Mechanism of the Severe Inhibition of Tetrachlorohydroquinone Dehalogenase by Its Aromatic Substrates†

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Received September 27, 2006; Revised Manuscript Received November 27, 2006

ABSTRACT: Tetrachlorohydroquinone (TCHQ) dehalogenase catalyzes the conversion of TCHQ to 2,6-dichlorohydroquinone during degradation of pentachlorophenol by *Sphingobium chlorophenolicum*. TCHQ dehalogenase is a member of the glutathione *S*-transferase superfamily. Members of this superfamily typically catalyze nucleophilic attack of glutathione upon an electrophilic substrate to form a glutathione conjugate and contain a single glutathione binding site in each monomer of the typically dimeric enzyme. TCHQ dehalogenase, in contrast to most members of the superfamily, is a monomer and uses 2 equiv of glutathione to catalyze a more complex reaction. The first glutathione is involved in formation of a glutathione conjugate, while the second is involved in the final step of the reaction, a thiol-disulfide exchange reaction that regenerates the free enzyme and forms GSSG. TCHQ dehalogenase is severely inhibited by its aromatic substrates, TCHQ and trichlorohydroquinone (TriCHQ). TriCHQ acts as a noncompetitive inhibitor of the thiol-disulfide exchange reaction required to regenerate the free form of the enzyme. In addition, dissociation of the GSSG product is inhibited by TriCHQ. The thiol-disulfide exchange reaction is the rate-limiting step in the reductive dehalogenation reaction under physiological conditions.

Tetrachlorohydroquinone (TCHQ) dehalogenase catalyzes the reductive dehalogenation of TCHQ to trichlorohydroquinone (TriCHQ) and then to 2,6-dichlorohydroquinone (DCHQ)[1] in the pathway for degradation of pentachlorophenol (PCP) by the soil bacterium *Sphingobium chlorophenolicum*. Large-scale introduction of PCP into the environment began in the 1930s (*1*). *S. chlorophenolicum* appears to have assembled a new metabolic pathway to degrade PCP by recruiting previously existing enzymes from at least two other metabolic pathways (*2*). The unusual reductive dehalogenation reactions catalyzed by TCHQ dehalogenase are critical for biodegradation of PCP. The presence of multiple electron-withdrawing substituents substantially decreases the rate of ring cleavage by intradiol dioxygenases (*3*−*5*); similar effects are likely to occur for the extradiol dioxygenase that cleaves the aromatic ring in the PCP degradation pathway (*6*).

TCHQ dehalogenase may have arisen from a double-bond isomerase such as maleylacetoacetate (MAA) or maleylpyruvate (MP) isomerase (*7*), enzymes involved in degradation of tyrosine and benzoate, respectively. MAA and MP isomerases, like TCHQ dehalogenase, are members of the glutathione *S*-transferase (GST) superfamily. Although the overall sequence identity between TCHQ dehalogenase and known MAA and MP isomerases is low (<33%), the sequence of the active site region in the N-terminal domain is very similar to those of the isomerases (*7*). Furthermore, TCHQ dehalogenase catalyzes isomerization of the double bond in maleylacetone at the same active site used for the dehalogenation reaction, as mutation of Cys13 affects both activities (*7*). The recruitment of such an enzyme to serve as a reductive dehalogenase is intriguing because GSTs typically utilize only 1 equiv of glutathione and thus have a single glutathione binding site in each monomer of the typically dimeric enzymes. TCHQ dehalogenase, however, utilizes 2 equiv of glutathione. The first is involved in formation of a glutathione conjugate at the active site (see Figure 1), a reaction typical of GST superfamily members. The second glutathione is involved in the thiol-disulfide exchange reaction that regenerates the active site Cys13 and forms glutathione disulfide (GSSG) (*8*−*11*). This reaction is not typical of GSTs in general, or MAA and MP isomerases in particular, and may have been "added on" to the ancestral catalytic capabilities of the progenitor. It is of particular interest to determine how well the enzyme is carrying out the additional steps required for the dehalogenation reaction. We recently found that the glutathione

†This work was supported by NSF Grant MCB-0077569.

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[1] Abbreviations: DCHQ, 2,6-dichlorohydroquinone; DNPS-SG, 2,4-dinitrophenyl-glutathionyl disulfide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; ESSG, TCHQ dehalogenase Cys13-glutathionyl disulfide; GS-DCHQ, 3,5-dichloro-2-*S*-glutathionylhydroquinone; GSH, glutathione; GS-*β*ME, glutathionyl 2-hydroxyethyl disulfide; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; IPTG, isopropyl-*β*-D-galactopyranoside; MAA, maleylacetoacetate; *β*-ME, 2-mercaptoethanol; MP, maleylpyruvate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PCP, pentachlorophenol; TCHQ, tetrachlorohydroquinone; TriCHQ, T, trichlorohydroquinone.

10.1021/bi0620104 CCC: $37.00
Published on Web 03/14/2007

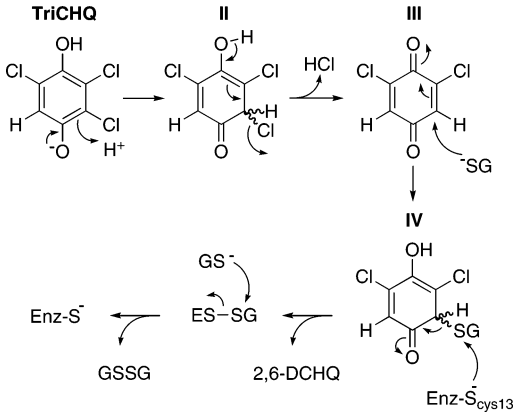


FIGURE 1: Postulated mechanism for TCHQ dehalogenase.

involved in the thiol-disulfide exchange reaction binds very weakly, and the enzyme does not alter its pK_a to improve its nucleophilicity (*11*). Furthermore, the thiol-disulfide exchange reaction is nonspecific; other thiols undergo thiol-disulfide exchange with ESSG at rates comparable to that of glutathione. We report here another surprising feature. TCHQ dehalogenase is subject to severe inhibition by its aromatic substrates (TCHQ and TriCHQ). The aromatic substrates interfere with both the thiol-disulfide exchange reaction and the dissociation of GSSG required to complete the catalytic cycle.

EXPERIMENTAL PROCEDURES

Reagents. Dithiothreitol (DTT) and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) were purchased from Research Products International Corp. Glutathione (glycine-2-^{3}H) (50 Ci/mmol) was from Perkin-Elmer Life and Analytical Sciences, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), glutathione, glutathione disulfide, glutathione reductase from baker's yeast (185 U/mg), and 2-mercaptoethanol (β-ME) were purchased from Sigma-Aldrich. All other reagents were purchased from common commercial sources.

Enzyme Preparation. C156S TCHQ dehalogenase was expressed and purified using an *N*-linked glutathione-Sepharose affinity matrix as previously described (*11*) with the following changes. The cells were shaken at 25 °C overnight after addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM), instead of for 4 h. Purification was carried out in buffers whose pH was adjusted to 8.0 rather than 7.5. This procedure results in typical yields of 200 mg of pure TCHQ dehalogenase from a 1 L culture. Aliquots of purified enzyme were frozen in liquid nitrogen and stored at −80 °C. 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.

