A Trade-Off between Catalytic Power and Substrate Inhibition in TCHQ Dehalogenase[†]

Joseph R. Warner, Linda S. Behlen, and Shelley D. Copley*

Department of Molecular, Cellular, and Developmental Biology and Cooperative Institute for Research in Environmental Sciences, University of Colorado at Boulder, Boulder, Colorado 80309

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ABSTRACT: Tetrachlorohydroquinone (TCHQ) dehalogenase is profoundly inhibited by its aromatic substrates, TCHQ and trichlorohydroquinone (TriCHQ). Surprisingly, mutations that change Ile12 to either Ser or Ala give an enzyme that shows no substrate inhibition. We have previously shown that TriCHQ is a noncompetitive inhibitor of the thiol−disulfide exchange reaction between glutathione and ESSG, a covalent adduct between Cys13 and glutathione formed during dehalogenation of the substrate. Substrate inhibition of the thiol−disulfide exchange reaction is less severe in the I12S and I12A mutant enzymes, primarily due to weaker binding of TriCHQ to ESSG. These mutations also result in a decrease in the rate of dehalogenation. Because the rate-limiting step in the I12S and I12A enzymes is dehalogenation, rather than the thiol−disulfide exchange reaction, the relatively modest inhibition of the thiol−disulfide exchange reaction does not affect the overall rate of turnover.

Pentachlorophenol (PCP)[1] was introduced into the environment in large quantities as a pesticide in the 1930s. Various strains of *Sphingobium chlorophenolicum* have been isolated from soil that was heavily contaminated with PCP (*1–3*). *S. chlorophenolicum* appears to have patched together a novel pathway for degradation of PCP (see Figure 1) (*4, 5*). Although the ability of *S. chlorophenolicum* to completely degrade a novel and toxic pesticide is remarkable, degradation is slow, and the cells cannot tolerate high levels of PCP (*6*).

Tetrachlorohydroquinone (TCHQ) dehalogenase catalyzes the third and fourth steps in the degradation of PCP, the reductive dehalogenation of TCHQ and trichlorohydroquinone (TriCHQ). The mechanism of TCHQ dehalogenase is shown in Figure 2 (*7*). The substrate binds primarily as $TriCHQ^-$ (the predominant form in solution at pH 7.0) and is rapidly deprotonated to $TriCHQ^{2-}$ at the active site. Subsequently, $TriCHQ^{2-}$ is converted to its tautomer (TriCHQ*), which is attacked by glutathione. Cys13 then attacks the glutathione conjugate, releasing the reduced product and forming a covalent bond between Cys13 and glutathione. Finally, the free enzyme is regenerated by a thiol−disulfide exchange reaction with a second molecule of glutathione.

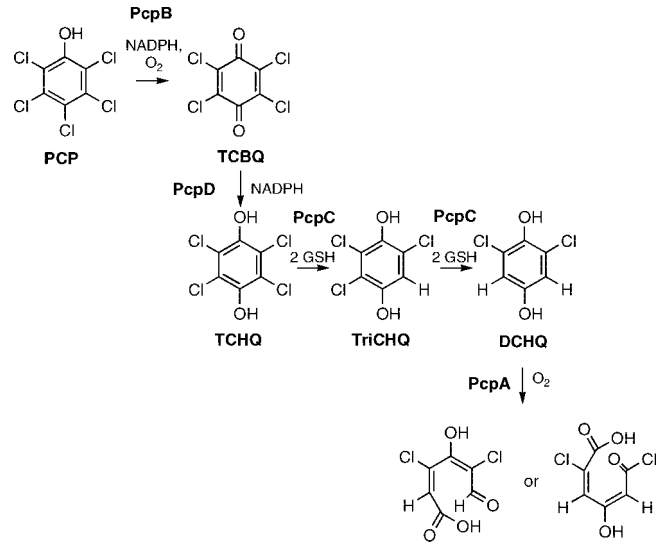

FIGURE 1: The pathway for degradation of PCP in *S. chlorophenolicum*. Abbreviations: PcpB, PCP hydroxylase; PcpD, TCBQ reductase; PcpC, TCHQ dehalogenase; PcpA, DCHQ dioxygenase; GSH, glutathione.

TCHQ dehalogenase exhibits a peculiar characteristic; it is profoundly inhibited by its aromatic substrates (see Figure 3) (*8*). The reaction catalyzed by TCHQ dehalogenase is formally a ping-pong reaction (see Scheme 1). Ping-pong reactions must be carefully orchestrated so that substrates bind to the correct form of the enzyme so as to avoid formation of nonproductive complexes. TCHQ dehalogenase does not do this well. When the covalent adduct between Cys13 and glutathione (ESSG) is formed, the next event should be attack of a second molecule of glutathione upon the disulfide bond. However, if TCHQ or TriCHQ binds to ESSG, the thiol−disulfide exchange reaction is prevented (*8*). We have previously shown that the inhibition of the

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* To whom correspondence should be addressed. Phone: (303) 492-6328. Fax: (303) 492-1149. E-mail: shelley@cires.colorado.edu.

[1] Abbreviations: DCHQ, 2,6-dichlorohydroquinone; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESSG, TCHQ dehalogenase Cys13-glutathionyl disulfide; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; GS-DCHQ, 3,5-dichloro-2-*S*-glutathionylhydroquinone; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; MAA, maleylacetoacetate; MP, maleylpyruvate; PCP, pentachlorophenol; TCHQ, tetrachlorohydroquinone; TriCHQ and T, trichlorohydroquinone.

