at the active site of most GSTs (*10*, *11*). The resulting thiolate is stabilized by a hydrogen-bonding interaction with the hydroxyl group of a serine or tyrosine in most classes of GSTs. Thiolate formation causes an increase in absorbance at 240 nm. In the case of TCHQ dehalogenase, no change in absorbance at 240 nm was observed when the enzyme was mixed with glutathione (data not shown).

Scheme 1

$$\mathrm{E} \underset{k_{-1}}{\overset{k_1\,[\mathrm{GSH}]}{\rightleftharpoons}} \mathrm{E{\cdot}GS(H)}$$

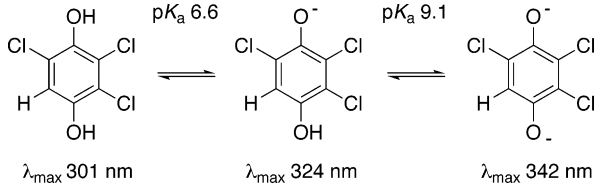
Binding of TriCHQ to TCHQ Dehalogenase. TriCHQ has three possible protonation states; each state has a characteristic absorption spectrum (see Figure 3). The pK_a values for the successive titrations of the hydroxyl groups and the λ_{max} for each protonation state are summarized in Scheme 2.

The apparent dissociation constant for TriCHQ was obtained by following the change in fluorescence when TriCHQ was titrated into a solution of TCHQ dehalogenase at pH 8.0 or 7.0 (parts a and b, respectively, of Figure 4) and in the absence or presence of GS-Me (1 mM). The data in Figure 4a show a monotonic decrease in fluorescence intensity as the concentration of TriCHQ is increased. The apparent dissociation constants for TriCHQ in the absence and presence of saturating GS-Me obtained by fitting the data to eq 3 are given in Table 1. The presence of GS-Me at

Table 1: Rate and Equilibrium Constants for Binding of Glutathione and TriCHQ to C156S TCHQ Dehalogenase

variable ligand	saturating ligand	pH	K_D (μM)	k_1 ($\mu M^{-1} s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})
TriCHQ		7.0	~4				
			130 ± 25				
		8.0	12 ± 2.0	1.0 ± 0.1	83 ± 10	25 ± 5	3.4 ± 5
TriCHQ	GS-ME	7.0	18 ± 2.0				
		8.0	4.0 ± 1.0	2.4 ± 0.4	41 ± 14	36 ± 10	10 ± 5
GSH		7.0	90 ± 10	0.76 ± 0.1	68 ± 8.0		
		8.0	17 ± 1.5	1.1 ± 0.1	16 ± 7.0		

Scheme 2



the active site causes a small but significant decrease in $K_{D,TriCHQ}$.

The data obtained at pH 7.0 (Figure 4b) are markedly different, showing a biphasic change in fluorescence intensity as the concentration of TriCHQ is increased. These data are consistent with either the existence of two forms of the enzyme, one that binds TriCHQ tightly and one that binds TriCHQ weakly, or differential binding of the two forms of TriCHQ in solution at pH 7.0 (TriCHQ and $TriCHQ^-$). Notably, when GS-Me is present at the active site, only the tighter binding form is evident ($K_{D,TriCHQ} = 18$ μM). This observation suggests that there are two conformational states of the enzyme and that the presence of GS-Me helps to order the active site in such a way that binding of TriCHQ is stronger. An alternative but unlikely possibility is that the presence of GS-Me allows binding of only the $TriCHQ^-$ isomer, while both isomers can bind in its absence.

The data for binding of TriCHQ to the enzyme at pH 7.0 were fit to eq 2 to obtain dissociation constants for the two states of ~4 and 130 ± 25 μM. Since the total enzyme concentration was 2 μM, it would have been preferable to fit the data to a sum of two quadratic functions rather than a sum of two hyperbolic functions, as use of a quadratic equation is appropriate when the concentration of the enzyme is greater than one-fifth the K_D. However, the quadratic equation for ligand binding (see eq 3) contains a term for enzyme concentration; we cannot determine the concentrations of the two different forms of the enzyme from our data because the amplitudes of the fluorescence changes may be different in the two different forms. Thus, the value of the lower K_D of 4 μM is only an approximation.

Because of the existence of two protonation states of the substrate at pH 7.0 and the apparent existence of two forms of the enzyme at pH 7.0, further investigation of the kinetics of TriCHQ binding was carried out at pH 8.0. Binding of TriCHQ to TCHQ dehalogenase results in complex spectroscopic changes (see Figure 5). The kinetic data described below suggest that these changes reflect conversion of $TriCHQ^-$, the predominant species in solution, to two additional species at the active site. Both species are formed when GS-Me is present at the active site, as well. Indeed, the spectroscopic changes observed are more pronounced when the enzyme is saturated with GS-Me.[2] We have

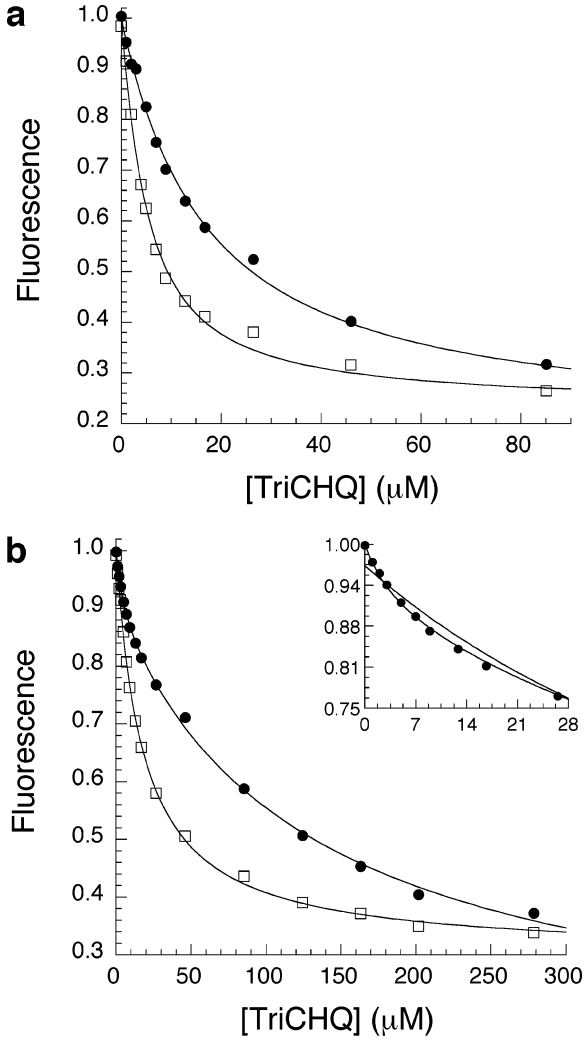


FIGURE 4: Fluorescence change due to binding of TriCHQ to TCHQ dehalogenase. The decrease in fluorescence was monitored as TriCHQ was added in small increments (≤0.4 vol %) to TCHQ dehalogenase (2.0 μM) in 200 mM potassium phosphate, pH 8.0 (a) or pH 7.0 (b), containing 0.005% ascorbate and in the absence (●) or presence (□) of GS-Me (1 mM). The data in (a) were fit to eq 3. The data in (b) were fit to eqs 1 (□) and 2 (●). The inset shows that the data at low concentrations of TriCHQ and in the absence of GS-Me fit poorly to eq 1 but well to eq 2.

previously shown that these species also form at the active site of ESSG (*7*).

