Characterization of the Initial Steps in the Reductive Dehalogenation Catalyzed by Tetrachlorohydroquinone Dehalogenase†

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ABSTRACT: Tetrachlorohydroquinone dehalogenase catalyzes the reductive dehalogenation of tetrachlorohydroquinone and trichlorohydroquinone during the degradation of pentachlorophenol by *Sphingbium chlorophenolicum*. Remarkably, the same active site catalyzes the glutathione-dependent isomerization of a double bond in maleylacetone (an analogue of maleylacetoacetate and maleylpyruvate) [Anandarajah, K. et al. (2000) *Biochemistry 39*, 5303−5311]. The mechanism of the initial steps in the reaction has been probed using the C13S mutant enzyme, which catalyzes the reaction only to the point at which Cys13 is required. The reaction proceeds by a rapid equilibrium random sequential kinetic mechanism. Substrate analogues that lack a second hydroxyl group cannot be turned over to products, although they can bind to the active site. The rate of the reaction is strongly influenced by the number of electron-withdrawing substituents on the substrate. These findings are consistent with a mechanism that begins with ketonization of the deprotonated substrate to form 2,3,5,6-tetrachloro-4-hydroxycyclohexa-2,4-dienone, followed by 1,4-elimination of HCl to from trichlorobenzoquinone. Subsequently, trichlorobenzoquinone is attacked by glutathione to form a glutathione conjugate that, in the absence of Cys13, decomposes to a mixture of products, either at the active site or after release into solution. Possible similarities between this mechanism and the mechanism for isomerization of maleylacetoacetate and maleylpyruvate are discussed.

Tetrachlorohydroquinone dehalogenase catalyzes the reductive dehalogenation of tetrachlorohydroquinone (TCHQ)[1] and trichlorohydroquinone (TriCHQ) during the degradation of pentachlorophenol (PCP) by *Sphingobium chlorophenolicum* (*1*), as well as the glutathione-dependent isomerization of a double bond in maleylacetone (*2*) (an analogue of maleylacetoacetate and maleylpyruvate). Studies described in the previous paper in this issue (*3*) eliminated the possibility that the dehalogenation reaction begins with a nucleophilic aromatic substitution reaction like those catalyzed by many other members of the glutathione *S*-transferase superfamily. In this paper, we describe further studies of the initial steps in the dehalogenation reaction. Two possible mechanisms are shown in Figure 1. Both begin with ketonization of the aromatic ring to form 2,3,5,6-tetrachloro-4-hydroxycyclohexa-2,4-dienone, but differ in the fate of this intermediate. In mechanism I, the thiolate form of glutathione (GS^-) displaces chloride in an S_N2 reaction to form 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone directly. In mechanism II, 1,4-elimination of HCl from 2,3,5,6-tetrachloro-4-hydroxycyclohexa-2,4-dienone forms trichlorobenzoquinone (TriCBQ), which is subsequently attacked by GS^- to form 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone. Here we present evidence consistent with the operation of mechanism II and discuss possible similarities between this mechanism and the mechanism for enzyme-catalyzed isomerization of maleylacetoacetate and maleylpyruvate.

MATERIALS AND METHODS

Enzyme Purification. C13S TCHQ dehalogenase was purified as described previously (*4*).

Enzyme Assays. HPLC assays of the turnover of TCHQ, TriCHQ, and 2,5-DCHQ were carried out in 200 mM potassium phosphate, pH 7.0. After addition of enzyme, aliquots were removed at various times and the reaction was quenched by addition of an equal volume of 1 N HCl. Reaction mixtures were analyzed by reverse-phase HPLC on a Rainin C18 column (4.6 × 50 mm). The column was eluted at 1 mL/min with 25% acetonitrile/75% 0.1% acetic acid in water for TCHQ, 20% acetonitrile/80% 0.1% acetic acid in water for TriCHQ, and 10% acetonitrile/90% 0.1% acetic acid in water for 2,5-DCHQ. The detector was set at 210 nm.

Continuous UV/vis assays of the conversion of TriCHQ to products by C13S TCHQ dehalogenase were carried out in 200 mM Tris-HCl, pH 7.0, 25 mM DTT and 0.1% ascorbate as described in the previous paper in this issue (*3*). The addition of 25 mM DTT was found to slightly increase the k_{cat} and to decrease the $K_{M,GSH}$ by 2.4-fold,

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[1] Abbreviations: 2,6-DCHQ, 2,6-dichlorohydroquinone; DTT, dithiothreitol; GS-DCHQ, 2,6-dichloro-5-*S*-glutathionylhydroquinone; GSH, glutathione; GS^-, thiolate form of glutathione; GSO_3^-, glutathione sulfonate; GST, glutathione *S*-transferase; GS-TriCHQ, 2,3,5-trichloro-6-*S*-glutathionylhydroquinone; MAA, maleylacetoacetate; MP, maleylpyruvate; PCP, pentachlorophenol; TCP, tetrachlorophenol; TriCBQ, trichlorobenzoquinone; TriCHQ, trichlorohydroquinone; TCHQ, tetrachlorohydroquinone.