Active site titration was carried out by quantifying the amount of DCHQ formed in a presteady-state burst experiment. TCHQ dehalogenase (10 μM) was mixed with TriCHQ (200 μM) and glutathione (1 mM) in 200 mM potassium phosphate, pH 7.0, containing 0.1% ascorbate. The reactions were quenched with an equal volume of 1 N HCl after various intervals in an Applied Photophysics rapid quench-flow instrument, and the amount of DCHQ was measured by HPLC analysis as previously described (*12*). The amount of DCHQ formed in the presteady-state burst was taken to be equal to the total concentration of active enzyme.

Unless otherwise noted, all experiments were carried out using C156S TCHQ dehalogenase, which has only one Cys residue at position 13 in the active site, in order to avoid complications in the synthesis of ESSG (see below) arising from reactions with Cys156, which is located on the surface at a distance from the active site. The substitution of Ser for Cys at position 156 does not appear to affect any properties of the enzyme (*12*). The C13S mutant enzyme showed no affinity for the glutathione-Sepharose affinity matrix. Therefore, it was purified as described previously (*12*). Enzymes were treated for 30 min with 5 mM DTT and exchanged into various buffers prior to experiments using 2-4 5 mL HiTrap Desalting columns (Amersham Biosciences) in series connected to an ÄKTA FPLC system (Amersham Biosciences). The following buffers were used: (Buffer A) 200 mM potassium phosphate, pH 7.0; (Buffer B) 200 mM Tris-HCl, pH 7.5, containing 5 mM EDTA; (Buffer C) 200 mM Tris-HCl, pH 8.7, containing 10 mM ascorbate; and (Buffer D) 200 mM potassium phosphate, pH 8.0.

Preparation of TriCHQ. TriCHQ was prepared from trichlorobenzoquinone as previously described (*7*). 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.

Preparation of TCHQ Dehalogenase Cys13-(^{3}*H-2-glycine)-glutathionyl Disulfide (*^{3}*H-ESSG*). ^{3}H-ESSG was prepared as previously described (*11*) with some modifications. C156S TCHQ dehalogenase (6 nmol) in 100−500 μL of degassed Buffer B at 4 °C was added to solid DNPS-[^{3}H]-SG (8 nmol, 1.4 Ci/ mmol). The yellow solution was allowed to sit for 30 min at 4 °C. ^{3}H-ESSG was then purified by gel filtration using three 5 mL HiTrap Desalting columns and used immediately. If necessary, ^{3}H-ESSG was concentrated with an Amicon Ultra 15 5-kDa cutoff spin filter (Millipore). Nonradiolabeled ESSG was prepared as previously (*11*) except that TCHQ dehalogenase and DNPSSG were allowed to react for 10 min, rather than 4 h, prior to removal of excess reagent by buffer exchange.

Kinetic Studies of the Thiol-Disulfide Exchange Reaction. Rapid quench-flow experiments were carried out using an Applied Photophysics rapid quench-flow instrument by mixing equal volumes of ^{3}H-ESSG (0.05−0.2 μM, 70 μL, 1.4 Ci/ mmol) and variable concentrations of glutathione (1−50 mM) or β-ME (1−20 mM) in Buffer A containing 0.1% ascorbate. The concentrations of enzyme and substrates described below are those obtained after mixing. The concentrations of thiol reagents were determined just prior to experiments using Ellman's assay (*13*). For inhibition studies, TriCHQ (5−600 μM) was added. After a delay time, the reaction was quenched with 1 N HCl (70 μL) and collected. The enzyme was removed by centrifugal filtration in a Microcon YM10 (Millipore). Radiolabeled GSSG or glutathionyl 2-hydroxyethyl disulfide (GS-βME) in the filtrate was quantified by scintillation counting. In general, 7−12 samples were collected after delay times between 12 ms and 30 s. The data were fit with a single-exponential

equation using Kaleida Graph 3.6 (Synergy Software). Experiments were performed at 20 °C. All results are the average of at least two determinations.

These reaction conditions permit multiple turnovers due to conversion of ESSG to free enzyme, which can then catalyze dehalogenation of TriCHQ. However, over the time course of the reaction, the change in concentration of TriCHQ was negligible, and thus the concentration of TriCHQ could be treated as a constant for the purpose of kinetic analysis.

Kinetic Studies of the Release of GSSG. ESSG (10 μM) was mixed with TriCHQ (100 μM) and glutathione (10 mM) in Buffer D in a rapid quench-flow instrument, in the presence or absence of glutathione reductase (30 μM) and NADPH (300 μM). (Prior to reactions, GSSG present in the glutathione stock (typically 1%) was reduced to glutathione using 1.5 equiv of DTT). The amount of glutathione reductase was chosen such that the rate of GSSG reduction was 10-fold faster than the rate of the steady-state production of GSSG during turnover of TCHQ dehalogenase. The reactions were quenched with 1 N HCl after various delay times (12 ms−15 s), and the amount of GSSG produced was 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 over 12 min was developed. GSSG eluted at 10 min and was detected at 210 nm. The amount of GSSG produced vs time was fit to a single exponential or a single exponential followed by a linear steady-state. All results are the average of three determinations. The error bars are the standard deviation.

Steady-State Velocities for C156S and C13S TCHQ Dehalogenase. The conversion of TriCHQ to DCHQ by C156S TCHQ dehalogenase was followed using an HP 8452A diode array spectrophotometer or an Applied Photophysics SX17.MV stopped-flow instrument. TCHQ dehalogenase (50 nM or 100 nM) was added to a solution of Buffer A containing ascorbate (0.1%), glutathione (0.2−25 mM), and TriCHQ (4−800 μM). In some instances, β-ME was included (50 mM). 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}). For experiments at pH 8.7, Buffer C containing β-ME (50 mM) and glutathione (4 mM) was used, and the conversion of TriCHQ to DCHQ was followed at 346 nm ($\Delta\epsilon_{346} = -1.3$ mM^{-1} cm^{-1}). For experiments at pH 8.0, Buffer D containing glutathione (10 mM) was used, and the conversion of TriCHQ to DCHQ was followed at 346 nm ($\Delta\epsilon_{346} = -0.7$ mM^{-1} cm^{-1}). The data were fit by linear regression during the first 5−10% conversion of TriCHQ.

All experiments were performed at 20 °C. Each reported initial velocity is the average obtained from at least three experiments. Error bars reflect the sample standard deviation between these experiments. Velocities obtained in this way were identical to those obtained by a discontinuous HPLC assay (*12*).

The conversion of TriCHQ to 3,5-dichloro-2-*S*-glutathionylhydroquinone catalyzed by C13S TCHQ dehalogenase (*9*) was followed by measuring the increase in absorbance at 346 nm as a function of time ($\Delta\epsilon_{346} = 2.9$ mM^{-1} cm^{-1}). The reaction was initiated by the addition of C13S TCHQ dehalogenase (100 nM) to Buffer A containing ascorbate (0.1%), glutathione (3 mM), and TriCHQ (4−800 μM).