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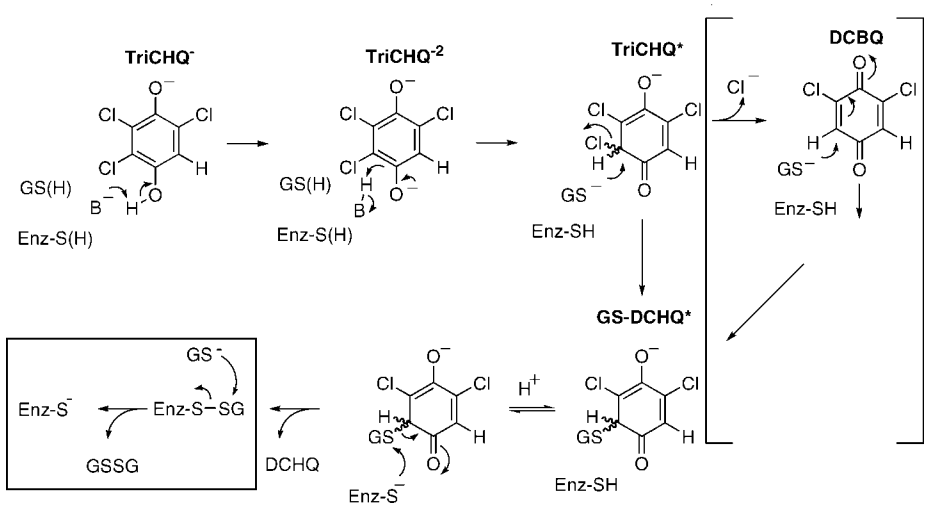


FIGURE 2: The mechanism of TCHQ dehalogenase. The thiol−disulfide exchange reaction that is inhibited by TCHQ or TriCHQ is enclosed in a box. The brackets indicate an alternative, although less likely, pathway for generating GS-DCHQ*. "(H)" indicates that the protonation state of the enzyme or glutathione is unknown. Since Cys13 and the thiol of glutathione are in close proximity, we assume that only one can be deprotonated at a time.

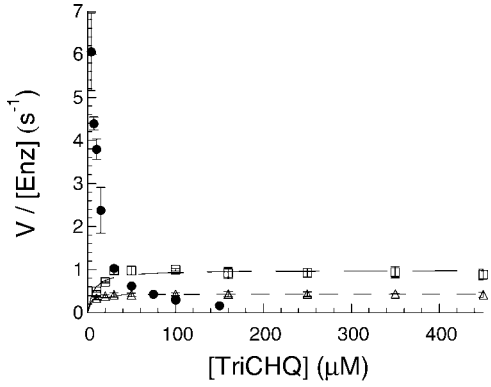
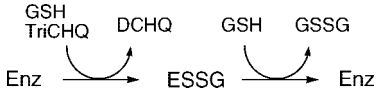
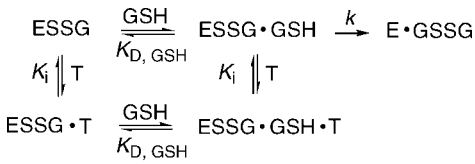


FIGURE 3: Effect of TriCHQ on the steady-state rate of the dehalogenation reaction catalyzed by C156S TCHQ dehalogenase and the I12S C156S and I12A C156S mutants. Initial velocities for conversion of TriCHQ were monitored by following the change in absorbance at 320 nm. Velocities are the average of values obtained from at least three measurements. Assay mixtures contained 200 mM potassium phosphate, pH 7.0, 3 mM glutathione, 0.1% ascorbate, and (●) C156S TCHQ dehalogenase, (□) I12A C156S TCHQ dehalogenase, or (△) I12S C156S TCHQ dehalogenase. The curves are a best fit of the data to the Henri−Michaelis–Menten equation.

Scheme 1



Scheme 2



T: TriCHQ

thiol−disulfide exchange reaction by TriCHQ is pure noncompetitive (see Scheme 2). Thus, TriCHQ does not compete with glutathione for access to the active site of ESSG, but when it is bound, the thiol−disulfide exchange reaction cannot occur.

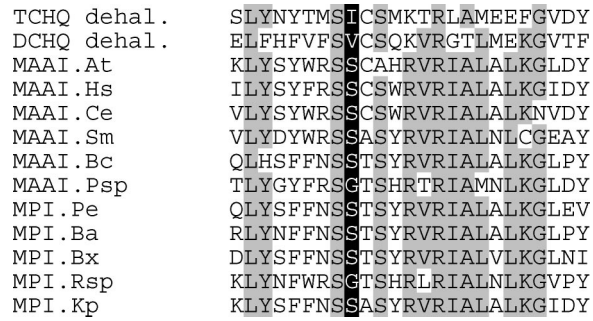


FIGURE 4: Multiple sequence alignment of the region of the active site contributed by the N-terminal domain in TCHQ dehalogenase (gi29429205), dichlorohydroquinone (DCHQ) dehalogenase from *Sphingomonas paucimobilis* (gi24935289), and representative maleylacetoacetate and maleylpyruvate isomerases. Highly conserved residues are shaded in gray, and the position corresponding to Ile12 in TCHQ dehalogenase is highlighted in black. Abbreviations: MAAI, maleylacetoacetate isomerase; MPI, maleylpyruvate isomerase; MAAI.At, *Arabidopsis thaliana* (gi15226952); MAAI.Hs, *Homo sapiens* (gi15988159); MAAI.Ce, *Caenorhabditis elegans* (gi17551302); MAAI.Sm, *Sinorhizobium meliloti* (gi11133647); MAAI.Bc, *Burkholderia cenocepacia* MC0-3 (gi118711904); MAAI.Psp, *Psychrobacter* sp. PRwf-1 (gi148652544); MPI.Pe, *Pseudomonas entomophila* L48 (gi104781695); MPI.Ba, *Bordetella avium* 197N (gi115422864); MPI.Bx, *Burkholderia xenovorans* LB400 (gi91783170); MPI.Rsp, *Ralstonia* sp. U2 (gi4220435); MPI.Kp, *Klebsiella pneumoniae* (gi58041828).