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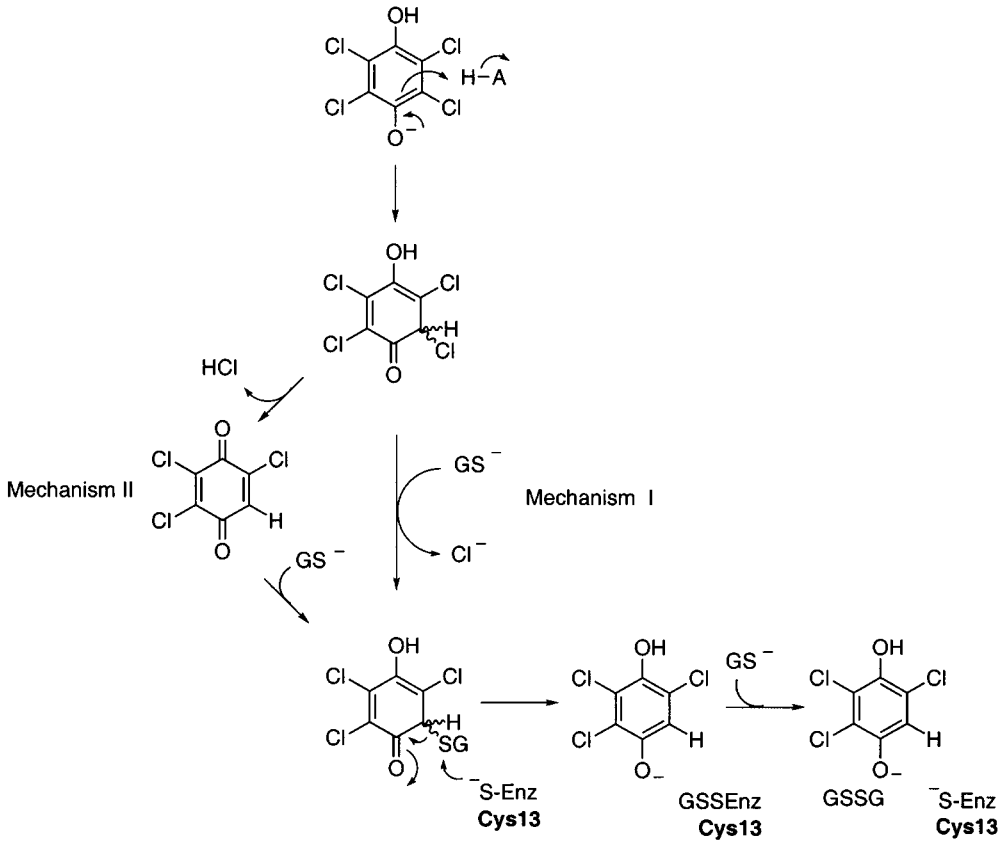


FIGURE 1: Two possible mechanisms for TCHQ dehalogenase.

presumably by maintaining glutathione in its reduced state during the reaction and thereby minimizing product inhibition from glutathione disulfide. For inhibition studies with 2,5-DCHQ, the 2,5-DCHQ concentration was varied from 0 to 1.95 mM while (a) the TriCHQ concentration was varied from 10 μM to 1 mM and the glutathione concentration was held constant at 2 mM, and (b) the glutathione concentration was varied from 10 μM to 20 mM and the TriCHQ concentration was held constant at 120 μM. For studies of inhibition by GSO_3^-, the GSO_3^- concentration was varied from 0 to 360 μM while (a) the TriCHQ concentration was varied from 10 μM to 1 mM and the glutathione concentration was held constant at 60 μM, and (b) the glutathione concentration was varied from 10 μM to 20 mM and the TriCHQ concentration was held constant at 305 μM. For inhibition studies with PCP or TCP, the inhibitor concentration was varied from 0 to 400 μM (PCP) or 0 to 2.2 mM (TCP) while the TriCHQ concentration was varied from 10 μM to 1 mM and the glutathione concentration was held constant at 10 mM.

Solvent Kinetic Isotope Effect Studies. Solvent kinetic isotope effects were determined as described in the previous paper in this issue (*3*). The $\Delta\epsilon_{346}$ values for this buffer system are as follows: 2.2 mM^{-1} cm^{-1} (pH 7.0); 2.9 mM^{-1} cm^{-1} (pH 7.5); 3.3 mM^{-1} cm^{-1} (pH 8.0); 3.0 mM^{-1} cm^{-1} (pH 8.5), 2.0 mM^{-1} cm^{-1} (pH 9.0).

RESULTS

Steady-State Kinetic Studies of C13S TCHQ Dehalogenase. Kinetic studies of the initial steps of the TCHQ dehalogenase reaction were performed using the C13S mutant enzyme. This system has the advantage of isolating the steps under consideration from the later steps in the reaction, and of avoiding the severe substrate inhibition that makes kinetic studies of the wild-type enzyme impossible (Anandarajah, Kiefer, and Copley, unpublished results). To characterize the kinetic mechanism of the enzyme, we carried out initial velocity determinations while varying the TriCHQ concentration from 33 to 495 μM and the glutathione concentration from 32 to 840 μM. The observed pattern of intersecting lines in the double-reciprocal plot (see Figure 2) is diagnostic of a sequential Bi Bi kinetic mechanism and excludes the possibility of a ping pong kinetic mechanism (*5*).