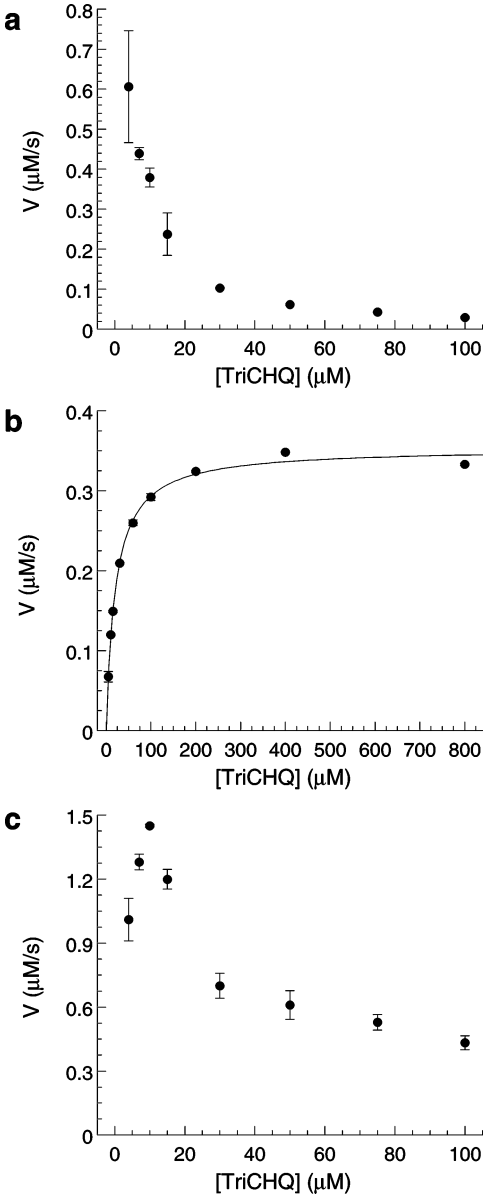


FIGURE 2: Effect of TriCHQ on the rate of the dehalogenation reaction catalyzed by TCHQ dehalogenase. Initial velocities for conversion of TriCHQ were monitored by following the change in absorbance at 320 nm. Assay mixtures contained 200 mM potassium phosphate buffer, pH 7.0, 3 mM glutathione, 0.1% ascorbate, and (a) 100 nM C156S TCHQ dehalogenase, (b) 100 nM C13S TCHQ dehalogenase, and (c) 100 nM C156S TCHQ dehalogenase, 50 mM β-ME. The curve in (b) is a best fit of the data to the Henri-Michaelis-Menten equation.

RESULTS AND DISCUSSION

C156S TCHQ Dehalogenase Is Inhibited by TriCHQ, but the C13S Enzyme Is Not. Initial velocities for TCHQ dehalogenase were measured at various concentrations of TriCHQ (see Figure 2a). C156S TCHQ dehalogenase is severely inhibited by TriCHQ. Similar results were obtained with TCHQ (data not shown). A phase in which the velocity increases with substrate concentration is not evident, even at the lowest concentrations at which the velocity can be measured. In contrast, C13S TCHQ dehalogenase is not subject to substrate inhibition (see Figure 2b). The plot of initial velocity vs TriCHQ concentration follows Henri-Michaelis-Menten kinetics, showing typical saturation behavior with $k_{cat} = 3$ s^{-1} and $K_{M,TriCHQ} = 20$ μM. Cys13

plays a key role in the catalytic cycle (see Figure 1). Replacement of Cys13 with Ser gives an enzyme that can carry out the initial steps in the reaction but cannot continue past the point at which Cys13 is required. The lack of substrate inhibition in the C13S enzyme suggests that the problematic step must occur after formation of 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone (IV in Figure 1) at the active site. TriCHQ might inhibit attack of Cys13 on 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone, the thiol-disulfide exchange reaction required to regenerate Cys13 at the active site, or release of products from the active site. High concentrations of thiols such as 2-mercaptoethanol (Figure 2c) decrease the severity of substrate inhibition. Similar results are obtained with glutathione or DTT (data not shown). These findings suggest that the substrate inhibition is likely to involve the thiol-disulfide exchange reaction between ESSG and glutathione, since this is the only step whose rate should be increased in the presence of high concentrations of thiol.

TriCHQ Inhibits the Thiol-Disulfide Exchange Reaction of ESSG with Glutathione. (^{3}H)ESSG was mixed with various concentrations of TriCHQ and a large excess of glutathione in a rapid quench-flow instrument, and the amount of (^{3}H)-GSSG formed was determined after various reaction times. Under these conditions, formation of GSSG follows pseudo first-order kinetics. Pseudo first-order rate constants were obtained by fitting the data to eq 1. (Rate constants that are labeled "obs" refer to processes that are inhibited by TriCHQ and are therefore dependent upon the concentration of TriCHQ.) TriCHQ indeed inhibits the thiol-disulfide exchange reaction (see Figure 3a).

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

Our initial hypothesis was that TriCHQ inhibited the reaction by competing with glutathione for access to the active site (see Scheme 1). Scheme 1 is consistent with our observation that the thiol-disulfide exchange reaction is irreversible under the conditions of the assays. We assume that rapid pre-equilibrium conditions apply. We see no lag in the appearance of GSSG that would indicate slow formation of ESSG•GSH and no slow onset of inhibition that would indicate slow binding of TriCHQ. For the situation shown in Scheme 1, the velocity of the reaction is given by eq 2, and k'_{obs} is given by eq 3. (T denotes TriCHQ and GSH denotes glutathione.)

$$v = k'_{obs}[\mathrm{ESSG}]_{tot} \quad (2)$$

$$k'_{obs} = \frac{k[\mathrm{GSH}]}{K_{D,GSH}(1+[T]/K_i) + [\mathrm{GSH}]} \quad (3)$$

Although this is a single-turnover experiment, eq 3 has the same form as the standard equation for competitive inhibition during steady-state turnover (*14*). The concentration of glutathione can be considered to be constant during the single-turnover experiment because we follow the disappearance of 0.1 μM radiolabeled ESSG in the presence of millimolar levels of glutathione.