Absorbance changes due to binding of TriCHQ to TCHQ dehalogenase in the absence and presence of GS-Me were

[2] Additional experiments shown in the Supporting Information were carried out to determine that the dissociation constant for GS-Me and TCHQ dehalogenase with TriCHQ in the active site was approximately 55 μM at pH 8.0.

Table 2: Observed Rate Constants for the Absorbance and Fluorescence Changes during Binding of TriCHQ to C156S TCHQ Dehalogenase at pH 8.0 in the Presence of Saturating GS-Me[a]

obsd rate constant (s^{-1})	absorbance, 320 nm	absorbance, 345 nm	absorbance, 370 nm	fluorescence[b]
$k_{obs,1}$	400 ± 100	300 ± 50	*c*	270 ± 15
$k_{obs,2}$	*c*	40 ± 20	25 ± 20	38 ± 3

[a] TCHQ dehalogenase (10 μM) in 200 mM potassium phosphate buffer, pH 8.0, containing GS-Me (500 μM) and ascorbate (0.005%) was mixed with an equal volume of TriCHQ (160 μM) in the same buffer. [b] Excitation at 292 nm, emission detected using a 310 nm high-pass filter. [c] Rate constants are not reported when the amplitude was too small to obtain an accurate fit.

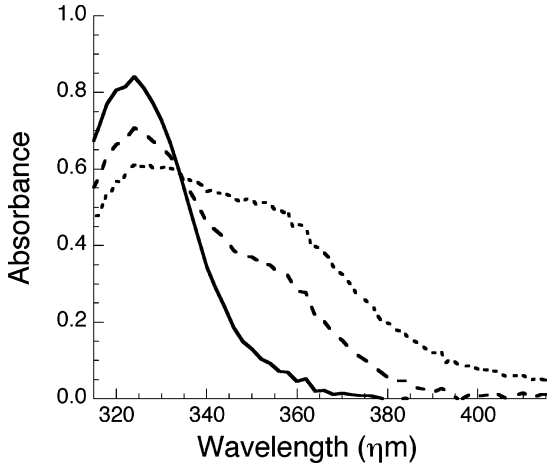


FIGURE 5: Spectra of 170 μM TriCHQ in 200 mM potassium phosphate, pH 8.0, 0.1% ascorbate in the absence (—) and presence (— — —) of 100 μM TCHQ dehalogenase and in the presence of 100 μM TCHQ dehalogenase and 800 μM GS-Me (---). The absorbance due to ascorbate and TCHQ dehalogenase, when present, was subtracted from each spectrum.

measured using a stopped-flow instrument. Representative traces are shown in Figure 6. At all concentrations of TriCHQ tested, the absorbance at 320 nm decreased and the absorbance at 345 and 370 nm increased. (Although Figure 5 suggests that a species with a λ_{max} of 360 nm is present, we monitored absorbance at 370 nm to minimize the contribution from the species absorbing at 345 nm.) A decrease in the fluorescence of the enzyme was also observed (see Figure 6b). All absorbance and fluorescence changes could be fit with double exponentials. Control experiments were conducted in the absence of enzyme and showed no change in absorbance or fluorescence. Table 2 shows the observed rate constants for the two phases in each case.

We attribute the immediate decrease in absorbance at 320 nm and concomitant increase in absorption at 345 nm to conversion of $TriCHQ^-$ to $TriCHQ^{-2}$. The rate constants for loss of the signal at 320 nm and the increase of the signal at 345 nm during the fast phase are nearly identical. The small change in absorbance at 370 nm during the initial phase may reflect the conversion of $TriCHQ^-$ to $TriCHQ^{-2}$. However, most of the change in absorbance at 370 nm occurs during the second phase. We designate the species that absorbs at 360 nm TriCHQ*. These results are consistent with the two-step model shown in Scheme 3, in which binding and deprotonation of $TriCHQ^-$ occur during a fast initial step and formation of TriCHQ* occurs in a slower subsequent step. Although formation of $TriCHQ^{-2}$ likely follows formation of a complex between $TriCHQ^-$ and TCHQ dehalogenase, a signal indicating such a complex was not detected. (Evidence for such a complex would have been observation of a three-phase absorbance or fluorescence change or a hyperbolic dependence of the fast phase of the observed biphasic absorbance or fluorescence change on the concentration of substrate.) It is possible that binding occurred during the dead time of the instrument or that formation of the initial enzyme—$TriCHQ^-$ complex did not result in an observable spectroscopic change. Similar data were observed for the binding of TriCHQ to ESSG (data not shown).

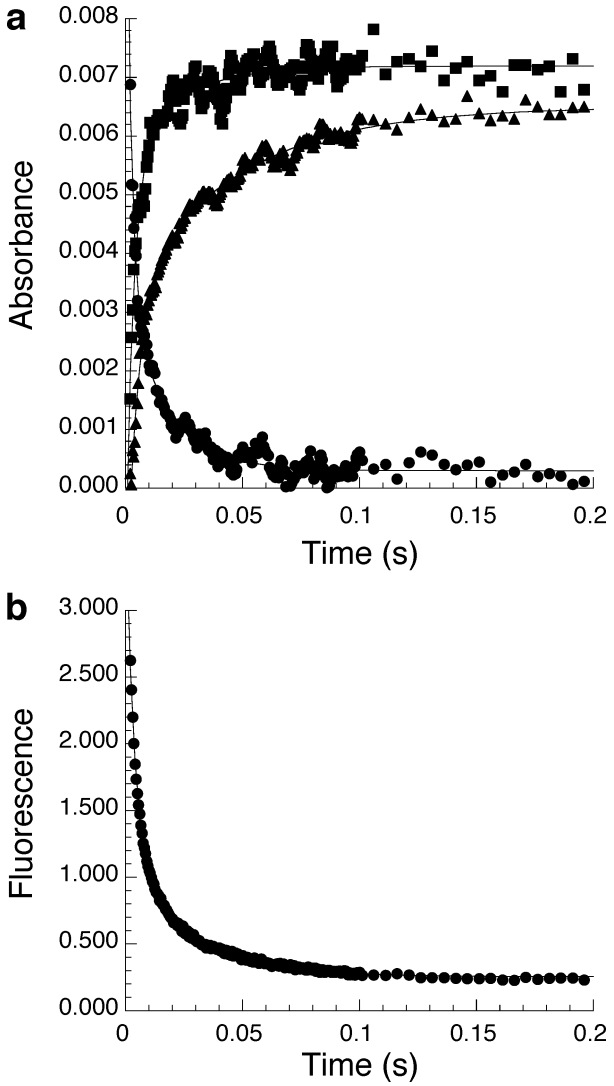


FIGURE 6: Representative traces from stopped-flow experiments in which TCHQ dehalogenase (10 μM) in 200 mM potassium phosphate buffer (pH 8.0) containing GS-Me (500 μM) and ascorbate (0.005%) was mixed with an equal volume of TriCHQ (160 μM) in the same buffer. The data at each wavelength were fit with a double exponential. (a) Absorbance change at 320 nm (●), 345 nm (■), and 370 nm (σ). (b) Fluorescence change with 292 nm excitation, emission detected beyond a 310 nm high-pass filter.