The observed data are consistent with both a rapid equilibrium random sequential kinetic mechanism and a steady state ordered kinetic mechanism, since the equations describing the dependence of velocity on substrate concentrations for these mechanisms are kinetically equivalent (*5*). To distinguish between these possibilities, we examined the inhibition of the reaction by dead-end inhibitors (*5*) that are analogues of TriCHQ and glutathione. 2,5-DCHQ was used as an analogue of TriCHQ. This compound is a very slow substrate for the enzyme (see below) and therefore can be treated as a dead-end inhibitor for this purpose. Glutathione sulfonate (GSO_3^-) was used as an unreactive analogue of glutathione. For a rapid equilibrium random sequential kinetic mechanism, 2,5-DCHQ should show competitive inhibition with respect to TriCHQ and noncompetitive or mixed inhibition with respect to glutathione. Similarly, GSO_3^- should show competitive inhibition with respect to glutathione and noncompetitive or mixed inhibition with respect to TriCHQ. Thus, the lines in double-reciprocal plots of $1/v$

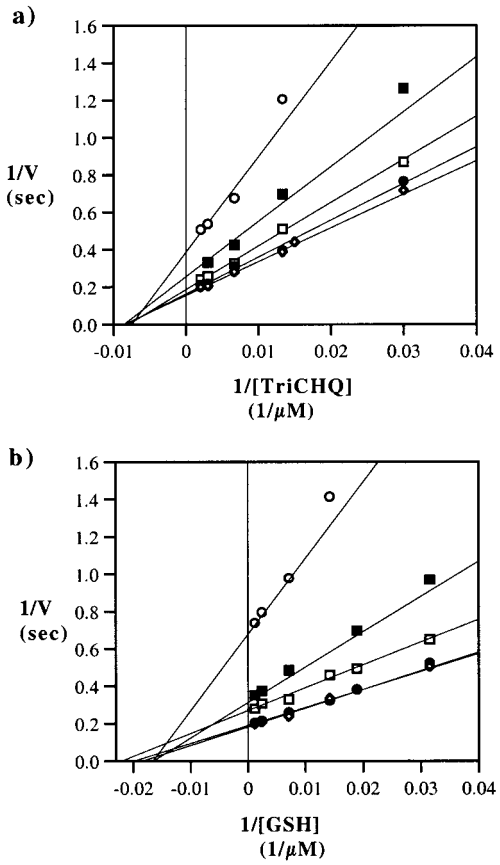


FIGURE 2: (a) $1/v$ vs 1/[TriCHQ] at varied levels of GSH. 846 μM (◇), 423 μM (●), 141 μM (□), 54 μM (■), and 32 μM (○). (b) $1/v$ vs 1/[GSH] at varied levels of TriCHQ. 495 μM (◇), 330 μM (●), 150 μM (□), 75 mM (■), and 33 μM (○). Each data point is an average of multiple observations. The lines are fits of each data set to the Michaelis−Menten equation ($v = V_{max}[S]/(K_M + [S])$. Error bars are not shown for clarity. However, each data point is within experimental error of the line fit to the data set.

vs 1/[S] at different inhibitor concentrations should intersect for both inhibitors. For a steady state ordered kinetic mechanism, the inhibition patterns would be different because a nonreactive analogue of the substrate that binds second will be an uncompetitive inhibitor with respect to the substrate that binds first. Thus, the lines in double reciprocal plots of $1/v$ vs the reciprocal of the concentration of the substrate that binds first at different inhibitor concentrations should be parallel rather than intersecting. In contrast, a nonreactive analogue of the substrate that binds first to the enzyme should show competitive inhibition with respect to the substrate that binds first, and noncompetitive or mixed inhibition with respect to the substrate that binds second. Therefore, the lines in a double reciprocal plot of $1/v$ vs the reciprocal of the concentration of the substrate that binds second at different inhibitor concentrations should intersect.

We carried out kinetic studies in which the initial velocity of the reaction of C13S TCHQ dehalogenase with TriCHQ was measured in the presence of either 2,5-DCHQ or GSO_3^-. Concentrations of 2,5-DCHQ and GSO_3^- were varied over a wide range (0−1950 μM and 0−260 μM, respectively). The data (see Supporting Information) indicate that DCHQ is a competitive inhibitor with respect to TriCHQ ($K_i = 1.03 \pm 0.15$ mM), and a noncompetitive inhibitor with respect to glutathione. GSO_3^- is a competitive inhibitor with respect to glutathione ($K_i = 70 \pm 20$ μM) and a noncompetitive inhibitor with respect to TriCHQ. The fact that double reciprocal plots of $1/v$ vs 1/[S] at various inhibitor concentrations show only intersecting lines rather than parallel lines eliminates the possibility of an ordered kinetic mechanism and indicates that the rapid equilibrium random sequential kinetic mechanism is correct. Random sequential kinetic mechanisms have been found for a number of other enzymes in the GST superfamily that catalyze more typical conjugation reactions (*6*−*10*). Thus, even though TCHQ dehalogenase catalyzes a reaction that is much more complex than those catalyzed by most members of the GST superfamily, it retains the random sequential substrate binding typical of the superfamily.

The equations describing the double-reciprocal plots for a rapid equilibrium random sequential kinetic mechanism are given below. The parameter α reflects the change in the K_D of one substrate caused by prior binding of the other substrate.

$$\frac{1}{v} = \frac{\alpha K_A}{V_{max}}\left(1 + \frac{K_B}{[B]}\right)\frac{1}{[A]} + \frac{1}{V_{max}}\left(1 + \frac{\alpha K_A}{[B]}\right) \quad (1)$$

$$\frac{1}{v} = \frac{\alpha K_B}{V_{max}}\left(1 + \frac{K_A}{[A]}\right)\frac{1}{[B]} + \frac{1}{V_{max}}\left(1 + \frac{\alpha K_A}{[A]}\right) \quad (2)$$

The family of lines for $1/v$ vs 1/A intersects at a point to the left of the *y*-axis and pivots clockwise around this point as the concentration of B is increased. The same pattern is seen for plots of $1/v$ vs 1/B as the concentration of A is increased. The height of the intersection point is $1/V_{max}(1 - \alpha)$. Thus, the lines will intersect on the *x*-axis if $\alpha = 1$. The plots shown in Figure 2 shows patterns of lines intersecting approximately on the *x*-axis to the left of the *y*-axis. Thus, α is approximately equal to one, and therefore binding of either substrate to the active site does not affect binding of the other substrate.