TCHQ dehalogenase is not closely related to enzymes of known function. The closest homologues are maleylacetoacetate (MAA) and maleylpyruvate (MP) isomerases, enzymes that catalyze glutathione-dependent isomerization of double bonds during degradation of tyrosine and benzoate, respectively (*9*). These enzymes have no more than 26% pairwise identity to TCHQ dehalogenase, but the region of the active site contributed by the N-terminal domain is quite similar (see Figure 4). Further, TCHQ dehalogenase has very low isomerase activity with MAA and MP (Behlen and Copley, unpublished results). Some positions in the active site are highly conserved in the double bond isomerases but different in TCHQ dehalogenase, suggesting that they might be involved in skewing the activity of an ancestral active site toward the dehalogenation reaction. In the course of investigating the roles of these residues, we made the unexpected

discovery that replacement of Ile12 with Ser or Ala completely alleviates the substrate inhibition (see Figure 3). Here we describe an examination of the mechanistic basis for the lack of substrate inhibition in the I12S C156S and I12A C156S mutant enzymes. The ESSG forms of these mutant enzymes have a diminished affinity for TriCHQ, resulting in less severe inhibition of the thiol−disulfide exchange reaction. However, the mutant enzymes are less efficient at low levels of TriCHQ, revealing a trade-off between catalytic power and substrate inhibition that depends upon the nature of the amino acid at position 12.

EXPERIMENTAL PROCEDURES

Reagents. All reagents were purchased from common commercial sources. Glutathione ([2-^{3}H]glycine) (50 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences.

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. C156S TCHQ dehalogenase was expressed and purified using an *N*-linked glutathione−Sepharose affinity matrix as previously described (*8, 10*). 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. Prior to experiments the enzyme was treated for 30 min with 5 mM DTT and exchanged into various buffers by dialysis or size exclusion chromatography using a Sephadex G25 column (Amersham Biosciences).

The genes encoding I12S C156S and I12A C156S TCHQ dehalogenase were generated by mutagenesis of the gene for C156S TCHQ dehalogenase in pET-21b (*10*) (Novagen) using the QuikChange II mutagenesis kit (Stratagene). The I12S C156S mutant was expressed and purified using an *N*-linked glutathione−Sepharose affinity column as previously described (*8*). The I12A C156S mutant protein was expressed as previously described for the C13S mutant enzyme (*8*). 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 (*8*), 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 I12A C156S TCHQ dehalogenase were combined and concentrated. Samples of protein were stored at 4 °C for no longer than 1 week.

The ESSG forms of C156S, I12S C156S, and I12A C156S TCHQ dehalogenase, in which glutathione is covalently linked to Cys13, were prepared as previously described using either glutathione or ([2-^{3}H]glycine)glutathione (1.4 Ci/mmol) (*8*).

Preparation of TriCHQ. TriCHQ was prepared from trichlorobenzoquinone as previously described (*8*). 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.

Steady-State Velocities for C156S, I12S C156S, and I12A C156S TCHQ Dehalogenase. The conversion of TriCHQ to DCHQ by TCHQ dehalogenase was followed using an HP 8452A diode array spectrophotometer as previously described (*8*). C156S TCHQ dehalogenase (0.1 μM), I12S C156S TCHQ dehalogenase (0.3 μM), or I12A C156S TCHQ dehalogenase (0.3 μM) was added to a solution of 200 mM potassium phosphate, pH 7.0, containing ascorbate (0.1%), glutathione (3 mM), and TriCHQ (5−450 μM). Loss of TriCHQ was measured by following the absorbance at 320 nm as a function of time ($\Delta\epsilon_{320} = -1.8$ mM^{-1} cm^{-1}). The data were fit by linear regression during the first 5–10% of the reaction. All experiments were performed at 20 °C. Each reported initial velocity is the average obtained from at least three experiments.

Measurement of the Rate of the Thiol−Disulfide Exchange Reaction. The rate of the thiol−disulfide exchange reaction of the ^{3}H-ESSG forms of I12S C156S and I12A C156S TCHQ dehalogenase with glutathione (1 mM) in the presence of TriCHQ (0−400 μM) was measured as previously described (*8*).

Equilibrium Binding Experiments. Binding of TriCHQ to the ESSG forms of C156S, I12S C156S, and I12A C156S was monitored by observation of the change in fluorescence (*7*) (excitation at 290 nm using a 2 nm band-pass filter; emission measured at 330 nm using an 8 nm band-pass filter) upon addition of TriCHQ to ESSG (1 or 2 μM) in 200 mM potassium phosphate, pH 7.0, containing 0.005% ascorbate. The data were corrected for dilution, which was less than 7%. The small inner-filter effect caused by TriCHQ was corrected using the method of Gauthier et al. (*11*). The magnitude of the fluorescence signal was plotted versus the concentration of TriCHQ ($[T]_{tot}$), and the data were fit to eq 1 to obtain the total change in fluorescence (ΔF), initial fluorescence (F_0), and the dissociation constant ($K_{D,TriCHQ}$). In the figure shown, the fluorescence intensities were normalized by dividing the observed fluorescence by F_0. The reported K_D's are the averages of values obtained from four individual titrations.