Scheme 3

$$\text{Enz} \underset{k_{-1}}{\overset{k_1\ \text{TriCHQ}^-}{\rightleftharpoons}} \text{Enz}\cdot\text{TriCHQ}^{-2} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Enz}\cdot\text{TriCHQ}^*$$

Rate constants for the fast and slow changes in the fluorescence signal were measured as a function of TriCHQ concentration (see Figure 7). The dependence of the observed rate constant for the fast phase on the concentration of

TriCHQ was fit with eq 5, which applies for two-step

$$k_{obs,1} = k_1[\text{TriCHQ}] + k_{-1} + k_2 + k_{-2} \quad (5)$$

reversible reactions (*12*). The slope of the line for the concentration dependence of $k_{obs,1}$ in the absence of GS-Me gives $k_1 = 1.0 \pm 0.1\ \mu M^{-1}\ s^{-1}$. We attribute the slow phase to formation of TriCHQ*. The dependence of the observed rate constant for this phase on the concentration of TriCHQ can be fit with eq 6 (*12*). The maximum rate is $k_{-2} + k_2 =$

$$k_{obs,2} = \frac{k_1[\text{TriCHQ}](k_{-2} + k_2) + k_{-1}k_{-2}}{k_1[\text{TriCHQ}] + k_{-1} + k_2 + k_{-2}} \quad (6)$$

$28 \pm 4\ s^{-1}$. Using this value and the *y* intercept of the plot of k_{obs} for the fast phase ($k_{-1} + k_2 + k_{-2}$), we can estimate that $k_{-1} = 83 \pm 10\ s^{-1}$. Rate constants for binding of TriCHQ in the presence of GS-Me (see Figure 7b) were obtained similarly and are reported in Table 1. Estimates for k_2 and k_{-2} were calculated using eqs 7−9

$$K_{D,TriCHQ} = 1/K_1(1 + K_2) \quad (7)$$

$$K_1 = k_1/k_{-1} \quad (8)$$

$$K_2 = k_2/k_{-2} \quad (9)$$

(*13*) and the values of k_1, k_{-1}, and $K_{D,TriCHQ}$ determined as described above. The presence of GS-Me in the active site of TCHQ dehalogenase increases the rate of formation of $TriCHQ^{-2}$ and lowers the reverse rate of protonation of $TriCHQ^{-2}$ (see Table 1), leading to a higher concentration of $TriCHQ^{-2}$ in the active site.

*Efforts To Identify TriCHQ**. TriCHQ* is observed when TriCHQ binds to either the free enzyme or the enzyme−GS-Me complex (see Figure 5). Similar spectral changes are observed when TriCHQ binds to the ESSG form of TCHQ dehalogenase (*7*). The spectrum of TriCHQ* is considerably shifted relative to that of either $TriCHQ^-$ or $TriCHQ^{-2}$ in solution. It seems unlikely that the spectrum of $TriCHQ^{-2}$ is shifted due to the unique environment at the active site, as previous experiments have shown that the spectra of $TriCHQ^-$ and $TriCHQ^{-2}$ are not perturbed upon binding to the active site of C13S TCHQ dehalogenase (*8*). Although the substitution of a neutral serine for a possibly charged cysteine residue is an important change, it is unlikely to cause a 20 nm shift in the absorbance spectrum.

We attempted to identify TriCHQ* using chemical quench procedures. TriCHQ is recovered quantitatively when the enzyme-TriCHQ complex is denatured by heat, HCl, NaOH, or triethylamine, indicating that TriCHQ* is converted back to TriCHQ under these conditions.

The Thiol of Glutathione Is Required for Formation of DCHQ from TriCHQ. The mechanism shown in Figure 1 does not explicitly involve glutathione until after formation of DCBQ. Thus, formation of DCBQ might be expected to occur in the presence of GS-Me. However, no DCBQ was formed during incubation of the enzyme with TriCHQ and GS-Me (data not shown). These data suggest that the thiol or thiolate of glutathione is required for conversion of TriCHQ* to the next intermediate in the catalytic cycle.

DCHQ Formation Follows Burst Kinetics. The rate of turnover of TCHQ dehalogenase is limited by events that occur during the second part of the catalytic cycle, specifically the thiol−disulfide exchange reaction between ESSG and glutathione, which is severely inhibited by the aromatic substrates TCHQ and TriCHQ (*7*). To measure the rate of the actual dehalogenation reaction, we performed chemical quench-flow experiments to observe the pre-steady-state rate of production of DCHQ from TriCHQ. Experiments were carried out at pH 7.0 to facilitate comparisons with previous studies of the thiol−disulfide exchange reaction and pH 8.0 to allow comparisons with the rates of events occurring during binding of TriCHQ (see above). Equal volumes of C156S TCHQ dehalogenase (20 μM) and glutathione (1 mM) were mixed with TriCHQ (400 μM) at 20 °C in the rapid quench-flow instrument. The reaction was quenched by mixing with an equal volume of 1 N HCl after reaction times ranging from 0.01 to 2 s, and the amount of DCHQ was detected by HPLC. A burst of formation of DCHQ was observed (see Figure 8), as expected since the formation of DCHQ after the first turnover is limited by the slow rate of the thiol−disulfide exchange reaction.

The formation of DCHQ can be described by the model shown in Scheme 4. Binding of TriCHQ under these conditions is very fast ($>500\ s^{-1}$), so the reaction can be treated as effectively beginning from the enzyme−glu-