The initial velocity for a rapid equilibrium random sequential kinetic mechanism in which binding of each substrate does not affect binding of the other substrate is given by eq 3 (5), in which K_T and K_G are the dissociation constants for TriCHQ and GSH.

$$v = \frac{k_{cat}[E_T][TriCHQ][GSH]}{K_T K_G + K_T[GSH] + K_G[TriCHQ] + [TriCHQ][GSH]} \quad (3)$$

Fitting the data in Figure 2 to eq 3 gives $k_{cat} = 6.7 \pm 0.2$ s^{-1}, $K_T = 112 \pm 6$ μM, and $K_G = 49 \pm 3$ μM.

Inhibition of TCHQ Dehalogenase by PCP and Tetrachlorophenol (TCP). PCP and TCP, substrate analogues that lack a second hydroxyl group, are not turned over to products by TCHQ dehalogenase (data not shown). To determine whether these compounds bind to the active site of the enzyme, we examined their effects on the turnover of TriCHQ by C13S TCHQ dehalogenase. The data indicate that both PCP and TCP are competitive inhibitors with respect to TriCHQ, with K_i values of 240 ± 40 μM and 620 ± 100 μM, respectively (see Supporting Information).

Effect of Substituents on k_{cat} and k_{cat}/K_M. We examined the effect of substituents on the rate of the reaction catalyzed by C13S TCHQ dehalogenase using TCHQ, TriCHQ, and

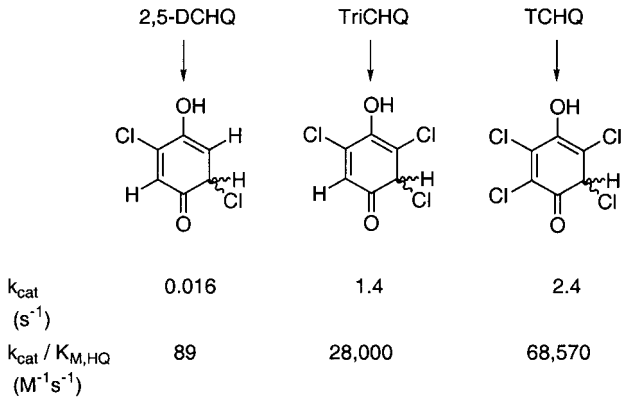


FIGURE 3: Kinetic parameters for turnover of TCHQ, TriCHQ, and 2,5-DCHQ, along with the structures of the intermediates formed by the initial ketonization reaction.

2,5-DCHQ. (The normal product of the reaction, 2,6-DCHQ, is neither a substrate nor an inhibitor of the enzyme, suggesting that it does not bind well to the active site.) Figure 3 summarizes values for k_{cat} and $k_{cat}/K_{M,HQ}$ for each of the three chlorinated hydroquinones. (The value of k_{cat} for TriCHQ differs from that derived from the data in Figure 2 because a different buffer system was used.) For each substrate, the structure of the nonaromatic intermediate postulated to be formed by the initial ketonization reaction is also shown. The data show that k_{cat} and $k_{cat}/K_{M,HQ}$ for TriCHQ are decreased by 1.7- and 2.4-fold, respectively, compared to the corresponding parameters for TCHQ. However, 2,5-DCHQ is a much poorer substrate: k_{cat} and $k_{cat}/K_{M,HQ}$ are decreased by 150- and 770-fold, respectively, compared to the corresponding parameters for TCHQ.

Attempts to Detect Partial Reaction in the Absence of Glutathione. Reactions of C13S TCHQ dehalogenase were carried out with TriCHQ (100 μM) in the absence of glutathione and in the presence of GSO_3^- (10 mM) to determine whether partial reaction of TriCHQ to form TriCBQ could be observed in the absence of glutathione. In neither case was any reaction of TriCHQ observed.

Distribution of Products formed by C13S TCHQ Dehalogenase as a Function of pH. C13S TCHQ dehalogenase cannot carry out the complete catalytic cycle but catalyzes the reaction up to the point at which Cys13 is required (*4*). We previously reported that the C13S enzyme produces a mixture of products from TCHQ that are formed by decomposition of 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone, either at the active site or in solution (*4*). We investigated the pH dependence of the product distribution to characterize the 1,4-elimination reaction responsible for forming one of the products. Figure 4 shows the distribution of products formed from TriCHQ by C13S TCHQ dehalogenase as a function of pH between pH 5 and pH 10. GS-DCHQ is the predominant product over most of the pH range, but a substantial amount of DCHQ begins to appear above pH 9. Above pH 9.5, DCHQ is the major product. The increased amount of DCHQ formed at high pH is consistent with the reactions shown in Scheme 1. The 1,4-elimination of glutathione from 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone should become more facile at higher pH due to removal of the hydroxyl proton. Since the tautomerization reaction leading to GS-DCHQ should be pH-independent, the shifting product

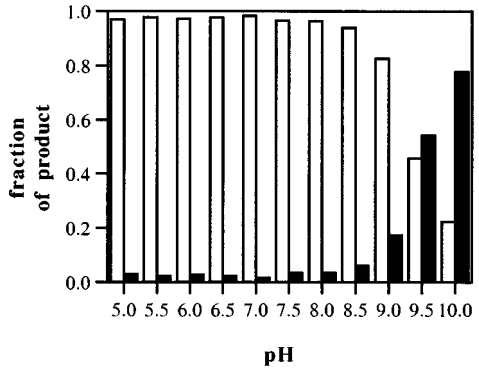


FIGURE 4: Distribution of products formed from TriCHQ by C13S TCHQ dehalogenase as a function of pH. White bars, GS-DCHQ; black bars, DCHQ.