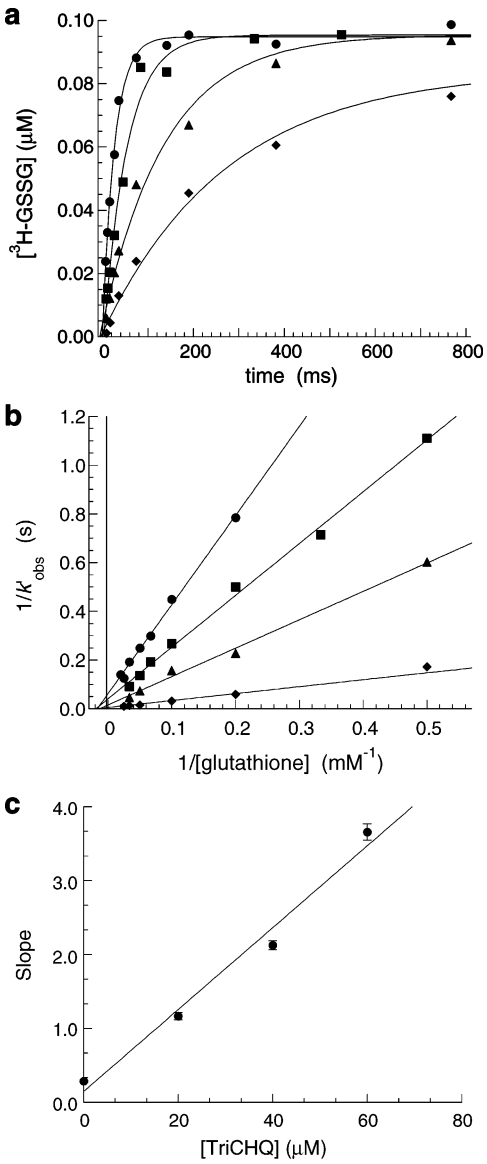
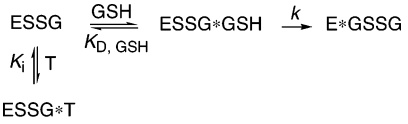


FIGURE 3: Effect of TriCHQ on the rate of the thiol-disulfide exchange reaction. (a) Formation of ^{3}H-GSSG vs time in reactions of ^{3}H-ESSG with 1 mM glutathione in the absence (●) and presence of TriCHQ; 10 μM (■), 35 μM (▲), and 75 μM (◆). Reaction mixtures contained 200 mM potassium phosphate, pH 7, and 0.1% ascorbate. The curves shown are fits of the data to a single exponential. (b) Plots of $1/k'_{obs}$ vs 1/[glutathione] for thiol-disulfide exchange reactions between ESSG and glutathione in the presence of various concentrations of TriCHQ; 60 μM (●), 40 μM (■), 20 μM (▲), and 0 μM (◆). (c) Replot of the slopes from Figure 3b as a function of [TriCHQ].

Scheme 1:

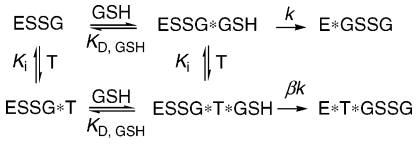


The reciprocal of k'_{obs} is given by eq 4.

$$\frac{1}{k'_{obs}} = \frac{K_{D,GSH}(1+[T]/K_i)}{k}\left(\frac{1}{[\mathrm{GSH}]}\right) + \frac{1}{k} \quad (4)$$

If inhibition is competitive, as shown in Scheme 1, then the inhibition should be overcome at high levels of glutathione, and a double reciprocal plot of $1/k'_{obs}$ vs 1/[GSH]

Scheme 2:



would show a pattern of lines intersecting on the *y*-axis, as the rate in every case will converge on the same maximal value. Figure 3b shows that this is not the case; the lines intersect on the *x*-axis. The pattern in Figure 3b suggests that inhibition of the thiol-disulfide exchange reaction by TriCHQ is noncompetitive (see Scheme 2). Noncompetitive inhibition would occur if the inhibitor binds to both ESSG and ESSG•GSH and sequesters a fraction of the enzyme in a less reactive or nonreactive form. The velocity of the reaction shown in Scheme 2 is given by eq 5, and k'_{obs} is given by eq 6.

$$v = k'_{obs}[ESSG]_{tot} = k[ESSG*GSH] + \beta k[ESSG*GSH*T] \quad (5)$$

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

A noncompetitive inhibitor can completely prevent reaction at the active site or merely slow the reaction (see Scheme 2). Pure noncompetitive inhibition occurs when $\beta = 0$ in Scheme 2. Partial noncompetitive inhibition occurs when $0 > \beta > 1$. We can differentiate between pure and partial noncompetitive inhibition by measuring the rate of thiol-disulfide exchange at high concentrations of TriCHQ, conditions under which all of the enzyme will have TriCHQ bound at the active site. The rate constant for the thiol-disulfide exchange reaction approaches 0 ± 0.17 s^{-1} at high concentrations of TriCHQ (see Figure 4), which is consistent with $\beta = 0$ and pure noncompetitive inhibition. When $\beta = 0$, $1/k'_{obs}$ is given by eq 7.

$$\frac{1}{k'_{obs}} = \frac{K_{D,GSH}(1+[T]/K_i)}{k}\left(\frac{1}{[GSH]}\right) + \frac{1+[T]/K_i}{k} \quad (7)$$

The *x*-intercept from the double reciprocal plot in Figure 3b is equal to $-1/K_{D,GSH}$. Thus, $K_{D,GSH}$ for the thiol-disulfide exchange reaction is 60 mM. The slope of the line for the experiment carried out in the absence of TriCHQ is equal to $K_{D,GSH}/k$. Thus, the rate constant for the thiol-disulfide exchange reaction, k, is 220 s^{-1}. (These values differ somewhat from those previously published ($K_M = 20$ mM, and $k = 100$ s^{-1}) that were obtained at pH 7.7 instead of pH 7.0 and at 5 °C instead of 20 °C (*11*).) A replot of the slopes of the lines in Figure 3b vs TriCHQ concentration provides a line for which the *x*-intercept is equal to $-K_{i,TriCHQ}$ (Figure 3c). The data indicate that $K_{i,TriCHQ} = 3$ μM. This value is close to the value of $K_{M,TriCHQ}$ for the C13S enzyme (20 μM). (The data show a slight upward curvature, which may indicate that the level of inhibition is increased by binding of a second molecule of TriCHQ.)

We have also considered other types of inhibition. Uncompetitive inhibition can be eliminated because this type

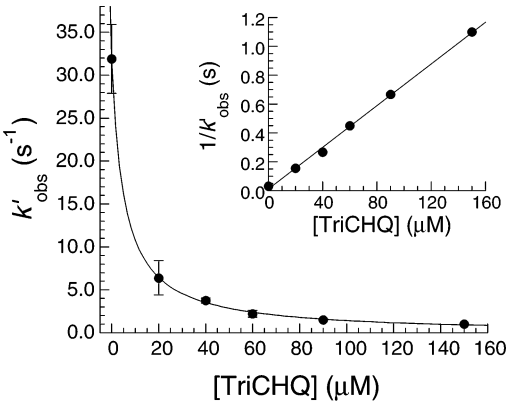


FIGURE 4: Effect of TriCHQ on the rate of the thiol-disulfide exchange reaction between ^{3}H-ESSG and 10 mM glutathione. Reaction mixtures contained 200 mM potassium phosphate, pH 7.0, 0.1% ascorbate, ^{3}H-ESSG, and variable concentrations of TriCHQ. k'_{obs} was obtained from a fit of the data to a single exponential. The curve shown is the best fit of the data to eq 6. The inset shows a replot of $1/k'_{obs}$ vs [TriCHQ].

of inhibition gives a pattern of parallel lines in a double reciprocal plot like that shown in Figure 3b. Hyperbolic mixed-type inhibition, which would occur if the binding constant for glutathione were altered in the presence of TriCHQ (and vice versa), can be eliminated because the lines in double reciprocal plots intersect above the *x*-axis, rather than on the *x*-axis.