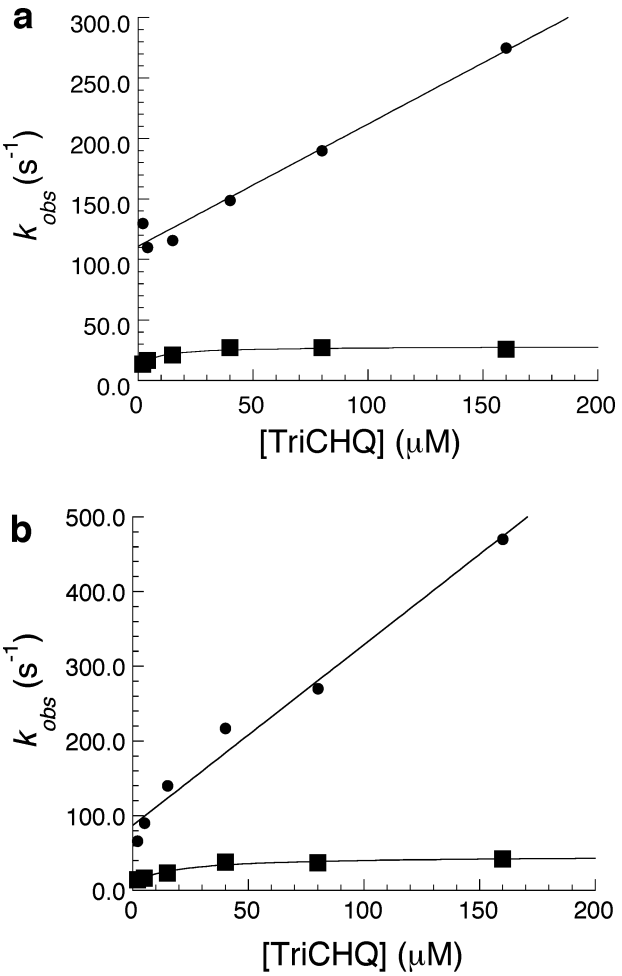


FIGURE 7: (a) Concentration dependence of the observed rate constants for the fast and slow phases of binding of TriCHQ to TCHQ dehalogenase (0.2−10 μM) in 200 mM potassium phosphate, pH 8.0, containing 0.005% ascorbate. The concentration dependence of the fast phase (●) was fit to eq 5 and that of the slow phase (■) to eq 6. (b) Same as (a), but the enzyme was saturated with 500 μM GS-Me prior to being mixed with a solution of TriCHQ containing 500 μM GS-Me.

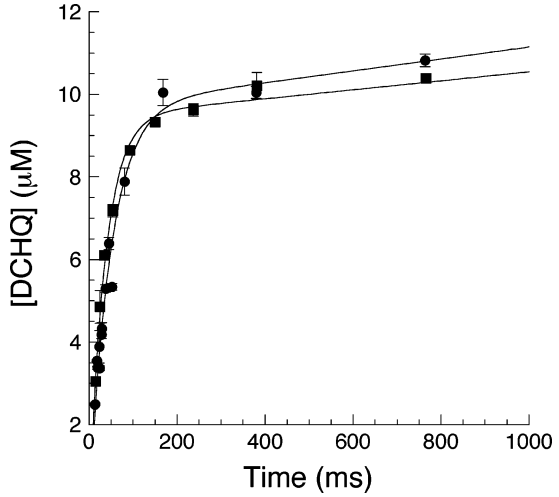


FIGURE 8: Formation of DCHQ from TriCHQ catalyzed by TCHQ dehalogenase (10 μM) in the presence of TriCHQ (200 μM) and glutathione (500 μM) at pH 7.0 (■) and 8.0 (●) and 20 °C. The data were fit to eq 10.

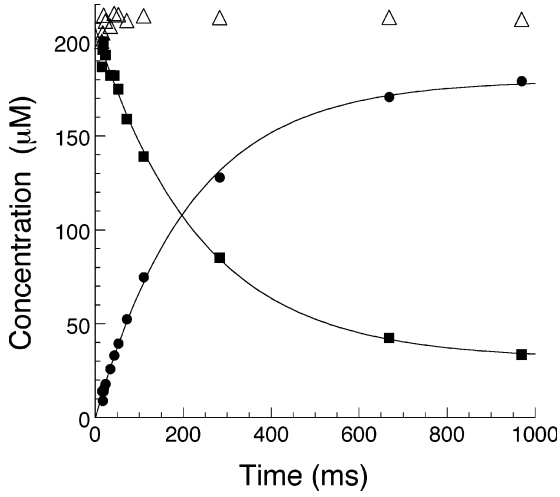
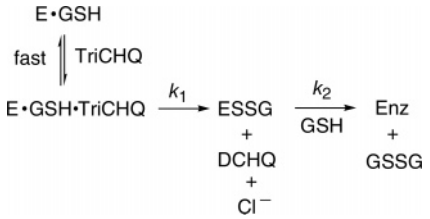


FIGURE 9: Formation of DCHQ (●) from TriCHQ (210 μM) (■) catalyzed by TCHQ dehalogenase (180 μM) at 5 °C in the presence of 1 mM glutathione. The triangles indicate the sum of the concentrations of TriCHQ and DCHQ. The data were fit with a single exponential.

Scheme 4



tathione−TriCHQ complex. The model assumes that both steps are irreversible on the basis of experimental evidence. Incubation of ESSG with DCHQ and chloride does not yield any TriCHQ (data not shown), indicating that the dehalogenation reaction is irreversible. We have previously shown that the thiol−disulfide exchange between ESSG and glutathione is also essentially irreversible in the presence of excess glutathione (*6*, *7*). The treatment of the second step as a second-order reaction is justified because the K_M for glutathione in the thiol−disulfide exchange reaction is very high (>20 mM) and the concentration of glutathione in the reaction mixture is only 500 μM. The rate of production of DCHQ according to Scheme 4 is described by eq 10 (*13*),

$$[\mathrm{DCHQ}]_t = [\mathrm{Enz}]_{tot}\, A(1 - e^{-k_{obs}t}) + [\mathrm{Enz}]_{tot}\, k_{cat}\, t + [\mathrm{DCHQ}]_0 \quad (10)$$

in which the first term describes the approach to the steady state and the second term the steady-state formation of DCHQ. The rate constants and amplitude (A) are defined by

$$k_{obs} = k_1 + k_2[\mathrm{GSH}] \quad (11)$$

$$A = \left(\frac{k_1}{k_1 + k_2[\mathrm{GSH}]}\right)^2 \quad (12)$$

$$k_{cat} = \frac{k_1 k_2[\mathrm{GSH}]}{k_1 + k_2[\mathrm{GSH}]} \quad (13)$$

The fit of the data to eq 10 at pH 7.0 gave $A[\mathrm{Enz}]_{tot} = 9.4 \pm 0.5$ μM, $k_{obs} = 28 \pm 5$ s^{-1}, $[\mathrm{Enz}]_{tot}k_{cat} = 1.1 \pm 0.2$ μM s^{-1}, and $k_{cat} = 0.10$ s^{-1} and at pH 8.0 gave $A[\mathrm{Enz}]_{tot} = 9.7 \pm 0.5$ μM, $k_{obs} = 23 \pm 5$ s^{-1}, $[\mathrm{Enz}]_{tot}k_{cat} = 1.5 \pm 0.6$ μM s^{-1}, and $k_{cat} = 0.15$ s^{-1}. Since the steady-state rate of DCHQ formation is much slower than the pre-steady-state rate, k_2[GSH] must be much smaller than k_1 and the k_2[GSH] term does not significantly contribute to the amplitude and rate constant for the burst phase. Therefore, k_{obs} is approximately equal to k_1, A is approximately equal to 1, and the amplitude of the burst ($A[\mathrm{Enz}]_{tot}$) is equal to the concentration of active TCHQ dehalogenase. Furthermore, k_{cat} is approximately equal to k_2[GSH].