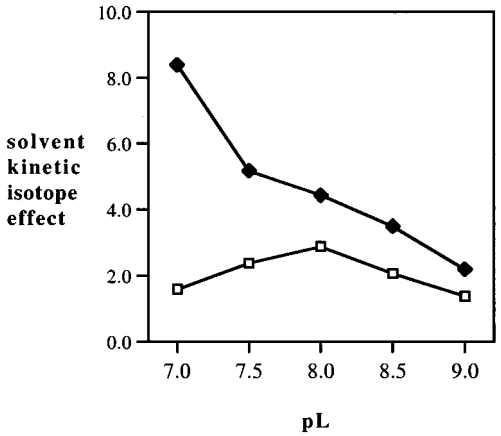
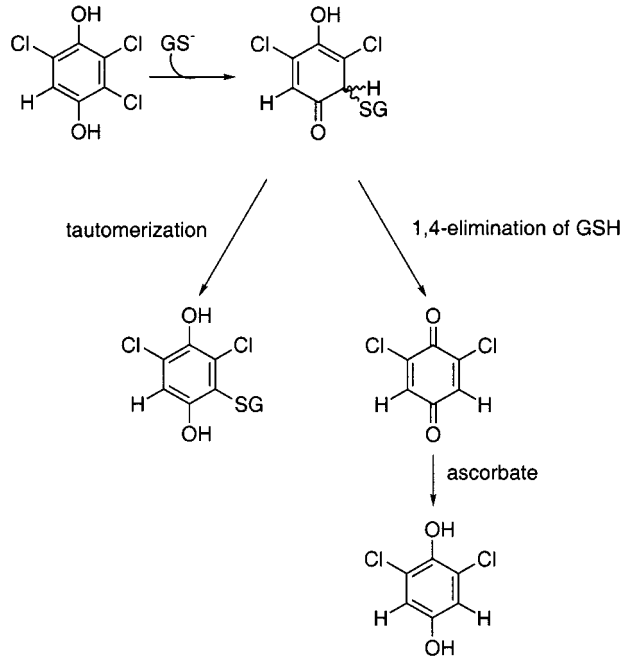


FIGURE 5: Solvent kinetic isotope effects on k_{cat} (□) and k_{cat}/K_M (■) as a function of pL.

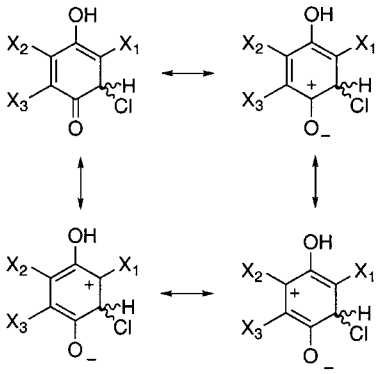
Scheme 1



distribution can be attributed to the increase in the rate of the 1,4-elimination reaction as the pH increases.

Solvent Kinetic Isotope Effect Studies. The solvent kinetic isotope effects on k_{cat} and $k_{cat}/K_{M,TriCHQ}$ were determined over a range of pL values from 7.0 to 9.0. Figure 5 shows that there is a significant solvent kinetic isotope effect on both

Scheme 2

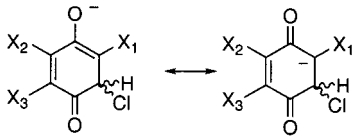


kinetic parameters over the entire pH range. The solvent kinetic isotope effect on $k_{cat}/K_{M,TriCHQ}$ decreases from 8.4 to 2.2 between pL 7.0 and 9.0. The solvent kinetic isotope effect on k_{cat} increases from 1.5 to 2.9 between pL 7.0 and 8.0, and then decreases again to 1.4 by pL 9.0.

DISCUSSION

Evidence Supporting Mechanism II. An important distinction between the two mechanisms under consideration (see Figure 1) is that both hydroxyl groups are required for mechanism II, while only one is required for mechanism I. Our kinetic studies show that both hydroxyl groups are required for substrate turnover. PCP and TCP, substrate analogues that lack a second hydroxyl group, are not turned over to products. To determine whether this was due to poor binding to the active site or to the enzyme's inability to carry out the required chemical steps, we carried out steady-state kinetic studies of the reaction of C13S TCHQ dehalogenase with TriCHQ in the presence and absence of PCP and TCP. PCP and TCP were good competitive inhibitors of the reaction, with K_i values of 240 and 620 μM, respectively. For comparison, the K_D for TriCHQ is 112 μM. Clearly, PCP and TCP can bind to the active site but cannot turn over to product. The most straightforward interpretation of this result is that both hydroxyl groups are required for substrate turnover. We have also considered the possibility that PCP binds "upside down" in the active site with a chlorine substituent in the position normally occupied by the hydroxyl that is required for the initial ketonization reaction. This possibility seems unlikely. TCHQ and TriCHQ are largely deprotonated at the active site of the enzyme. We postulate that the deprotonated hydroxyl group in the "down" position (see Scheme 2) is the one which initiates the ketonization reaction because evidence from the product distribution and solvent kinetic isotope effect studies to be discussed below suggests that the hydroxyl in the "up" position is protonated and the proton is removed only after the ketonization step. It seems most reasonable that PCP and TCP would bind with their deprotonated hydroxyl groups in the site that binds the deprotonated hydroxyl group of TriCHQ, rather than placing a hydrophobic chlorine substituent in that site and placing the deprotonated hydroxyl in a site that normally accommodates a chlorine substituent. Thus, it is likely that PCP and TCP bind in the proper orientation for the ketonization reaction, but the absence of the second hydroxyl prevents subsequent steps required for substrate turnover. This result implies that the second hydroxyl group participates in a required chemical step, as in mechanism II, or is required for a key interaction with the active site that permits the chemical steps to occur.