$$F = F_0 + \Delta F[T]_{tot}/([T]_{tot} + K_{D,TriCHQ}) \quad (1)$$

UV/Vis Spectra of TriCHQ in Solution and Bound to TCHQ Dehalogenase. The spectrum of TriCHQ (150 μM) in 200 mM potassium phosphate, pH 7.0, containing 0.005%

ascorbate was measured in a 1 cm cuvette. The spectrum of TriCHQ was also measured under the same conditions in the presence of the ESSG forms of the C156S, I12S C156S, and I12A C156S mutants (150 μM). The absorbance due to ascorbate and enzyme, when present, was subtracted from that of TriCHQ. The concentration of bound TriCHQ (including $TriCHQ^-$, $TriCHQ^{2-}$, and TriCHQ*) was obtained from eq 2 using the measured values of $K_{D,TriCHQ}$ and the total concentrations of enzyme and TriCHQ (150 μM). Subtracting the concentration of bound TriCHQ from the total concentration of TriCHQ yielded the concentration of free TriCHQ. Spectra of bound species were obtained by subtracting the contribution of free TriCHQ from each spectrum. Finally, the spectra were normalized by dividing the signal by the total concentration of bound TriCHQ.

$$[ESSG\cdot T] = \frac{(K_D + [ESSG]_{tot} + [T]_{tot}) - \sqrt{(K_D + [ESSG]_{tot} + [T]_{tot})^2 - 4[ESSG]_{tot}[T]_{tot}}}{2} \quad (2)$$

Rapid Quench-Flow Experiment. I12S C156S TCHQ dehalogenase (20 μM) in 200 mM Hepes, pH 7.0, containing 150 mM sodium chloride, 2 mM glutathione, and 0.1% ascorbate, was mixed with an equal volume of TriCHQ (400 μM) in the same buffer lacking glutathione at 20 °C in an Applied Photophysics 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 min, DCHQ at 14.0 min, and TriCHQ at 16 min. The concentration of DCHQ was plotted versus time. The data were fit to a line.

RESULTS

The Severe Substrate Inhibition of TCHQ Dehalogenase Is Alleviated in the I12S C156S and I12A C156S Mutants. Figure 3 shows a plot of steady-state velocity vs substrate concentration for the C156S enzyme (which can be considered to be equivalent to the wild-type enzyme) and the I12S C156S and I12A C156S mutant enzymes. As previously described (*8*), the C156S enzyme is severely inhibited by TriCHQ; a phase in which the velocity increases as a function of substrate concentration is not seen even at substrate concentrations as low as 5 μM. In contrast, the I12S C156S and I12A C156S enzymes display standard Michaelis–Menten behavior. For the I12S C156S enzyme, $k_{cat} = 0.4 \pm 0.1$ s^{-1} and $K_{M,TriCHQ} = 3$ μM. For the I12A C156S enzyme, $k_{cat} = 1.0 \pm 0.2$ s^{-1} and $K_{M,TriCHQ} = 6$ μM. (Values for $K_{M,TriCHQ}$ are approximate due to limitations of the assay that prevent measurement of initial velocities at concentrations of TriCHQ below 5 μM.) The C156S enzyme is more effective than the I12S C156S and I12A C156S enzymes at low substrate concentrations. However, turnover of the I12S C156S and I12A C156S enzymes is faster at high substrate concentrations at which the activity of the C156S enzyme is dramatically reduced.

Inhibition of the Thiol−Disulfide Exchange Reaction Is Diminished in the I12S C156S and I12A C156S Enzymes.

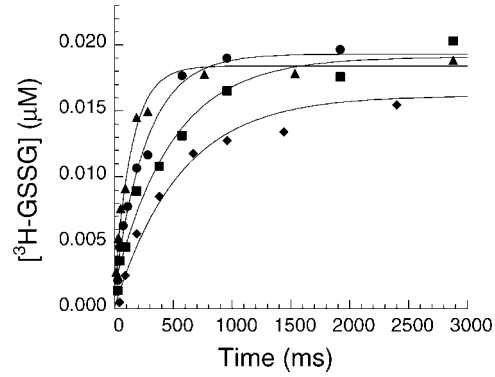


FIGURE 5: Formation of ^{3}H-GSSG in reactions of the ^{3}H-ESSG form of I12A C156S TCHQ dehalogenase and glutathione (1 mM) in the presence of variable concentrations of TriCHQ: 40 μM (▲), 100 μM (●), 160 μM (■), and 400 μM (◆). The curves shown are fits of the data to eq 2. (Additional data points at longer reaction times were used in the curve fits but are not shown here in order to show detail at shorter reaction times.)

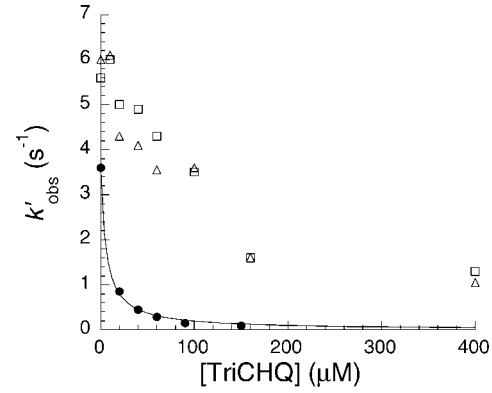
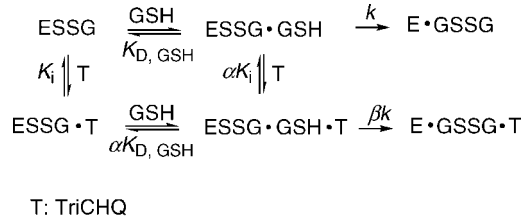


FIGURE 6: Effect of TriCHQ on the rate of the thiol−disulfide exchange reaction. Reaction mixtures contained 200 mM potassium phosphate, pH 7.0, 0.1% ascorbate, 1 mM glutathione, 0−400 μM TriCHQ, and 0.02 μM (●) C156S ^{3}H-ESSG, (△) I12S C156S ^{3}H-ESSG, or (□) I12A C156S ^{3}H-ESSG. Rate constants are the average of values obtained from two to four experiments. Standard errors for experiments carried out three or more times were <20% of the value plotted. The data for the C156S enzyme were obtained in a previous study and fit to eq 5; from this fit, $K_i = 6 \pm 3$ μM.

