The Reaction Catalyzed by Tetrachlorohydroquinone Dehalogenase Does Not Involve Nucleophilic Aromatic Substitution†

Philip M. Kiefer, Jr., Darla L. McCarthy,‡ and Shelley D. Copley*

Department of Molecular, Cellular, and Developmental Biology and Cooperative Institute for Research in Environmental Sciences, Campus Box 216, University of Colorado at Boulder, Boulder, Colorado 80309

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ABSTRACT: Tetrachlorohydroquinone dehalogenase catalyzes the reductive dehalogenation of tetrachlorohydroquinone and trichlorohydroquinone during the biodegradation of the xenobiotic compound pentachlorophenol by *Sphingobium chlorophenolicum*. The mechanism of this transformation is of interest because it is unusual and difficult, and because aerobic microorganisms rarely catalyze reductive dehalogenation reactions. Tetrachlorohydroquinone dehalogenase is a member of the glutathione *S*-transferase superfamily. Many enzymes in this superfamily are capable of catalyzing nucleophilic aromatic substitution reactions. On the basis of this precedent, we have considered a mechanism for tetrachlorohydroquinone dehalogenase that involves a nucleophilic aromatic substitution reaction, either via an S_NAr mechanism or an $S_{RN}1$-like mechanism, in the initial part of the reaction. Mechanistic studies were carried out with the wild type enzyme and with the C13S mutant enzyme, which catalyzes only the initial steps in the reaction. Three findings eliminate the possibility of a nucleophilic aromatic substitution reaction. First, the product of such a reaction, 2,3,5-trichloro-6-*S*-glutathionylhydroquinone, is not a kinetically competent intermediate. Second, the enzyme can carry out the reaction when the substrate is deprotonated at the active site. Nucleophilic aromatic substitution should not be possible when the substrate is negatively charged. Third, substantial normal solvent kinetic isotope effects on k_{cat} and $k_{cat}/K_{M,TriCHQ}$ are observed. Nonenzymatic and enzymatic nucleophilic S_NAr reactions typically show inverse solvent kinetic isotope effects.

Tetrachlorohydroquinone dehalogenase catalyzes the reductive dehalogenation of tetrachlorohydroquinone (TCHQ)[1] and trichlorohydroquinone (TriCHQ) during the degradation of pentachlorophenol (PCP) by *Sphingobium chlorophenolicum* [formerly known as *Sphingomonas chlorophenolica* (*1*, *2*)] (see Figure 1). PCP was first introduced as a pesticide in 1936 (*3*) and is not known to be a natural product. It appears that *S. chlorophenolicum* has assembled a pathway for degradation of this xenobiotic compound over a relatively short period of time (*4*). Consequently, it is likely that some of the enzymes in the pathway, particularly near its beginning, were not originally designed to carry out these particular reactions but were recruited from other functions in the cell to catalyze novel reactions in the evolving metabolic pathway. The origin and mechanism of the enzyme catalyzing the reductive dehalogenation steps are particularly interesting because aerobic microorganisms typically do not carry out reductive dehalogenation reactions. We have recently shown that TCHQ dehalogenase catalyzes isomerization of maleylacetone at the same site used for dehalogenation of TCHQ, suggesting that it may have evolved from a glutathione-dependent double bond isomerase such as maleylacetoacetate isomerase or maleylpyruvate isomerase (see Scheme 1) (*5*). We suspect that some catalytic residues in the active site participate in both reactions and that there are underlying mechanistic similarities between the two

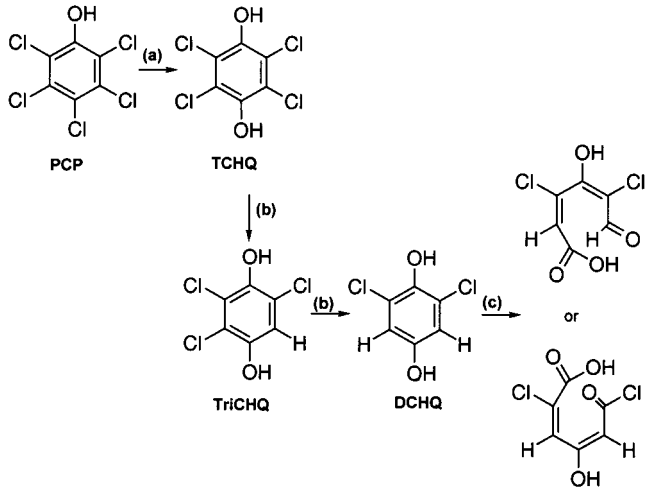

FIGURE 1: Pathway for degradation of PCP in *S. chlorophenolicum*. (a) pentachlorophenol hydroxylase, O_2, 2 NADPH; (b) tetrachlorohydroquinone dehalogenase, 2 GSH; (c) 2,6-dichlorohydroquinone dioxygenase, O_2.