Scheme 3



Additional evidence consistent with mechanism II comes from studies of the effects of substituents on k_{cat} and $k_{cat}/K_{M,HQ}$. The effects of substituents on each of the steps in mechanisms I and II can be predicted based upon principles of physical organic chemistry. The ketonization step, which is common to both mechanisms, should be more difficult with multiple electron-withdrawing chlorine substituents on the ring. As shown in Scheme 2, there are three high-energy resonance structures of the cyclohexadienone intermediate in which positive charge is located on one of the ring carbons. Chlorine substituents at X_1 and X_2 should exert a destabilizing effect and slow the rate of the ketonization reaction. However, this effect should be rather small, since the high-energy resonance structures will have only a minor contribution to the overall electronic structure. The second step in mechanism I is an S_N2 reaction in which glutathione displaces chloride. Generally, only a small amount of charge develops on the central carbon in an S_N2 reaction, and substituents have little effect on the rate of the reaction (*11*). The second step in mechanism II is a 1,4-elimination reaction, which requires removal of the hydroxyl proton. The resonance structures in Scheme 3 show that an electron-withdrawing chlorine at X_1 should stabilize the negative charge in the deprotonated species and therefore accelerate the rate of proton removal. Chlorine atoms at X_2, and to a lesser extent at X_3, should also provide stabilization via inductive effects. TCHQ and TriCHQ have chlorine substituents at X_1 and X_2, while 2,5-DCHQ has a chlorine substituent only at the less important X_2. Consequently, the 1,4-elimination reaction should be slower for 2,5-DCHQ.

Figure 3 summarizes the kinetic parameters for turnover of three chlorinated hydroquinones, along with a depiction of the cyclohexadienone intermediate that would be produced from each by the initial ketonization reaction and which would undergo either an S_N2 reaction (mechanism I) or 1,4-elimination of HCl (mechanism II). These data show that both k_{cat} and $k_{cat}/K_{M,HQ}$ decrease as the number of chlorine substituents decreases, with a particularly striking difference between the parameters for TriCHQ and 2,5-DCHQ. The magnitude of these effects is inconsistent with what could reasonably be expected for substituent effects on an S_N2 reaction. However, these findings are consistent with the expected effect of substituents on the 1,4-elimination reaction. A chlorine substituent at X_3 should have a small effect on the rate. In accordance with this expectation, k_{cat} and $k_{cat}/K_{M,HQ}$ for TriCHQ are slightly lower than those for TCHQ. However, a chlorine substituent at X_1 should have a much larger effect on the rate. Indeed, k_{cat} and $k_{cat}/K_{M,HQ}$ for 2,5-DCHQ are 88- and 315-fold smaller than those for TriCHQ.

The findings described here are most consistent with mechanism II. We have shown that two hydroxyl groups are required for substrate turnover. In mechanism I, only one hydroxyl is required for the initial ketonization reaction. In contrast, the second hydroxyl is required for the 1,4-

elimination reaction in mechanism II. We have also found that the direction and magnitude of the effects of substituents on k_{cat} and $k_{cat}/K_{M,HQ}$ are consistent with a mechanism in which substantial negative charge accumulates in the ring during a kinetically significant step. This would be expected for the 1,4-elimination reaction in mechanism II but not for the S_N2 reaction in mechanism I.

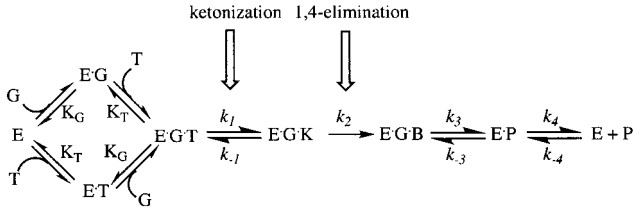
Attempts to Detect Chemical Changes in the Absence of Glutathione. Mechanism II postulates that TCHQ is converted to TriCBQ at the active site prior to attack by glutathione. Support for this mechanism would be provided by evidence that the enzyme could convert TCHQ to TriCBQ in the absence of glutathione or in the presence of a glutathione analogue lacking the thiol moeity. The most conservative glutathione analogue is one in which the Cys residue is replaced with Ser (*12*). We synthesized this analogue, but found that it does not bind to the enzyme (Anandarajah, Kiefer, and Copley, unpublished results), presumably because ionization of the sulfhydryl is required for good binding. Therefore, we used an alternative analogue, glutathione sulfonate (GSO_3^-), which is a good competitive inhibitor with respect to glutathione ($K_i = 70\ \mu M$).

We were unable to demonstrate any formation of TriCBQ in the absence of glutathione or in the presence of GSO_3^-. Assuming that mechanism II is correct, these results suggest that glutathione itself is required for the initial steps to take place, either because it participates in catalysis or because it is required to properly organize the active site. The thiol of glutathione might be required for catalysis if it transfers a proton to the ring during the ketonization step. We consider this possibility unlikely because the kinetic data indicate that the reaction proceeds by a rapid equilibrium random sequential kinetic mechanism. Since ionization of glutathione at the active site of GSTs is typically very rapid (*1*, *14*), the thiol proton is likely to be lost at the active site of TCHQ dehalogenase before the aromatic substrate binds. If the thiol were required to serve as the general acid for the ketonization step, then the reaction would not be able to occur when glutathione bound first, and a random sequential mechanism would not be observed. The alternative possibility that glutathione is required to orient the active site is certainly plausible. Substrate-induced conformational transitions that are required for catalytic turnover have been detected in alpha (*13*, *15*), theta (*14*), and pi (*16*) GSTs. While we would expect that GSO_3^- should be an adequate substitute for glutathione, it is possible that the bulky sulfonate group perturbs binding sufficiently to prevent participation of functional groups in catalysis or proper orientation of a mobile region of the active site.