There are three ways in which TriCHQ could act as a noncompetitive inhibitor of thiol-disulfide exchange. A noncompetitive inhibitor need not bind to the active site; binding at a different site that alters the conformation of the active site can result in inhibition. However, in this case, it is most likely that TriCHQ binds to the active site, since it is a substrate for the enzyme. Furthermore, we have previously shown that binding of TriCHQ and glutathione occurs in a rapid equilibrium random sequential fashion (*9*), which indicates that the presence of glutathione in the active site does not preclude access of TriCHQ to its normal binding site. In addition, K_i for inhibition of the thiol-disufide exchange reaction is similar to the $K_{M,TriCHQ}$ for the C13S enzyme (for which accurate measurement of a K_M is possible because of the lack of substrate inhibition.) TriCHQ is negatively charged in the active site of C13S TCHQ dehalogenase (*10*) and TCHQ dehalogenase (unpublished data). This negative charge could prevent formation of the thiolate form of glutathione needed to attack ESSG. Alternatively, it could destabilize the developing negative charge on Cys13 in the transition state for thiol-disulfide exchange. TriCHQ could also interfere sterically with attack of the thiolate on the disulfide bond, although it apparently does not interfere with binding of glutathione to the active site.

When TriCHQ binds to ESSG, a shift in the UV/visible spectrum occurs immediately upon mixing. The λ_{max} of TriCHQ in solution at pH 7 is 322 nm (see Figure 5). At the active site of the enzyme, a new absorbance with a λ_{max} of about 360 nm appears. Thus, an additional species is likely to be contributing to the UV/vis spectrum at the active site of ESSG. Formation of this species is reversible, as TriCHQ can be quantitatively recovered when ESSG is denatured by treatment with heat or acid (1 N HCl). The substrate inhibition must therefore involve this species, in equilibrium with TriCHQ itself, at the active site of ESSG. Efforts to

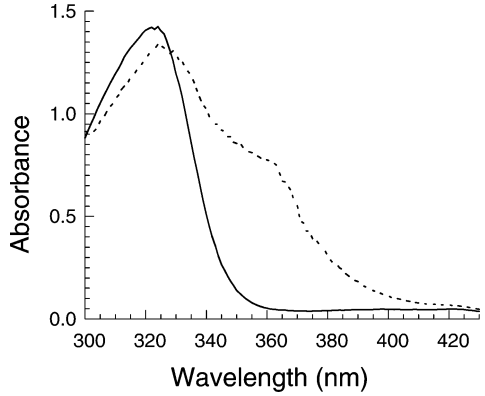


FIGURE 5: UV/vis spectra of (—) TriCHQ (300 μM) in 200 mM potassium phosphate, pH 7 and (---) TriCHQ (300 μM) at the active site of ESSG (400 μM). Under these conditions all the TriCHQ is bound to the enzyme.

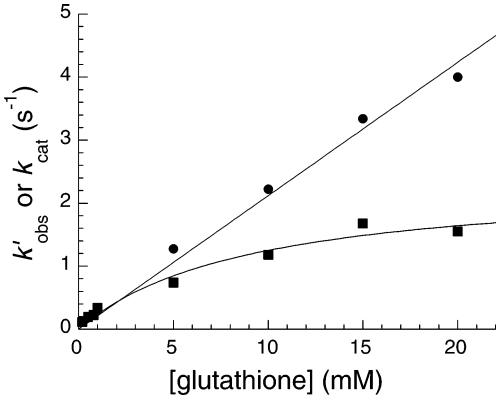


FIGURE 6: The effect of glutathione concentration on the rates of overall turnover and the thiol-disulfide exchange reaction. Reaction mixtures contained 200 mM potassium phosphate, pH 7.0, 0.1% ascorbate, and 60 μM TriCHQ. (●) k'_{obs} for the thiol-disulfide exchange reaction between ^{3}H-ESSG and glutathione. Error bars are not shown, but the standard error for each point was less than 20%. (■) k_{cat} for steady-state turnover monitored by following the conversion of TriCHQ to DCHQ at 320 nm ($\Delta\epsilon_{320} = -1.8$ mM^{-1} cm^{-1}). The data were fit to the Henri-Michaelis-Menten equation.

identify this species are underway. A possibility is that this unknown species is 3,5,6-trichloro-4-hydroxycyclohexa-2,4-dienone (II in Figure 1), the tautomeric form of TriCHQ that is formed during the first step of the dehalogenation reaction.

Thiol-Disulfide Exchange Is Only Partially Rate-Limiting during Turnover of TCHQ Dehalogenase at High Concentrations of Glutathione. TriCHQ is a noncompetitive inhibitor of the thiol-disulfide exchange reaction. The release of GSSG is another step that is not part of the normal turnover of enzymes in the GST superfamily. We investigated the rate of this step to determine whether it is also subject to inhibition by TriCHQ. Figure 6 shows a comparison of the rate constants for the thiol-disulfide exchange reaction (k'_{obs}) and the overall dehalogenation reaction (k_{cat}) in reactions containing 60 μM TriCHQ and a range of glutathione concentrations. The rate of the thiol-disulfide exchange reaction is very similar to that of the overall reaction at glutathione concentrations less than 1.5 mM, suggesting that it is partially or completely rate-limiting under these conditions. At higher concentrations of glutathione, the rate of the thiol-disulfide exchange reaction is greater than that of the overall reaction, suggesting that a different step, possibly release of GSSG, becomes rate-limiting. Similar results are obtained using 20 and 40 μM TriCHQ (data not shown).

GSSG Release Is Rate-Limiting at High Concentrations of Glutathione. The rate of GSSG release was determined under conditions in which the rate of the thiol-disulfide exchange reaction was increased 10-fold by increasing the pH from 7.0 to 8.0, conditions chosen to make the unknown step cleanly rate-limiting (*11*). The following experiments demonstrate that release of GSSG is rate-limiting under these conditions. ESSG (10 μM) was mixed with TriCHQ (100 μM) and glutathione (10 mM) at pH 8.0 in the presence of glutathione reductase and NADPH in a rapid quench-flow experiment. (ESSG was used rather than free enzyme to enhance our ability to detect a burst of formation of GSSG in the first turnover. After the release of the first equiv of GSSG, the enzyme begins turning over substrate, so the steady state after the burst reflects events occurring during normal turnover.) GSSG released from the enzyme was rapidly reduced to glutathione by glutathione reductase. Thus, only GSSG sequestered at the active site of the enzyme could be detected after the reaction was quenched. The data show that the amount of GSSG bound to the enzyme rapidly approaches a steady state in which the amount of GSSG bound to the enzyme is 85% of the total enzyme present (Figure 7a). The rate constant for approach to the steady state is 30 $\pm$ 7 s^{-1}, which corresponds approximately to the rate of the thiol-disulfide exchange reaction under these conditions.[2] At the conclusion of the reaction, the amount of GSSG declines to zero. The accumulation of nearly stoichiometric amounts of GSSG at the active site indicates that release of GSSG is slow under these circumstances.