We have previously shown that the substrate inhibition of the C156S enzyme is due to noncompetitive inhibition of the thiol−disulfide exchange reaction (*8*). We measured the rate of the thiol−disulfide exchange reaction of glutathione with the ^{3}H-ESSG forms of the I12S C156S and I12A C156S enzymes. Representative traces for the I12A C156S enzyme are shown in Figure 5. Pseudo-first-order rate constants were obtained from the fit of the concentration of the product, ^{3}H-GSSG, versus time to eq 3. The rate constants for both the I12S C156S and I12A C156S enzymes, as well as the previously reported data for the C156S enzyme, are shown in Figure 6.[2] For the general case of mixed-type inhibition shown in Scheme 3, the velocity of the reaction is defined by eq 4, in which k'_{obs} is given by eq 5 (*12*). [α is the factor by which the binding constant for the substrate (glutathione) is affected by the presence of the inhibitor (TriCHQ), and vice versa, and β is the factor by which the rate of the

[2] The data for the I12S C156S and I12A C156S enzymes at low concentrations of TriCHQ are somewhat less reliable than those for the C156S enzyme because there is more variability in the measured values when the rate is high.

Scheme 3



thiol−disulfide exchange reaction is reduced in the presence of TriCHQ.] For the C156S enzyme, we have previously shown that the inhibition is pure noncompetitive, and therefore $\alpha = 1$ and $\beta = 0$. In such a case, k'_{obs} is defined by eq 6 (*8*). [Since the concentration of glutathione is in excess, it can be treated as effectively constant. Hence, k_{eff} can be defined as k[GSH]/($K_{D,GSH}$ + [GSH]).] The data for the C156S enzyme were fit to this equation, giving a value of K_i of 6 ± 3 μM. The data for the I12S C156S and I12A C156S enzymes were not fit to an equation. As we cannot identify the mode of inhibition from these data, there is no justification for using eq 6, and there are too many undefined parameters in eq 5 to allow a unique and reliable fit.

$$[GSSG] = [GSSG]_i + [ESSG]_0(1 - e^{-k'_{obs}t}) \quad (3)$$

$$v = k'_{obs}[ESSG]_{tot} \quad (4)$$

$$k'_{obs} = \frac{k[GSH]}{\alpha K_{D,GSH}\left(\frac{[T] + K_i}{\beta[T] + \alpha K_i}\right) + [GSH]\left(\frac{[T] + \alpha K_i}{\beta[T] + \alpha K_i}\right)} \quad (5)$$

$$k'_{obs} = \frac{k[GSH]}{(K_{D,GSH} + [GSH])\left(1 + \frac{[T]}{K_i}\right)} = \frac{k_{eff}}{\left(1 + \frac{[T]}{K_i}\right)} \quad (6)$$

The thiol−disulfide exchange reaction in the I12S C156S and I12A C156 enzymes is inhibited by TriCHQ, but to a lesser extent than that in the C156S enzyme. Notably, the rate constants for the thiol−disulfide exchange reactions of the I12S C156S and I12A C156S enzymes were approximately 2-fold greater than that for the C156S enzyme in the absence of TriCHQ (see Figure 6).

$K_{D,TriCHQ}$ Is Increased for the ESSG Forms of the I12S C156S and I12A C156S Enzymes. Binding of TriCHQ to the ESSG forms of the C156S, I12S C156S, and I12A C156S enzymes was assessed by following the change in intrinsic protein fluorescence upon addition of TriCHQ (see Figure 7). The $K_{D,TriCHQ}$ is an apparent dissociation constant because binding of TriCHQ, which is present primarily as the monoanionic form at pH 7.0, is followed by deprotonation and tautomerization (see Scheme 4 and further discussion below). The apparent dissociation constant is thus equal to $1/K_1K_2K_3$. The $K_{D,TriCHQ}$ for the ESSG form of the C156S enzyme is 13 ± 3 μM. Binding of TriCHQ to the I12S C156S and I12A C156S enzymes is weaker; $K_{D,TriCHQ}$ is 45 ± 10 μM for the I12S C156S enzyme and 65 ± 15 μM for the I12A C156S enzyme.

Deprotonation and Tautomerization of TriCHQ Occurs at the Active Sites of the I12S C156S and I12A C156S ESSG. We have previously shown that $TriCHQ^{2-}$ and TriCHQ* (see Scheme 4) form when $TriCHQ^-$ binds to the active site of the ESSG form of the C156S enzyme (*8*). $TriCHQ^{2-}$ absorbs at 345 nm and TriCHQ* at 360 nm. Because $TriCHQ^{2-}$ and/or TriCHQ* may contribute to the inhibition of the thiol−disulfide exchange reaction, we investigated the formation of these species in the ESSG forms of the I12S C156S and I12A C156S enzymes. Figure 8a compares the absorbance spectrum of TriCHQ in solution with spectra of TriCHQ in the presence of the ESSG forms of the C156S, I12S C156S and I12A C156S enzymes. Since the $K_{D,TriCHQ}$ is higher for the I12S C156S and I12A C156S enzymes, less TriCHQ is bound under these conditions. Figure 8b shows normalized spectra of TriCHQ bound to the active sites of the ESSG forms of the three enzymes. These spectra indicate that both $TriCHQ^{2-}$ and TriCHQ* form at the active sites of the ESSG forms of all three enzymes, although at somewhat lower levels for the I12A C156S enzyme.