An alternative test of the mechanism would be to determine whether TCHQ dehalogenase can convert TriCBQ to 2,6-DCHQ in a kinetically competent fashion. Unfortunately, this experiment cannot be done because of the rapid decomposition of TriCBQ in aqueous solution.

Insights into the Individual Steps of Mechanism II. Mechanism II begins with a ketonization reaction that results in loss of aromaticity in the ring. Although this is energetically unfavorable, there are precedents for enzymic catalysis of such reactions. There is a large family of flavin monooxygenases that hydroxylate phenols (*17*). The hydroxylation step takes place by nucleophilic attack of the phenol upon a C(4a)hydroperoxyflavin at the active site. The hydroxyl group of the phenol donates electron density to the ring and thus allows attack upon the electrophilic C(4a)hydroperoxyflavin. A similar tautomerization reaction is also believed to occur in the reaction catalyzed by a mammalian reductive dehalogenase, thyroxine deiodinase (*18*).

Scheme 4



The second step of mechanism II is a 1,4-elimination of HCl. Some insights into the energetics of this step are provided by the distribution of products formed from TriCHQ by C13S TCHQ dehalogenase as a function of pH (see Figure 4). The C13S mutant enzyme catalyzes the conversion of TriCHQ to 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone, which decomposes, either at the active site or after release into solution, to a mixture of products (see Scheme 1). Between pH 5 and pH 8.5, GS-DCHQ accounts for over 90% of the product. GS-DCHQ is formed by tautomerization of 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone. At pH values higher than 8.5, a significant amount of DCHQ appears, and it is the predominant product at pH 10. DCHQ can be formed by 1,4-elimination of GSH from 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone, followed by reduction of dichlorobenzoquinone by ascorbate in the reaction mixture. These data suggest that the 1,4-elimination reaction is extremely fast—faster even than the tautomerization reaction that restores the aromaticity of the ring—when the proton is removed from the hydroxyl group. The data in Figure 4 indicate that the 1,4-elimination reaction depends on the protonation state of a group with a pK_a of approximately 9.5. This might correspond to the hydroxyl group in 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone. Alternatively, if the intermediate decomposes at the active site, this value might reflect the pK_a of a basic group in the active site. The 1,4-elimination of GSH from 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone is very similar to the 1,4-elimination of HCl from 3,5,6-trichloro-4-hydroxycyclohexa-2,4-dienone that occurs in mechanism II. Consequently, we expect that this reaction will also be very facile if the hydroxyl proton is removed. We do not yet know whether the hydroxyl proton is removed by an enzymic group or a buffer molecule.

Insights into the Rate-Determining Steps. The solvent kinetic isotope effect data provide insights into the relative rates of the steps in the reaction. Figure 5 shows that there is a large solvent kinetic isotope effect of 8.4 on $k_{cat}/K_{M,TriCHQ}$ at pH 7.0. This value decreases to 2.2 at pH 9.0. This trend is consistent with a change in rate-limiting step over this pH range. Mechanism II involves at least three chemical steps. A minimal kinetic scheme for this mechanism is shown in Scheme 4, in which T denotes TriCHQ, K denotes the keto intermediate, and B denotes TriCBQ. This scheme does not include explicitly the ionization of GSH upon binding to the active site (which does not affect the derivation of the equations for the kinetic parameters under consideration here), and is vague in terms of the processes involved in k_3, since we do not know whether decomposition of 3,5-

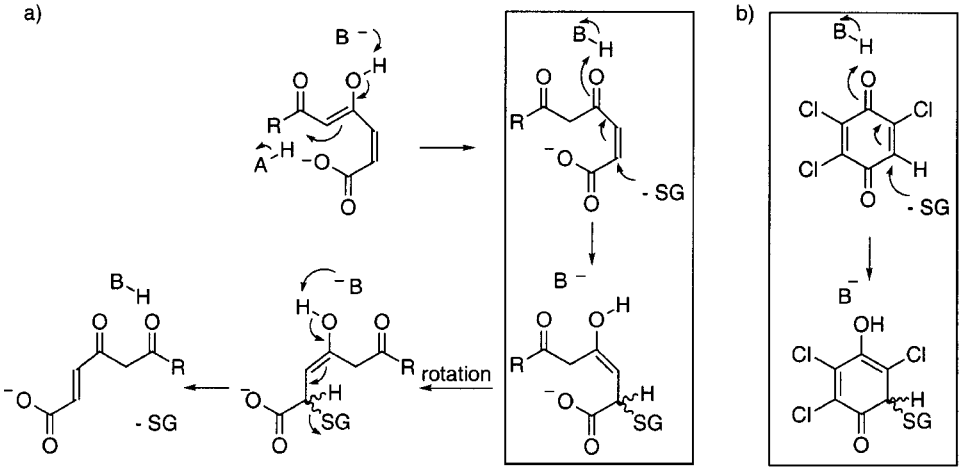


FIGURE 6: (a) Possible mechanism of MAA and MP isomerases. The 4,5-double-bond of the substrate is shown in the *Z* configuration for clarity, although it is not yet known which isomer binds to the enzyme. The box encloses the step involving nucleophilic attack of GS^- upon an electrophilic enone form of the substrate. (b) Step in the dehalogenation reaction catalyzed by TCHQ dehalogenase that involves a comparable nucleophilic attack of GS^- upon an electrophilic enone form of the substrate.

dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone occurs before or after product release. The 1,4-elimination reaction (corresponding to k_2) should be an irreversible step, since chloride is a good leaving group but a poor nucleophile. Thus, the rate constants for the ketonization and 1,4-elimination steps, but not the subsequent steps, will contribute to $k_{cat}/K_{M,TriCHQ}$ in the absence of products (see eq 4). Both the ketonization and 1,4-elimination steps involve proton-transfer reactions and should be subject to substantial solvent kinetic isotope effects.

$$\frac{k_{cat}}{K_{M,TriCHQ}} = \frac{k_1 k_2}{(k_{-1} + k_2)K_T} \quad (4)$$

$$k_{cat} = \frac{k_1 k_2 k_3 k_4}{k_2 k_3 k_4 + k_{-1} k_3 k_4 + k_1 k_3 k_4 + k_1 k_2 k_{-3} + k_1 k_2 k_4 + k_1 k_2 k_3} \quad (5)$$

Upon the basis of our studies of product distribution as a function of pH, we expect the rate of the 1,4-elimination reaction to be very high at high pH, and to decrease at lower pH. Thus, at high pH, if $k_2 \gg k_{-1}$, then $k_{cat}/K_{M,TriCHQ} = k_1/K_T$, and the solvent kinetic isotope effect will be due to effects on k_1 and K_T. At low pH, the 1,4-elimination reaction will be much slower. When $k_2 \ll k_{-1}$, then $k_{cat}/K_{M,TriCHQ} = (k_1/k_{-1})k_2/K_T$, and the observed solvent isotope effect will be a function of the equilibrium isotope effects on the binding of TriCHQ and the ketonization step and the kinetic isotope effect on the 1,4-elimination reaction. Thus, we expect that the trend in the solvent kinetic isotope effect on $k_{cat}/K_{M,TriCHQ}$ reflects a change in the magnitude of k_2 relative to that of k_{-1}.

Figure 5 shows that the solvent kinetic isotope effect on k_{cat} is significantly lower than that on $k_{cat}/K_{M,TriCHQ}$ at all pH values. This finding suggests that a step or steps occurring after the 1,4-elimination reaction that have either no or an inverse solvent kinetic isotope effect are kinetically significant and diminish the observed solvent kinetic isotope effect. Such steps could include attack of GS^-, which would be expected to contribute an inverse solvent kinetic isotope effect (*19*), chemical steps involved in decomposition of 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone if it occurs at the active site, and conformational transitions required for product release. The trend in the solvent kinetic isotope effect on k_{cat} as a function of pL is complex, suggesting changes in kinetically significant steps over the range of pL from 7.0 to 9.0. Since k_{cat} is a complex function of several rate constants (see eq 5), we have not attempted to interpret the trends in Figure 5.

CONCLUSION

The studies reported here complete the description of the chemical steps involved in reductive dehalogenation of TCHQ. Our recent discovery that TCHQ dehalogenase has a significant level of maleylacetone isomerase activity (*2*) has cast our mechanistic studies in a new light. It appears likely that a glutathione-dependent double bond isomerase originally involved in degradation of tyrosine (MAA isomerase) or benzoate (MP isomerase) was recruited to provide a reductive aromatic dehalogenase. Our challenge, therefore, is not only to understand the mechanism of TCHQ dehalogenase, but to understand the mechanism of glutathione-dependent double bond isomerases and why an active site suitable for catalysis of the isomerization reaction provided a suitable starting place for evolution of a reductive dehalogenase. We suspect that there are underlying similarities in the mechanisms of these two seemingly very different reactions such that key catalytic residues in the active site can facilitate both reactions.

We have very little information about the mechanism of glutathione-dependent double bond isomerases. A possible mechanism for the MAA and MP isomerase reactions is shown in Figure 6a. This mechanism is inspired by the precedent for catalysis of glutathione attack upon enone substrates in alpha class GSTs (*20*) and our mechanistic studies of TCHQ dehalogenase. The mechanism begins with an enol form of the substrate that is likely to be the most stable form in solution. Tautomerization of the substrate to produce the enone would activate the substrate for nucleophilic attack by glutathione. Formation of the glutathione conjugate (shown in the box in Figure 6a) is the typical reaction catalyzed by enzymes in the GST superfamily. Rotation about the 2,3 carbon−carbon bond, followed by 1,4-elimination of glutathione, would form the trans isomer.

If this mechanism is correct, then the underlying mechanistic similarities between the dehalogenase and isomerase reactions could include (1) protonation of the substrate by an active site acid and removal of a hydroxyl proton by an active site base to "prepare" the electrophilic substrate for attack by glutathione; and (2) facilitation of the nucleophilic attack of glutathione upon an "enone" substrate by ionization of glutathione at the active site and possibly electrophilic assistance at the carbonyl group. Perhaps the most striking possible similiarity between the two proposed mechanisms is the structure of the electrophile that is attacked by glutathione (see boxes in Figure 6). Further studies are underway to investigate the chemical and physical steps required for catalysis of the isomerase reaction and to identify the catalytic residues that participate in the dehalogenase and isomerase reactions.

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SUPPORTING INFORMATION AVAILABLE

Kinetic data for the inhibition of the dehalogenation of TriCHQ by GSO_3^-, 2,5-DCHQ, PCP, and TCP. This material is available free of charge via the Internet at http://pubs.acs.org.

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