The observed slow rate of release of GSSG could be due to an intrinsically slow release of GSSG (Scheme 3a) or to a situation in which DCHQ or chloride must leave the active site before GSSG can be released, and release of one of these products is rate-limiting (Scheme 3b). Scheme 3b supposes that the thiol-disulfide exchange reaction can occur while DCHQ is bound at the active site, which is unlikely given that TCHQ and TriCHQ completely inhibit the thiol-disulfide exchange reaction. To differentiate between these possibilities, we carried out a similar experiment in which glutathione reductase and NADPH were omitted so the amount of GSSG measured after quenching included both GSSG at the active site and GSSG that had been released to solution. If Scheme 3b were an accurate representation, 2 equiv of GSSG would be formed during the initial burst under these experimental conditions. The first would be formed during the initial reaction of ESSG with glutathione, and the second during the first turnover. (If DCHQ or chloride release were rate-limiting, the appearance of GSSG in later turnovers would be slower.) The data shown in Figure 7b show a burst of formation of GSSG as ESSG is converted to GSSG with a rate constant of 40 $\pm$ 10 s^{-1}, followed by a steady state with a rate constant of 1.0 $\pm$ 0.5 s^{-1}. Notably, the magnitude of

[2] An appropriate mathematical treatment for presteady-state burst kinetics is described in Johnson, K. A. (1992) *Transient-State Kinetic Analysis of Enzyme Reaction Pathways, "The Enzymes"*, Vol. XX, pp 1−61. The rate constant for the burst is approximately that of the thiol-disulfide exchange because 1) the thiol-disulfide exchange reaction is irreversible (*11*); 2) GSSG release is irreversible in the presence of high concentrations of glutathione (especially when glutathione reductase and NADPH are present); and 3) the subsequent steady-state rate is much slower.

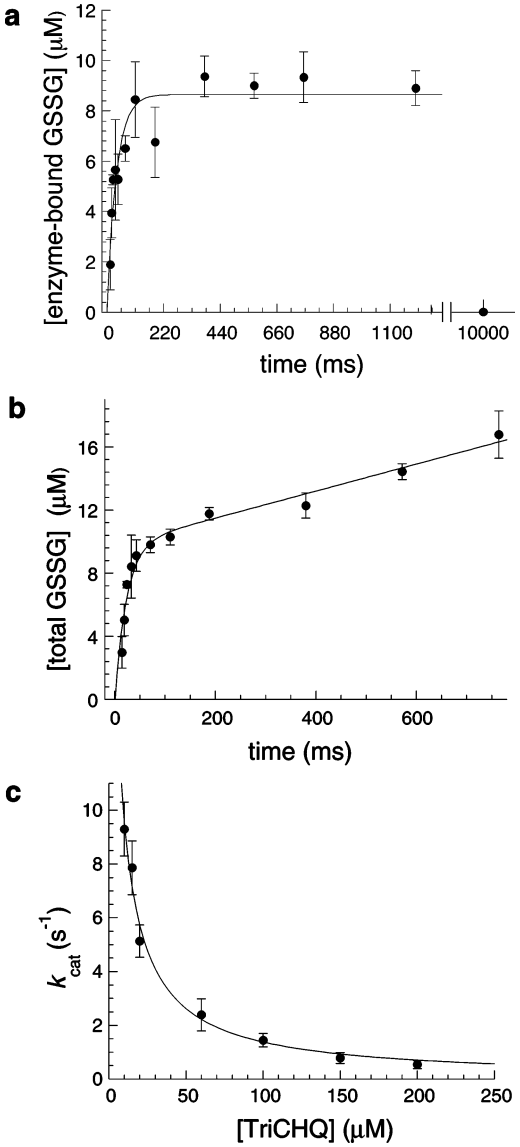


FIGURE 7: Kinetic studies of the rate of release of GSSG. (a) Concentration of enzyme bound-GSSG as a function of time in reaction mixtures containing ESSG (10 μM), TriCHQ (100 μM), glutathione (10 mM), 0.1% ascorbate, glutathione reductase (30 μM), and NADPH (300 μM) in 200 mM potassium phosphate, pH 8.0. (b) Concentration of GSSG as a function of time in a reaction mixture similar to that used in (a) but lacking glutathione reductase and NADPH. (c) Inhibition of the rate of overall turnover at pH 8.0 by TriCHQ. Reaction mixtures contained TCHQ dehalogenase (0.2 μM), TriCHQ (10−200 μM), glutathione (10 mM), and 0.1% ascorbate in 200 mM potassium phosphate, pH 8.0. Initial rates were determined by following the change in absorbance at 346 nm ($\Delta\epsilon_{346} = -0.7$ mM^{-1} cm^{-1}). The data were fit to eq 8.

the burst corresponds to only 1 equiv of GSSG; thus the possibility that release of DCHQ or chloride limits the rate of GSSG release can be ruled out. After release of the first GSSG formed at the active site, the free enzyme undergoes additional turnovers at a steady-state rate limited by the rate of GSSG release. Thus, we conclude that the rate constant for GSSG release at pH 8 in the presence of 100 μM TriCHQ is 1 s^{-1}. Since k_{cat} is independent of pH between pH 7 and pH 9 at high concentrations of glutathione (unpublished observations), we can conclude that the rate constant for GSSG release at pH 7 and in the presence of 100 μM TriCHQ is also 1 s^{-1}. Under these conditions, the rate constant for the thiol-disulfide exchange reaction is approximately 1.5 s^{-1} (see Figure 4). Thus, the rates of both the thiol-disulfide exchange and GSSG release limit the rate of overall turnover.

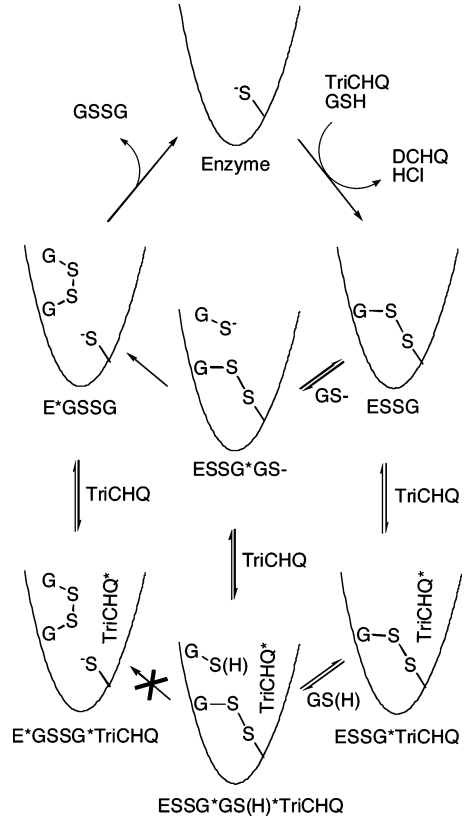


FIGURE 8: Model for the catalytic cycle of TCHQ dehalogenase showing inhibition of the thiol-disulfide exchange reaction and release of GSSG by TriCHQ. TriCHQ* represents a mixture of TriCHQ and an unidentified species (see Figure 5 and discussion in text).