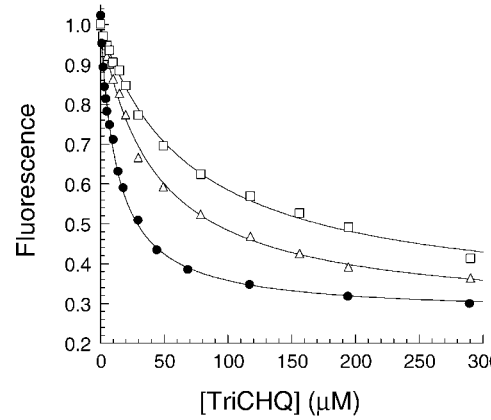
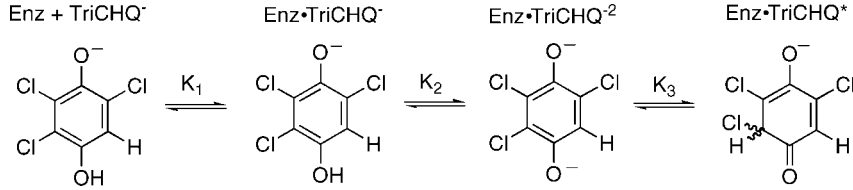


FIGURE 7: Fluorescence change due to binding of TriCHQ to (●) C156S ESSG, (□) I12A C156S ESSG, or (△) I12S C156S ESSG. The decrease in fluorescence was monitored as TriCHQ was added in small increments (≤0.4% volume) to 1 or 2 μM enzyme in 200 mM potassium phosphate, pH 7.0, containing 0.005% ascorbate. The data were fit to eq 1. The fluorescence intensity was normalized by dividing the fluorescence by the initial fluorescence.

The Thiol−Disulfide Exchange Reaction Is Not Rate-Limiting in the I12S C156S Enzyme. The thiol−disulfide exchange reaction catalyzed by the C156S enzyme is fast in the absence of TriCHQ, even though the enzyme binds the glutathione very weakly and does not alter its pK_a to potentiate its nucleophilicity (*10*). However, in the presence of TriCHQ, the thiol−disulfide exchange reaction becomes rate-limiting (*8*). Consequently, the C156S enzyme exhibits a burst in the formation of DCHQ. The rate constant in the pre-steady-state phase is 23 ± 5 s^{-1} at pH 7.0 when the C156S enzyme is saturated with glutathione at 20 °C (*7*).

We investigated the rate of formation of DCHQ in I12S C156S TCHQ dehalogenase by mixing equal volumes of enzyme (20 μM) and glutathione (2 mM) with TriCHQ (400 μM) at 20 °C in a rapid quench-flow instrument. The reaction was quenched by mixing with an equal volume of 1 N HCl after reaction times ranging from 0.02 to 2 s, and the amount of DCHQ was detected by HPLC. A burst in formation of DCHQ was not observed for the I12S C156S enzyme (see Figure 9). DCHQ was formed at a rate of 3 μM/s. Dividing the rate of DCHQ formation by the enzyme concentration gives a rate constant of 0.3 s^{-1}. This value agrees well with the k_{cat} of 0.4 ± 0.1 s^{-1} obtained from the continuous UV–vis assay (see Figure 3), suggesting that a step involved in dehalogenation, rather than thiol−disulfide exchange or

Scheme 4



dissociation of GSSG, is rate-limiting for the I12S C156S enzyme under these conditions.

DISCUSSION

The inhibition of TCHQ dehalogenase by its substrate is striking. Although many enzymes are inhibited at high substrate levels, it is unprecedented for an enzyme to be so severely inhibited in the physiological range of substrate concentration. The substrate inhibition is due to interference by TriCHQ in the thiol−disulfide exchange reaction required to regenerate the free form of the enzyme after dehalogenation of the substrate is accomplished. $TriCHQ^{2-}$ and TriCHQ*, which are intermediates in the normal catalytic cycle (*7*), also form at the active site of ESSG (*8*). These intermediates form at the active sites of the ESSG forms of the I12S C156S and I12A C156S enzymes, as well (see Figure 8), although the equilibrium concentrations of the various species are somewhat different. Thus, inhibition of the thiol−disulfide exchange reaction in all cases occurs in the presence of an equilibrating mixture of $TriCHQ^{-}$, $TriCHQ^{2-}$, and TriCHQ*. The observation that the inhibition is noncompetitive for the C156S enzyme rules out the possibility that the aromatic substrate simply competes with glutathione for access to the active site. The substrate inhibition may be due to the negative charges on any or all of the three forms of the substrate. The thiol−disulfide exchange reaction requires deprotonation of the incoming glutathione, as well as development of a negative charge upon the leaving group as the thiolate of glutathione attacks the disulfide bond of ESSG. A negative charge in the close vicinity would interfere with both of these processes.

Remarkably, a change from Ile to Ser at position 12 in the active site alleviates the severe inhibition of the dehalogenation reaction by the aromatic substrate (see Figure 3). In order to determine whether this effect is due to the smaller size of Ser relative to Ile, or to the introduction of a hydroxyl group that can interact with substrates or other residues in the active site via hydrogen-bonding interactions, we also generated the I12A C156S mutant enzyme. The I12A C156S mutant enzyme behaved similarly; no inhibition was seen. This result suggests that the alleviation of the substrate inhibition is due to the replacement of a bulky hydrophobic residue at position 12 with a small residue.