TriCHQ^{-2} Is the Only Intermediate Observed during Turnover of C156S TCHQ Dehalogenase. A rapid quench-flow experiment was carried out to look for intermediates that accumulate and disappear during turnover. C156S TCHQ dehalogenase (360 μM) and glutathione (2 mM) were mixed with TriCHQ (420 μM) at 5 °C on the quench-flow instrument. Reactions were quenched by mixing with an equal volume of 1 N HCl and analyzed by HPLC. Figure 9 shows the loss of TriCHQ and the formation of DCHQ. The data were fit with a single exponential. TriCHQ was lost with a rate constant of 4.3 ± 1.5 s^{-1}. DCHQ was produced with an observed rate constant of 4.8 ± 0.5 s^{-1}. There is no lag in the formation of DCHQ that would indicate accumulation of an intermediate. Furthermore, the total amount of TriCHQ and DCHQ recovered at each time point was always 210 ± 10 μM. No GS-DCHQ (which would form from intermediate **IV** upon acid quench) could be detected by HPLC. Furthermore, no evidence for DCBQ (intermediate **III**) was observed during the reaction. (Under the quench conditions, DCBQ is converted to a mixture of GS-DCHQ and DCHQ by reaction with glutathione and ascorbate in the reaction solution.) Intermediate **II** would be expected to revert to TriCHQ upon acid quench, so these results do not rule out accumulation of intermediate **II**, but do rule out accumulation of intermediates **III** and **IV**.

The formation and decay of TriCHQ^{-2} and TriCHQ* were monitored at 346 and 385 nm, respectively, during conversion of TriCHQ to DCHQ during a single turnover in the stopped-flow instrument. (TriCHQ* was monitored at 385 nm to minimize interference from the signal due to TriCHQ^{-2}). C156S TCHQ dehalogenase (180 μM) in 200 mM potassium

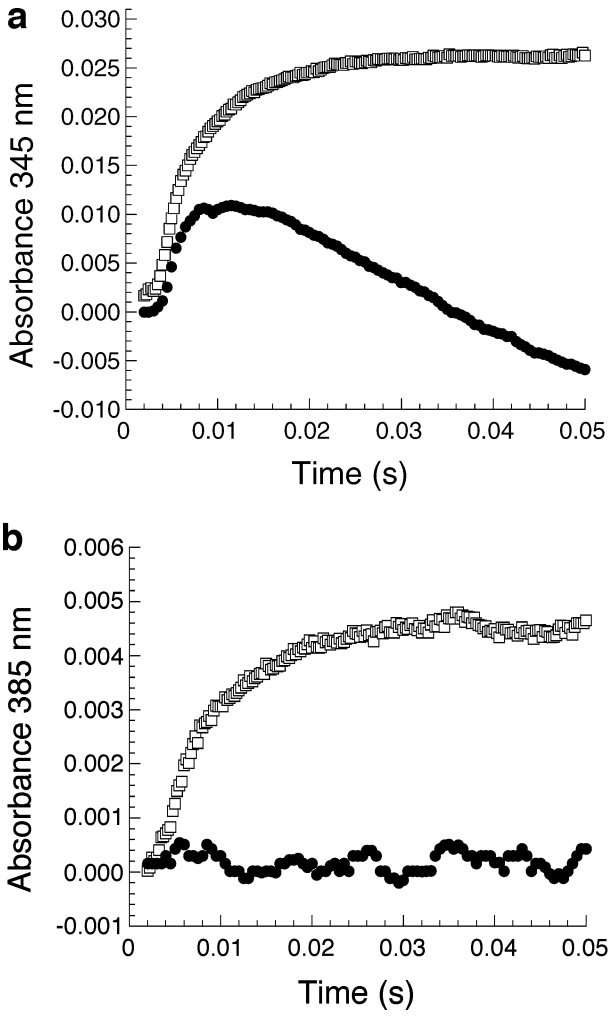


FIGURE 10: Observation of $TriCHQ^{-2}$ at 345 nm (a) and TriCHQ* at 385 nm (b) during binding of TriCHQ (□) or during dehalogenation of TriCHQ (●). Binding of TriCHQ was monitored at 345 and 385 nm after TCHQ dehalogenase (180 μM) in 200 mM potassium phosphate buffer, pH 8.0, containing GS-Me (1 mM) and ascorbate (0.005%) was mixed with an equal volume of TriCHQ (40 μM) in 200 mM potassium phosphate buffer, pH 8.0, containing GS-Me (1 mM) and ascorbate (0.005%). Dehalogenation of TriCHQ was monitored under the same conditions except that GS-Me was replaced by glutathione.

phosphate, pH 8.0, containing glutathione (1 mM) and ascorbate (0.005%) was mixed with an equal volume of TriCHQ (40 μM) in 200 mM potassium phosphate, pH 8.0, containing glutathione (1 mM) and ascorbate (0.005%). A comparable experiment was carried out using GS-Me in place of glutathione.

Observation at 345 nm (Figure 10a) shows that, following a lag phase, the concentration of $TriCHQ^{-2}$ increased rapidly in the presence of both glutathione and GS-Me. (The lag is likely due to binding events under these experimental conditions.) Under turnover conditions, the concentration of $TriCHQ^{-2}$ increased and then decreased as it was converted into DCHQ with a rate constant of 19 ± 4 s^{-1} (obtained by fitting the signal between 4 and 300 ms to a double exponential).

Figure 10b shows that the absorbance at 385 nm that we attribute to TriCHQ* appears when GS-Me is present, but not when glutathione is present. These data suggest that either TriCHQ* is an irrelevant side product that does not occur

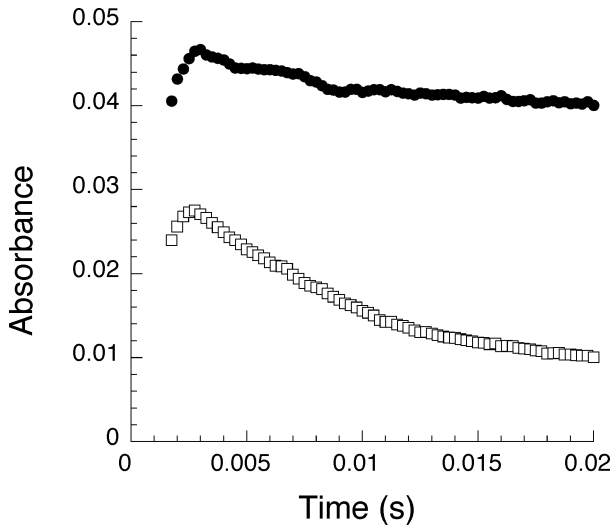


FIGURE 11: Detection of $TriCHQ^{-2}$ at 345 nm (●) and TriCHQ* at 385 nm (□) during turnover by K16A C156S TCHQ dehalogenase. K16A C156S TCHQ dehalogenase (80 μM) in 200 mM potassium phosphate buffer, pH 8.0, containing glutathione (5 mM) and ascorbate (0.005%) was mixed with an equal volume of TriCHQ (40 μM) in 200 mM potassium phosphate buffer, pH 8.0, containing glutathione (5 mM) and ascorbate (0.005%).

during turnover, or that does not accumulate during turnover because it is consumed immediately after it is formed. Given that TriCHQ* forms with a rate constant of 36 ± 10 s^{-1} in the presence of GS-Me and that the rate constant for formation of DCHQ reported above is 23 ± 5 s^{-1}, TriCHQ* is certainly a kinetically competent intermediate. Although TriCHQ* cannot be detected during turnover of TriCHQ by the C156S TCHQ dehalogenase, its formation and decay can be observed during turnover by the K16A C156S mutant enzyme under similar conditions (see Figure 11). Note that the increase in absorbance at 385 nm cannot be attributed to the long-wavelength absorbance of $TriCHQ^{-2}$. $TriCHQ^{-2}$ absorbs very weakly at 385 nm, but the magnitude of the decrease in the absorbance at 385 nm during the second phase is much greater than that at 345 nm. Thus, these signals clearly arise from two different species.