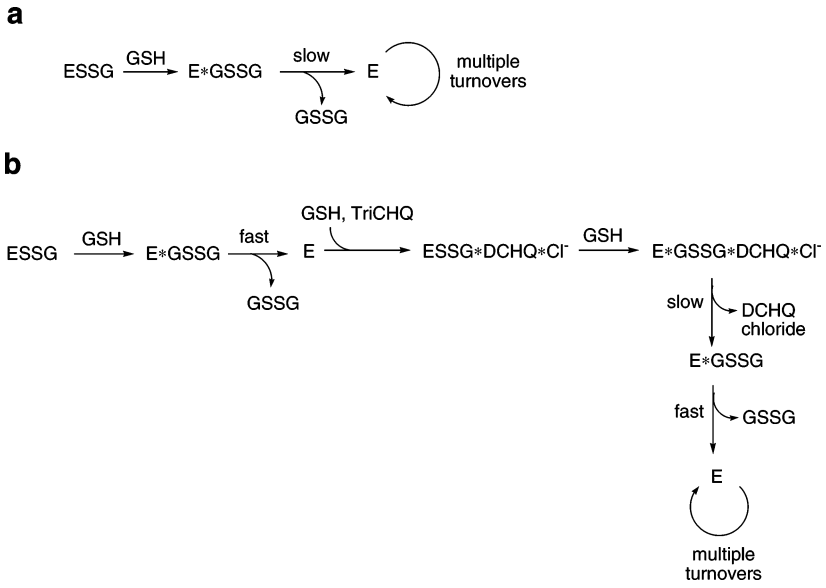
Release of GSSG Is Inhibited by TriCHQ. To determine whether release of GSSG is inhibited by TriCHQ, the rate of the reaction was measured in the presence of various concentrations of TriCHQ under conditions in which turnover is limited by the rate of GSSG release (pH 8 and 10 mM glutathione). Figure 7c shows the dependence of k_{off} (taken to be equal to k_{cat}) on the concentration of TriCHQ. Release of GSSG is inhibited by TriCHQ. Scheme 4 describes the dissociation of GSSG from E*GSSG and E*T*GSSG. Equation 8 can be used to fit the data in Figure 7c. The curve approaches a value of -0.1 ± 0.45 s^{-1}at high concentrations of TriCHQ, suggesting that dissociation of GSSG does not occur when TriCHQ is bound.

$$k_{cat} = \frac{k_{off}}{(1+[T]/K_i)} + \frac{\beta k_{off}}{(1+K_i/[T])} \quad (8)$$

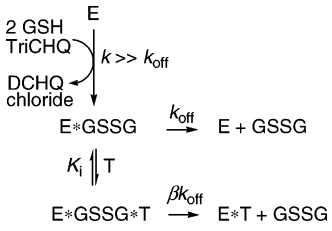
Figure 8 summarizes the interactions of TriCHQ with various forms of the enzyme. Binding of TriCHQ allows glutathione to bind to ESSG but prevents the thiol-disulfide exchange reaction. Only after TriCHQ dissociates can the thiol-disulfide exchange reaction proceed. Once thiol-disulfide exchange occurs, binding of TriCHQ before GSSG can be released again stops the catalytic cycle completely. Release of GSSG can only occur after TriCHQ dissociates.

Substrate Inhibition under Physiological Conditions Is Due to Inhibition of Thiol-Disulfide Exchange. We have previ-

Scheme 3:



Scheme 4:



ously estimated that the cytoplasmic concentration of glutathione is 1.6 mM (*11*), and the concentrations of TCHQ and TriCHQ are approximately 2 and 5 μM, respectively (*15*), in *S. chlorophenolicum* cells that are actively metabolizing PCP. If the cytoplasmic pH is 7.0 and TCHQ and TriCHQ have similar inhibition constants, the observed rate constant for the thiol-disulfide exchange reaction would be 1.7 s^{-1} (calculated using eq 7 and $K_{D,GSH} = 60$ mM, $k = 220$ s^{-1}, $K_i = 3$ μM, [GSH] = 1.6 mM, and [T] = [TriCHQ] + TCHQ] = 7 μM). The rate of dissociation of GSSG under these conditions would be approximately 9 s^{-1} (based upon the data in Figure 7c). Thus, the thiol-disulfide exchange reaction is the rate-limiting step in the turnover of TCHQ dehalogenase under physiological conditions, although the release of GSSG is only 5-fold faster and is also severely inhibited by TriCHQ.

Substrate Inhibition Is Less Significant at High pH and High Concentrations of Thiols. Habash et al. have reported that TCHQ dehalogenase from *Sphingomonas* sp. UG30 is not inhibited by TCHQ. This enzyme has 94% sequence identity to TCHQ dehalogenase from *S. chlorophenolicum* ATCC 39723. It would be surprising if such similar enzymes had dramatically different susceptibilities to substrate inhibition. However, the assay conditions used by Habash et al. were considerably different from those used here. They assayed activity at pH 8.7, 50 °C, in the presence of 4 mM glutathione and 25−50 mM DTT, conditions chosen because they gave optimal activity. We assay activity under more physiological conditions of pH 7, 20 °C, and without high concentrations of nonphysiological thiols such as DTT.

In order to resolve this apparent conflict in the kinetic properties of the two enzymes, we assayed the *S. chlorophenolicum* TCHQ dehalogenase under conditions similar to those used for the UG30 enzyme. Figure 9 shows that under these conditions the *S. chloropenolicum* enzyme shows little sign of substrate inhibition up to a TriCHQ concentration of 150 μM. The plot of velocity vs TriCHQ concentration shows saturation behavior with a $K_{M,TriCHQ} = 4$ μM, $k_{cat} = 14$ s^{-1}, and $k_{cat}/K_M = 3.5 \times 10^6$ M^{-1} s^{-1}. However, TCHQ dehalogenase still shows substrate inhibition at high concentrations of TriCHQ (see Figure 9 inset).

The mechanistic studies reported here suggest an explanation for the dependence of substrate inhibition on the assay conditions. Both the thiol-disulfide exchange reaction and the release of GSSG are inhibited by TriCHQ. Any factor that increases the rate of these steps will favor their completion before TCHQ or TriCHQ can bind and cause inhibition. We have previously shown that the thiol-disulfide exchange of ESSG can occur with many thiols, with little or no specificity (*11*). The rate of the thiol-disulfide exchange reaction with DTT is only slightly slower than that with glutathione (*11*). The thiol-disulfide exchange reaction with small thiols such as DTT and β-ME is inhibited by TriCHQ, just as is the normal thiol-disulfide exchange reaction with glutathione. The $K_{i,TriCHQ}$ obtained for thiol-disulfide exchange with β-ME (7 μM) is very close to that obtained for the thiol-disulfide exchange reaction with glutathione (3 μM) (data not shown). However, the very high levels of DTT (50 mM) included in the assays reported by Habash et al. will greatly increase the rate of the thiol-disulfide exchange reaction. Furthermore, the high pH of the buffer used by Habash et al. will increase the concentration of the reactive thiolate form of the nucleophile, which has a pK_a of 9.2, by 50-fold (Szajewski 1980), thus further increasing the rate of the thiol-disulfide exchange reaction. Both factors will promote completion of the thiol-disulfide exchange reaction before TCHQ or TriCHQ can bind and halt the catalytic cycle.