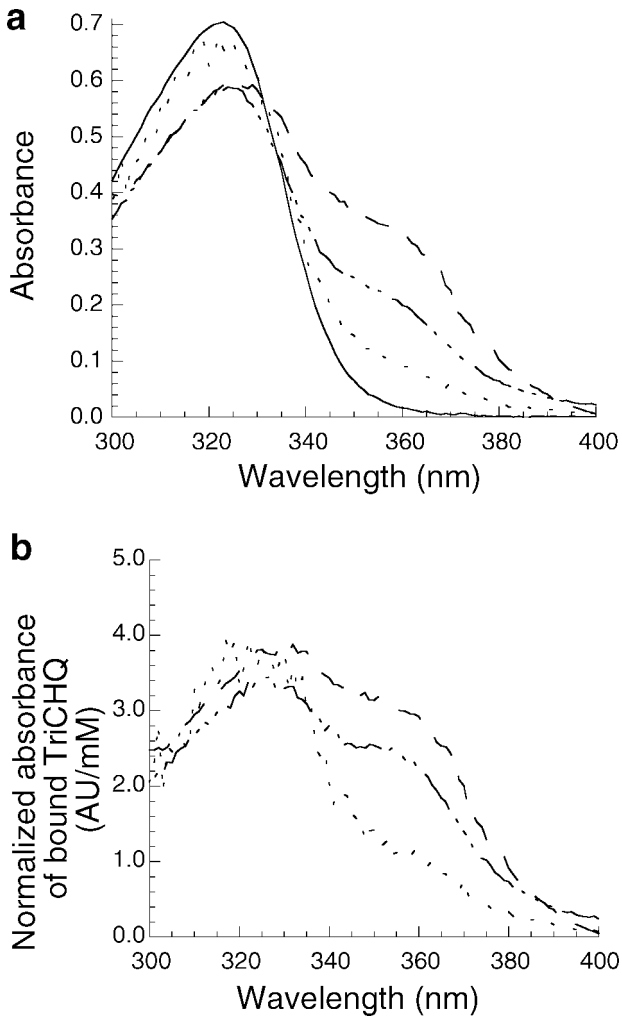


FIGURE 8: (a) UV/vis spectra of 150 μM TriCHQ in 200 mM potassium phosphate, pH 7, containing 0.005% ascorbate in the absence (—) and presence of 150 μM C156S ESSG (— —), 150 μM I12S C156S ESSG (- - - —), or 150 μM I12A C156S ESSG (- - -). The absorbance due to ascorbate and enzyme (when present) was subtracted from the spectrum. (b) UV/vis spectra of TriCHQ bound to the active site of C156S ESSG (— —), I12S C156S ESSG (- - - —), or I12A C156S ESSG (- - -). These spectra were obtained by subtracting the contribution of free TriCHQ from the spectra in panel a and then normalizing by dividing each spectrum by the concentration of bound TriCHQ.

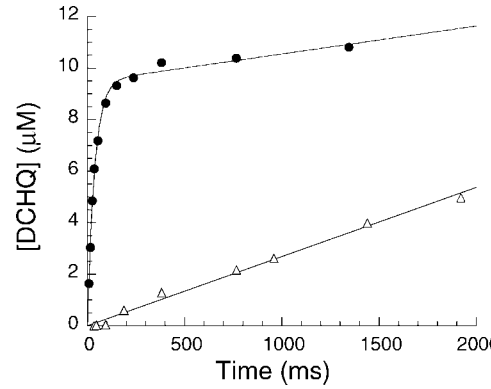


FIGURE 9: Formation of DCHQ from TriCHQ catalyzed by (△) I12S C156S TCHQ dehalogenase (10 μM) in the presence of TriCHQ (200 μM) and glutathione (1 mM) at pH 7.0 and 20 °C. Comparable data for formation of DCHQ from TriCHQ catalyzed by C156S TCHQ dehalogenase under similar conditions (●) are reproduced from Warner and Copley (*7*). The data were fit with an equation describing a single exponential followed by a line and show a burst in formation of DCHQ equal to the concentration of enzyme in the assay.

Since the I12S C156S and I12A C156S mutants showed no inhibition during steady-state turnover, we hypothesized that inhibition of the thiol−disulfide exchange reaction by the aromatic substrate might be reduced or relieved. Indeed, the rate of the thiol−disulfide exchange reaction is only modestly affected by TriCHQ in the I12S C156S and I12A C156S mutants (see Figure 6). In the C156S enzyme, the rate of the thiol−disulfide exchange reaction approaches 0 s^{-1} at 400 μM TriCHQ (*8*). In contrast, at 400 μM TriCHQ, the rate of the thiol−disulfide exchange reaction is decreased by only 5-fold in the I12S C156S and I12A C156S mutants. Under these conditions, the thiol−disulfide exchange reaction is not rate-limiting for the I12S C156S enzyme, as shown by the lack of a burst in formation of DCHQ (see Figure 9). Therefore, even though the rate of the thiol−disulfide exchange reaction is somewhat diminished when TriCHQ is bound, the effect is not seen in the overall rate of turnover.

The reduced substrate inhibition in the I12S C156S and I12A C156S enzymes cannot be attributed to a change in the speciation of TriCHQ in the active site. The spectra of bound TriCHQ (see Figure 8b) are similar for the C156S and I12S C156S enzymes, suggesting that the concentrations of $TriCHQ^-$, $TriCHQ^{2-}$, and TriCHQ* are similar in both cases, although the thiol−disulfide exchange reaction is inhibited more severely in the C156S enzyme (see Figure 3). The concentrations of $TriCHQ^{2-}$ and TriCHQ* appear to be lower for the I12A C156S enzyme. Nevertheless, the inhibition of the thiol−disulfide exchange reaction is similar for the I12S C156S and I12A C156S enzymes.