DISCUSSION

The pre-steady-state kinetic studies described here are consistent with the revised mechanism shown in Figure 12. This mechanism differs from the previously proposed mechanism in several ways: (1) Binding of $TriCHQ^{-}$ is proposed to occur with the negative charge at the top of the active site rather than at the bottom. (2) Deprotonation of $TriCHQ^{-}$ to $TriCHQ^{-2}$ is proposed to be the initial step in catalysis. (3) Attack of glutathione is proposed to most likely occur on TriCHQ*, rather than on DCBQ formed by elimination of HCl from TriCHQ*. The data supporting each feature of this mechanism will be discussed below.

Binding of Substrates Can Be Considered To Be Ordered under in Vivo Conditions. We have previously shown that binding of substrates to C13S TCHQ dehalogenase proceeds by a rapid equilibrium random sequential mechanism. Given the K_D for glutathione of 90 μM (at pH 7.0) and the in vivo concentration of glutathione (1.6 mM) (*6*), the enzyme should be saturated with glutathione in vivo. Furthermore, the enzyme−glutathione complex will form at a rate of 1200

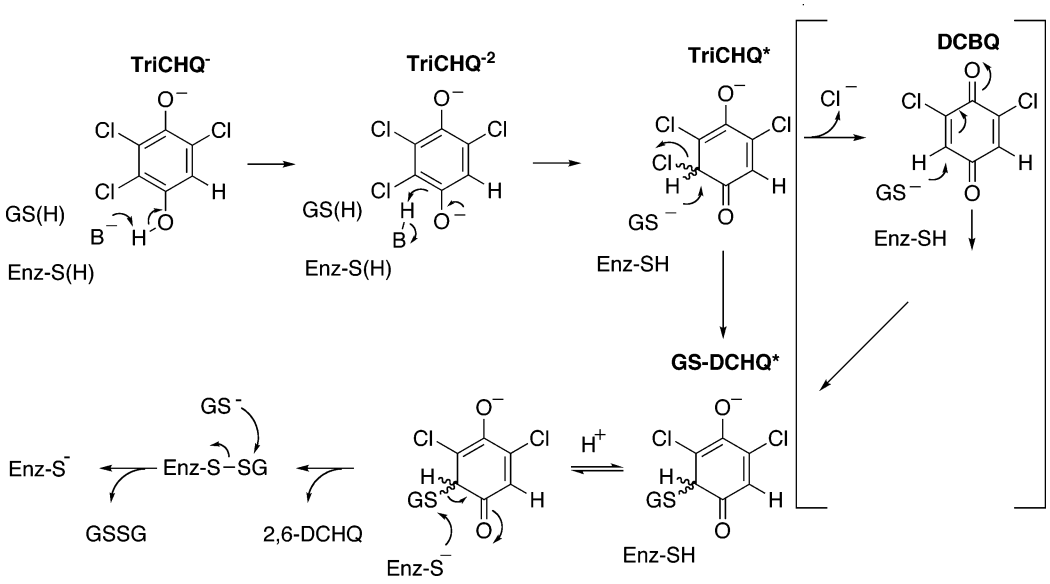


FIGURE 12: Revised mechanism for TCHQ dehalogenase. The protonation states of glutathione and Cys13 are not specified unless a particular protonation state is required for activity. We expect that one of the thiols will be deprotonated, but not both. An alternative and less likely pathway for formation of GS-DCHQ* is shown in brackets.

s^{-1}. A comparable calculation using the rate constant for binding of TriCHQ to the free enzyme ($1\ \mu M^{-1}\ s^{-1}$) and the estimated in vivo concentration ($<5\ \mu M$) (*14*) suggests that the enzyme−TriCHQ complex will form at a rate of $<5\ s^{-1}$. Thus, for practical purposes, the binding of substrates can be considered ordered, with binding of glutathione preceding binding of TriCHQ.

The presence of GS-Me (and by extension glutathione) influences the binding of TriCHQ. At pH 7.0, GS-Me appears to stabilize the conformation of the active site in a state that binds TriCHQ tightly. The presence of GS-Me also increases the levels of both $TriCHQ^{-2}$ and TriCHQ* at the active site.

Protonation State of Bound Glutathione. An increase in absorbance at 240 nm due to deprotonation of the thiol of glutathione is not observed when glutathione binds to the enzyme at pH 7.0. This is unusual, as ionization of the thiol of glutathione upon binding to the active site is characteristic of all classes of GSTs (*10*), including the zeta class human MAA isomerase (*15*). Formation of the thiolate enhances the ability of glutathione to attack electrophilic substrates. The lack of a change in absorbance at 240 nm could mean that the protonation state of glutathione does not change. Alternatively, deprotonation of the thiol of glutathione might occur concomitantly with protonation of the thiolate of Cys13, resulting in no net change in the absorbance at 240 nm. Cys13 is largely deprotonated in the free enzyme (Warner and Copley, unpublished results). The sulfur atoms of Cys13 and glutathione must be in close proximity, as they form a covalent bond in the second stage of the dehalogenation reaction. Therefore, it is unlikely that both can be deprotonated at the same time. During the catalytic cycle, each thiol acts as a nucleophile at different stages, so presumably the protonation states of the two thiols change during the reaction.

Binding and Deprotonation of TriCHQ⁻ at the Active Site. The predominant form of TriCHQ at pH 8.0 is $TriCHQ^-$. Upon binding to the active site, $TriCHQ^-$ is rapidly deprotonated to $TriCHQ^{-2}$. The identity of the base that removes the proton from the hydroxyl group of $TriCHQ^{-2}$ is unknown. Because $TriCHQ^{-2}$ forms rapidly at the active site of the free enzyme, the enzyme with GS-Me bound, ESSG, and the K16A enzyme, we conclude that neither Cys13, glutathione, or Lys16 removes the hydroxyl proton of $TriCHQ^-$. A multiple sequence alignment of TCHQ dehalogenase and maleylacetoacetate and maleylpyruvate isomerases provides little help, as only the regions near the active site Cys and the part of the N-terminal domain involved in glutathione binding are well-conserved.