The thiol-disulfide exchange reaction with β-ME results in formation of a product, GS-βME, whose rate of dissociation is faster than the rate of dissociation of GSSG. Figure 10 shows a comparison of the rate constants of the thiol-disulfide exchange reaction and the overall dehalogenation

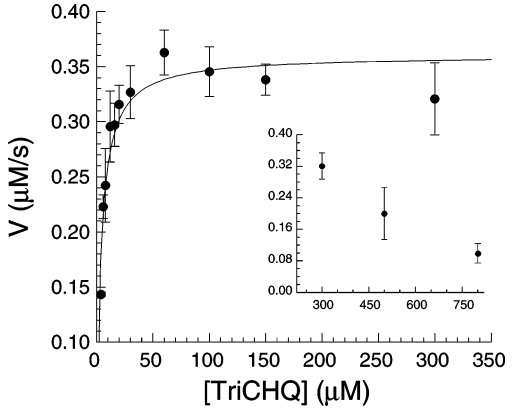


FIGURE 9: Effect of TriCHQ on the rate of the dehalogenation reaction at pH 8.7 in the presence of high concentrations of β-ME. Reaction mixtures contained 200 mM Tris-HCl, pH 8.7, 4 mM glutathione, 50 mM β-ME, 10 mM ascorbate, and 25 nM TCHQ dehalogenase. The curve is a best fit of the data to the Henri-Michaelis−Menten equation. The inset shows inhibition of activity at higher concentrations of TriCHQ.

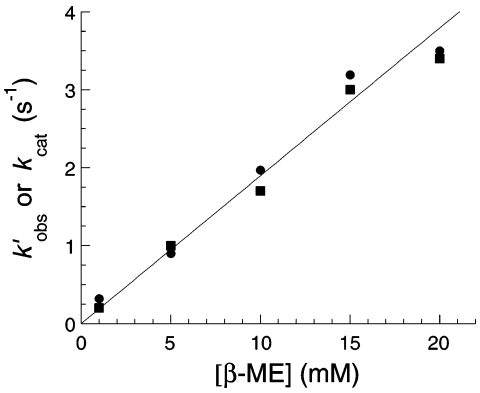


FIGURE 10: The effect of β-ME on the rate constants for overall turnover and the thiol-disulfide exchange reaction. Reaction mixtures contained 60 μM TriCHQ, 100 μM glutathione, and 0.1% ascorbate in 200 mM potassium phosphate, pH 7.0. (●) k'_{obs} for the thiol-disulfide exchange between ^{3}H-ESSG and β-ME. k'_{obs} is the pseudo first-order rate constant obtained from fits of [^{3}H-GS-βME] vs time. (■) k_{cat} for steady-state turnover of TCHQ dehalogenase monitored by following the conversion of TriCHQ to DCHQ at 320 nm ($\Delta\epsilon_{320} = -1.8$ mM^{-1} cm^{-1}).

reaction in reactions containing 60 μM TriCHQ, 100−200 μM glutathione, and a range of concentrations of β-ME. The glutathione was required for the initial steps of the dehalogenation reaction; 100−200 μM glutathione is sufficient to cause rapid formation of ESSG, but the large excess of β-ME in the reaction results in predominant attack upon ESSG by β-ME, generating GS-βME as the final product. The data show that the rate constant for the overall turnover under these conditions is the same as that of the thiol-disulfide exchange reaction, suggesting that release of the product GS-βME is not rate-limiting at high concentrations of thiol, in contrast to the situation when glutathione is used (see Figure 6). Thus, the rate constant for release of GS-βME must be considerably greater than 4 s^{-1}, while the rate constant for release of GSSG under comparable conditions is 2 s^{-1} (see Figure 7c). Dissociation of GS-βME may be faster than dissociation of GSSG because there are fewer interactions with the active site, or because TriCHQ interferes less with egress of the smaller product from the active site. The latter seems most likely, as the very high $K_{M,GSH}$ for the thiol-disulfide exchange step suggests that the glutathione involved in the thiol-disulfide exchange binds very weakly to the active site.

Based upon the evidence that TriCHQ inhibits both thiol-disulfide exchange reaction and the release of GSSG and these studies showing that high concentrations of small thiols such as DTT accelerate both thiol-disulfide exchange and release of the disulfide product, we conclude that inhibition of the thiol-disulfide exchange reaction and GSSG release by TCHQ and TriCHQ will be masked under the reaction conditions used by Habash et al.

CONCLUSION

We attribute the severe substrate inhibition of TCHQ dehalogenase to effects upon the last two steps in the reaction. TCHQ or TriCHQ binds to the ESSG form of the enzyme and prevents thiol-disulfide exchange by acting as a noncompetitive inhibitor. The hydroquinone substrates prevent thiol-disulfide exchange even though the active site is still accessible to glutathione. If the thiol-disulfide exchange reaction is completed, the dissociation of GSSG is inhibited when TCHQ or TriCHQ binds to the active site. Notably, both of the steps that are subject to substrate inhibition are atypical for members of the GST superfamily. These steps may have been "added on" to the ancestral function of the TCHQ dehalogenase progenitor. The enzyme has apparently not evolved a mechanism to prevent binding of the hydroquinone substrate to the active site before the catalytic cycle is completed.

The profound substrate inhibition observed for TCHQ dehalogenase is unusual, and perhaps unprecedented, in an enzyme that is "well-evolved". An important question raised by our findings is whether substrate inhibition occurs in vivo. We have previously shown that the levels of TCHQ and TriCHQ are in the low micromolar range because the slow rate of PCP hydroxylase limits the flux of material through the pathway (*15*). Our results indicate that substrate inhibition would occur in vivo; the rate constant for the rate-limiting thiol-disulfide exchange reaction would be decreased from 5.7 to 1.7 s^{-1}. However, the "design flaw" in TCHQ dehalogenase is physiologically irrelevant, since the flux through the pathway is limited by the poor performance of the first enzyme in the pathway. Thus, there is no selective pressure to evolve an enzyme that is not subject to substrate inhibition. If *S. chlorophenolicum* were to acquire mutations that improved the catalytic performance of the first two enzymes in the pathway (PCP hydroxylase and tetrachlorobenzoquinone reductase), the resulting higher levels of TCHQ and TriCHQ would cause more serious substrate inhibition, and the flux through the degradation pathway would become limited by the catalytic performance of TCHQ dehalogenase.

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BI0620104