The reduced inhibition of the I12S C156S and I12A C156S enzymes is due largely to weaker binding of TriCHQ to the ESSG forms of these enzymes. The $K_{D,TriCHQ}$ is increased from 13 μM for the C156S enzyme to 45 μM for the I12S C156S enzyme and 65 μM for the I12A C156S enzyme. We note, however, that the mode of inhibition is not necessarily the same for the I12S C156S and I12A C156S enzymes as for the C156S enzyme. The data shown in Figure 6 do not allow us to differentiate between pure noncompetitive and other modes of inhibition. Scheme 3 shows the general scheme for mixed-type inhibition. In this scheme, α describes the effect of binding of the substrate (glutathione) on the binding of the inhibitor (TriCHQ), and vice versa, and β describes the effect of the presence of TriCHQ at the active site on the rate of the thiol−disulfide exchange reaction. When $\alpha = 1$ and $\beta = 0$, as in the C156S enzyme, inhibition is pure noncompetitive (see Scheme 2). Other types of inhibition include competitive inhibition ($\alpha = \infty$ and $\beta = 0$), partial competitive inhibition ($\alpha > 1$ and $\beta = 1$), partial noncompetitive inhibition ($\alpha = 1$ and $0 < \beta < 1$), or mixed-type inhibition ($\alpha > 1$ and $0 < \beta < 1$). The extra room in the active site of the I12S C156S and I12A C156S enzymes might cause a change in the mechanism of inhibition if there is a shift in the position of incoming glutathione so that it binds in a new position that interferes with the binding of TriCHQ (and vice versa), introducing a competitive component to the inhibition. Alternatively, the introduction of extra water molecules might partially shield the reacting atoms from the negative charges on the nearby TriCHQ, allowing the thiol−disulfide exchange reaction to occur in the presence of TriCHQ. (This effect would increase β in Scheme 3 from 0 to something less than 1.)

Notably, the rate of the thiol−disulfide exchange reaction in the absence of TriCHQ is about 2-fold higher in the I12S C156S and I12A C156S enzymes than in the C156S enzyme. This increase in rate could be due to (1) a decrease in the $K_{D,GSH}$, resulting in a higher concentration of glutathione in the active site, (2) an increase in the concentration of the thiolate form of glutathione in the active site due to a change in its pK_a, (3) an alteration in the position of the glutathione that allows a more effective trajectory for attack on the disulfide bond, or (4) an increased ability to stabilize the developing negative charge on Cys13 as the disulfide bond breaks due to additional water molecules in the active site. Replacement of the bulky Ile12 with a smaller residue could lead to any of these effects.

The loss of substrate inhibition in the I12S C156S and I12A C156S mutant enzymes is associated with a decrease in the efficiency of the dehalogenation reaction. In the C156S enzyme, dehalogenation occurs with a rate constant of 25 s^{-1} (*7*). The thiol−disulfide exchange step limits the overall turnover due to the profound substrate inhibition. In the I12S C156S mutant enzyme, the lack of a burst in the formation of DCHQ indicates that a step involved in formation of DCHQ, rather than a subsequent step (such as release of DCHQ or chloride, thiol−disulfide exchange, release of GSSG, or a conformational change required to reset the active site), is rate-limiting. The value of k_{cat} (1.0 s^{-1} for the I12A C156S enzyme and 0.4 s^{-1} for the I12S C156S enzyme) is thus likely to reflect the rate of dehalogenation. Replacement of Ile12 with Ser or Ala could easily affect k_{cat} by altering the positioning of TriCHQ, or by diminishing the nucleophilicity of glutathione and Cys13, possibly due to the introduction of extra water molecules in the space created by the loss of the bulky hydrophobic side chain of Ile12.

The profound substrate inhibition and the enzyme's inability to exclude the aromatic substrate from the active site of ESSG suggest that TCHQ dehalogenase is in the process of evolving a new function and has not yet achieved the exquisite level of control of well-evolved enzymes. The physiological effect of the trade-off between substrate inhibition and catalytic power we report here will depend upon the concentration of the substrates *in vivo*. The concentrations of TCHQ and TriCHQ *in vivo* are estimated to be 2 and 5 μM, respectively (*13*). Since the $K_{i,TriCHQ}$ is 6 μM, the wild-type enzyme will be significantly inhibited *in vivo*. However, the rate will still be faster than could be achieved with an I12S or I12A mutant enzyme. Thus, the enzyme is appropriately "tuned" to the *in vivo* conditions. The flux through the pathway is currently limited by the poor performance of PCP hydroxylase, the first enzyme in the pathway (*13*). If the flux were to increase, leading to higher levels of TCHQ and TriCHQ, the substrate inhibition of TCHQ dehalogenase would come to limit the flux through the pathway. We have shown that it is unexpectedly easy to overcome the substrate inhibition; only a single mutation is needed to change Ile to Ser. (Two mutations are required to change Ile to Ala.) However, there is a trade-off between substrate inhibition and catalytic power; substitution of Ile by either Ser or Ala substantially decreases the rate of dehalogenation. The I12S mutant would function better than the wild-type enzyme only at substrate concentrations higher than 100 μM. The I12A mutant, which would be more difficult to reach because two mutations are required, would

function better than the wild-type enzyme at substrate concentrations higher than 40 μM. Certainly additional changes in the active site might compensate for the loss of catalytic power. However, an evolutionary trajectory that begins with a change of Ile12 to Ser, followed by a compensatory mutation, might be difficult to achieve *in vivo*. Future studies will explore the possibility of compensatory mutations that could restore catalytic power without reintroducing substrate inhibition.

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