$TriCHQ^-$ could bind in the active site with the deprotonated hydroxyl group either deep in the active site cleft or near the mouth of the cleft. We previously proposed that $TriCHQ^-$ binds with the deprotonated hydroxyl group deep in the active site cleft (see Figure 1), as that orientation would favor the ketonization reaction leading to intermediate **II**. However, it is now clear that a proton is removed from the hydroxyl group of $TriCHQ^-$ in the active site prior to dehalogenation. It is most likely that the protonated hydroxyl binds deep in the active site on the basis of the observation that $TriCHQ^{-2}$ does not form in the C13S enzyme, which suggests that the basic residue is in the vicinity of Cys13 in the bottom of the active site cleft. (Note that the conclusion from this observation that Cys13 is responsible for removal of the hydroxyl proton cannot be correct, as $TriCHQ^{-2}$ forms rapidly in the active site of ESSG, in which Cys13 is involved in a disulfide with glutathione and unable to act as a base.) Possibly formation of a hydrogen bond between the hydroxyl of Ser13 and the base alters its position or pK_a so that it is unable to remove the hydroxyl proton. However, the C13S enzyme is able to turn over TriCHQ to GS-DCHQ in the presence of glutathione, so binding of glutathione must somehow facilitate the deprotonation reaction.

Binding of $TriCHQ^-$ with the protonated hydroxyl deep in the pocket is consistent with the stereospecificity of the dehalogenation reaction, which produces 2,6-DCHQ from TriCHQ. The predominant form of $TriCHQ^-$ in solution would carry the negative charge on the oxygen between the two chlorine substituents. Binding of this form with the charge near the mouth of the active site would place the

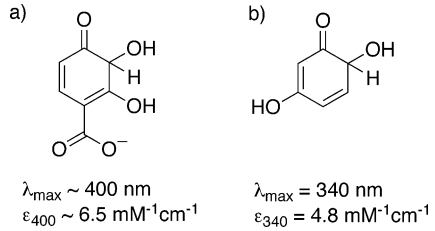


FIGURE 13: Spectroscopic characteristics of the cyclohexadieneone intermediates proposed for a) hydroxylation of 2,4-dihydroxybenzoate by *p*-hydroxybenzoate hydroxylase (*18*, *19*), and b) hydroxylation of phenol by phenol hydroxylase (*16*).

molecule in proximity to the catalytic machinery in a way that would produce 2,6-DCHQ if the chlorine substituent were oriented toward the thiol of glutathione (see Figure 12). (Binding in the orientation that places a hydrogen in a comparable position would not lead to a productive reaction.)

During turnover, TriCHQ^{-2} forms and then disappears. TriCHQ^{-2} could be a true intermediate in the reaction. Alternatively, it could be a side product in equilibrium with a true intermediate. However, its intermediacy is plausible from a chemical standpoint. The attack of an aromatic ring upon an electrophile is greatly facilitated by a hydroxyl group in the ortho or para position; the hydroxylation of phenols by flavin monooxygenases occurs by an analogous mechanism (*16*−*19*). In this case, formation of TriCHQ* may require a second strongly electron-donating substituent to counteract the effects of the three or four electron-withdrawing chlorine substituents. This would explain our previous finding that pentachlorophenol and tetrachlorophenol do not undergo reductive dehalogenation, although both bind at the active site (*4*).

*Conversion of TriCHQ^{-2} to TriCHQ**. TriCHQ^{-2} is converted to TriCHQ* in the enzyme−GS-Me complex. The assignment of TriCHQ* to the tautomer of TriCHQ^{-} is based upon the following reasoning. First, TriCHQ* reverts to TriCHQ upon quenching under various conditions. Thus, TriCHQ* is likely an unstable isomeric form of TriCHQ. Second, the long-wavelength absorbance of TriCHQ* is consistent with that expected for a cyclohexadienone intermediate. Similar intermediates form during the enzymatic hydroxylation of phenols by flavin monooxygenases (see Figure 13) (*16*, *18*, *19*). Third, TriCHQ* forms in TCHQ dehalogenase saturated with TriCHQ and GS-Me with a rate constant of 36 ± 10 s^{-1}. This rate constant is remarkably similar to that for formation of DCHQ (23 ± 5 s^{-1}) in rapid quench-flow experiments. Thus, the chemical and spectroscopic behavior of TriCHQ* is consistent with that expected for the tautomer of TriCHQ^{-}.

TriCHQ* is not observed during turnover by the C156S enzyme. This would be expected if the rate-limiting step is formation of TriCHQ* from TriCHQ^{-2} and its subsequent conversion to 2,6-DCHQ is rapid. The proposal that TriCHQ* is an intermediate in the reaction is supported by the observation of an intermediate that absorbs at 385 nm and could be either TriCHQ* or GS-DCHQ* during turnover of TriCHQ by the K16A C156S enzyme (see Figure 11). This enzyme is somewhat catalytically impaired because of the lack of a residue that likely interacts with Cys13. Apparently the resulting active site changes alter the rate of chemical steps such that one or both of these intermediates can be detected during turnover.

Further Reaction of TriCHQ Requires the Thiol/Thiolate of Glutathione.* The finding that the reaction stops at the stage of TriCHQ* when TriCHQ is incubated with the enzyme−GS-Me complex demonstrates that the thiol/thiolate group of glutathione is required for further reaction of TriCHQ*. Formation of GS-DCHQ* might proceed via either a direct S_N2 displacement of chloride by glutathione or initial 1,4-elimination of HCl to give 2,6-DCBQ, followed by attack of glutathione on the electrophilic benzoquinone. We previously favored the latter because of its similarity to other reactions catalyzed by GSTs; this mechanism was also consistent with the requirement for a second hydroxyl group for substrate conversion. However, the present data are more consistent with a direct attack of glutathione on TriCHQ*. The cyclohexadiene structure of TriCHQ* offers little steric hindrance to attack of glutathione upon the ring. We cannot exclude the possibility that the thiolate of glutathione affects the conformation or protonation state of a residue in the active site that is required for elimination of chloride, but a structural and mechanistic reason for this is not obvious. Thus, the most parsimonious interpretation of the data is that the thiolate of glutathione attacks TriCHQ* to form GS-DCHQ*.

Formation of DCHQ Is Fast Relative to the Subsequent Steps in the Reaction. The replacement of a chlorine substituent on an aromatic substrate with a hydrogen is an unusual chemical reaction. Remarkably, given the evidence that TCHQ dehalogenase is not well-evolved to serve as a reductive aromatic dehalogenase, the formation of DCHQ occurs with a rate constant of approximately 25 s^{-1} when the enzyme is saturated with both glutathione and TriCHQ. The effective catalytic performance of the enzyme in this early stage of the reaction is in stark contrast to its poor performance in the second stage of the reaction, in which binding of TriCHQ^{-} to the ESSG form of the enzyme prevents completion of the catalytic cycle.

SUMMARY

TCHQ dehalogenase appears to have been recently recruited to serve in the pathway for PCP degradation. It is constitutively expressed, in contrast to the other enzymes in the pathway, whose expression is induced in the presence of PCP (*25*). Constitutive regulation of an enzyme that normally serves another function is often an initial step in its recruitment to serve a new function (*20*−*24*). Furthermore, our previous work shows that the enzyme cannot control access to the active site during turnover and binding of the aromatic substrates TCHQ and TriCHQ to the ESSG form of the enzyme prevents the thiol−disulfide exchange reaction required to complete the catalytic cycle (*7*). Nevertheless, the enzyme is remarkably effective in certain aspects of catalysis. The steps leading to production of DCHQ are quite fast, producing DCHQ with a rate constant of 25 s^{-1} when the enzyme is saturated with both substrates. The thiol−disulfide exchange reaction is also fast in the absence of the aromatic substrates. We previously estimated that it is 10000-fold faster than a comparable reaction in solution (*6*). This dichotomy between impressive catalysis of some steps, but rather poor performance overall, offers a fascinating glimpse at the workings of an enzyme that may be in the process of evolving a new function, but has not yet evolved to the level of catalytic prowess of most extant enzymes.

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SUPPORTING INFORMATION AVAILABLE

Description of the determination of the K_D for GS-Me and TCHQ dehalogenase in the presence of saturating TriCHQ. This material is available free of charge via the Internet at http://pubs.asc.org.

REFERENCES

1. Xun, L., Topp, E., and Orser, C. S. (1992) Purification and characterization of a tetrachloro-*p*-hydroquinone reductive dehalogenase from a *Flavobacterium* sp., *J. Bacteriol. 174*, 8003−8007.
2. Anandarajah, K., Kiefer, P. M., and Copley, S. D. (2000) Recruitment of a double bond isomerase to serve as a reductive dehalogenase during biodegradation of pentachlorophenol, *Biochemistry 39*, 5303−5311.
3. McCarthy, D. L., Navarrete, S., Willett, W. S., Babbitt, P. C., and Copley, S. D. (1996) Exploration of the relationship between tetrachlorohydroquinone dehalogenase and the glutathione *S*-transferase superfamily, *Biochemistry 35*, 14634−14642.
4. Kiefer, P. M., Jr., and Copley, S. D. (2002) Characterization of the initial steps in the reductive dehalogenation catalyzed by tetrachlorohydroquinone dehalogenase, *Biochemistry 41*, 1315−1322.
5. McCarthy, D. L., Louie, D. F., and Copley, S. D. (1997) Identification of a covalent intermediate between glutathione and Cysteine13 during catalysis by tetrachlorohydroquinone dehalogenase, *J. Am. Chem. Soc. 119*, 11337−11338.
6. Warner, J. R., Lawson, S. L., and Copley, S. D. (2005) A mechanistic investigation of the thiol-disulfide exchange step in the reductive dehalogenation catalyzed by tetrachlorohydroquinone dehalogenase, *Biochemistry 44*, 10360−10368.
7. Warner, J. R., and Copley, S. D. (2007) Mechanism of the severe inhibition of tetrachlorohydroquinone dehalogenase by its aromatic substrates, *Biochemistry 46*, 4438−4447.
8. Kiefer, P. M., Jr., McCarthy, D. L., and Copley, S. D. (2002) The reaction catalyzed by tetrachlorohydroquinone dehalogenase does not involve nucleophilic aromatic substitution, *Biochemistry 41*, 1308−1314.
9. Gauthier, T. D., Shane, E. C., Guerin, W. F., Seitz, W. R., and Grant, C. L. (1986) Fluorescence quenching method for determining equilibrium constants for polycyclic aromatic hydrocarbons binding to humic materials, *Environ. Sci. Technol. 20*, 1162−1166.
10. Armstrong, R. N. (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases, *Chem. Res. Toxicol. 10*, 2−18.
11. Caccuri, A. M., Antonini, G., Nicotra, M., Battistoni, A., Lo Bello, M., Board, P. G., Parker, M. W., and Ricci, G. (1997) Catalytic mechanism and role of hydroxyl residues in the active site of theta class glutathione *S*-transferases: investigation of Ser-9 and Tyr-113 in a glutathione *S*-transferase from the Australian blowfly, *Lucilia cuprina*, *J. Biol. Chem. 272*, 29681−29686.
12. Johnson, K. A. (1986). Rapid kinetic analysis of mechanochemical adenosinetriphosphatases, in *Methods in Enzymology* (Vallee, R. B., Ed.) pp 677−705, Academic Press, New York.
13. Johnson, K. A. (1992). Transient-state kinetic analysis of enzyme reaction pathways, *The Enzymes* pp 1−61, Academic Press, New York.
14. McCarthy, D. L., Claude, A., and Copley, S. D. (1997) In vivo levels of chlorinated hydroquinones in a pentachlorophenol-degrading bacterium, *Appl. Environ. Microbiol. 63*, 1883−1888.
15. Ricci, G., Turella, P., De Maria, F., Antonini, G., Nardocci, L., Board, P. B., Parker, M. W., Carbonelli, M. G. F. G., and Caccuri, A. M. (2004) Binding and kinetic mechanisms of the zeta class glutathione transferase, *J. Biol. Chem. 279*, 33336−33342.
16. Maeda-Yorita, K., and Massey, V. (1993) On the reaction mechanism of phenol hydroxylase: new information obtained by correlation of fluorescence and absorbance stopped flow studies, *J. Biol. Chem. 268*, 4134−4144.
17. Powlowski, J., Ballou, D. P., and Massey, V. (1990) Studies of the oxidative half-reaction of anthranilate hydroxylase (deaminating) with native and modified substrates, *J. Biol. Chem. 265*, 4969−4975.
18. Schopfer, L. M., Wessiak, A., and Massey, V. (1991) Interpretation of the spectra observed during oxidation of *p*-hydroxybenzoate hydroxylase reconstituted with modified flavins, *J. Biol. Chem. 266*, 13080−13086.
19. Merényi, G., Lind, J., and Anderson, R. F. (1991) Spectral characteristics of 4-carboxy-5,6-dihydroxy-2,4-cyclohexadienone, a likely component of intermediate II in *p*-hydroxybenzoate hydroxylase, *J. Am. Chem. Soc. 113*, 9371−9372.
20. Doten, R. C., and Mortlock, R. P. (1984) Directed evolution of a second xylitol catabolic pathway in *Klebsiella pneuomiae*, *J. Bacteriol. 159*, 730−735.
21. Cocks, G. T., Aguilar, J., and Lin, E. C. C. (1974) Evolution of L-1,2-propanediol catabolism in *Escherichia coli* by recruitment of enzymes for L-fucose and L-lactate metabolism, *J. Bacteriol. 118*, 83−88.
22. Badia, J., Ibanez, E., Sabate, M., Baldoma, L., and Aguilar, J. (1998) A rare 920-kilobase chromosomal inversion mediated by IS1 transposition causes constitutive expression of the yiaK-S operon for carbohydrate utilization in *Escherichia coli*, *J. Biol. Chem. 273*, 8376−8381.
23. Clarke, P. H., and Drew, R. (1988) An experiment in enzyme evolution. Studies with *Pseudomonas aeruginosa* amidase, *Biosci. Rep. 8*, 103−120.
24. Schneider, K. H., Jakel, G., Hoffman, R., and Giffhorn, F. (1995) Enzyme evolution in *Rhodobacter sphaeroides*: selection of a mutant expressing a new galactitol dehydrogenase and biochemical characterization of the enzyme, *Microbiology 141*, 1865−1873.
25. Orser, C. S., Dutton, J., Lange, C., Jablonski, P., Xun, L., and Hargis, M. (1993) Characterization of a *Flavobacterium* glutathione-S-transferase gene involved in reductive dehalogenation. *J. Bacteriol. 175*, 2640−2644.

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