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* Corresponding author: phone 303-492-6328; e-mail copley@cires.colorado.edu.

‡ Current address: Department of Chemistry and Biochemistry, Calvin College, 3201 Burton St. SE, Grand Rapids, MI 49546.

[1] Abbreviations: 2,6-DCHQ, 2,6-dichlorohydroquinone; DTT, dithiothreitol; GSH, glutathione; GS^-, thiolate form of glutathione; GST, glutathione *S*-transferase; GS-TriCHQ, 2,3,5-trichloro-6-*S*-glutathionylhydroquinone; MAA, maleylacetoacetate; MP, maleylpyruvate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PCP, pentachlorophenol; TriCHQ, trichlorohydroquinone; TCHQ, tetrachlorohydroquinone.

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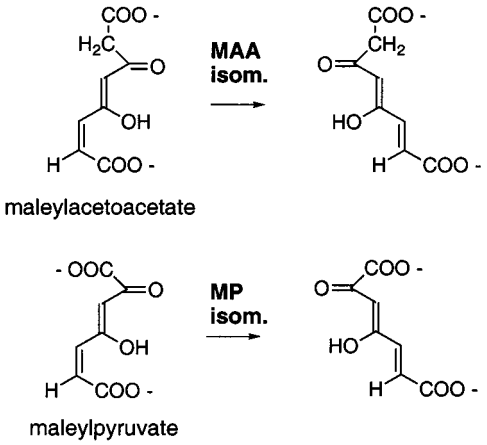
Scheme 1



reactions that are not obvious based upon the overall transformations. Here we report new mechanistic studies of the dehalogenation reaction that will contribute to our goal of understanding how the active site of TCHQ dehalogenase can effectively catalyze both reductive dehalogenation of an aromatic ring and glutathione-dependent isomerization of a double bond.

TCHQ dehalogenase is a member of the glutathione *S*-transferase (GST) superfamily (*6*, *7*). Most members of this superfamily catalyze the nucleophilic attack of glutathione upon an electrophilic substrate to form a glutathione conjugate. TCHQ dehalogenase catalyzes a reaction that is more complicated than is typical for this superfamily. However, we expect that formation of a glutathione conjugate, the reaction that this superfamily has evolved to catalyze, will still be part of the mechanism.

The initial mechanism that we proposed for TCHQ dehalogenase (see Figure 2) (*7*) begins with a nucleophilic aromatic substitution reaction, followed by a tautomerization that protonates the aromatic ring. Subsequently, Cys13 is proposed to attack the sulfur of the glutathionyl substituent, releasing the reduced substrate and forming a mixed disulfide adduct at the active site. In the final step, a thiol-disulfide exchange reaction with a second glutathione regenerates the active site cysteine residue and forms glutathione disulfide. The later steps of the proposed mechanism are well-supported. The products formed from the C13S mutant enzyme are consistent with the intermediacy of 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone

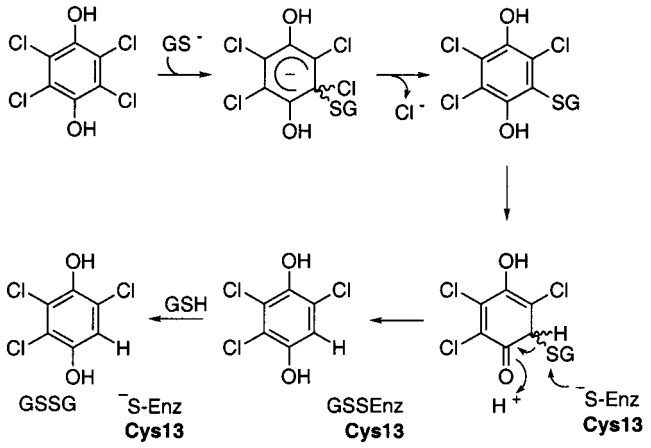
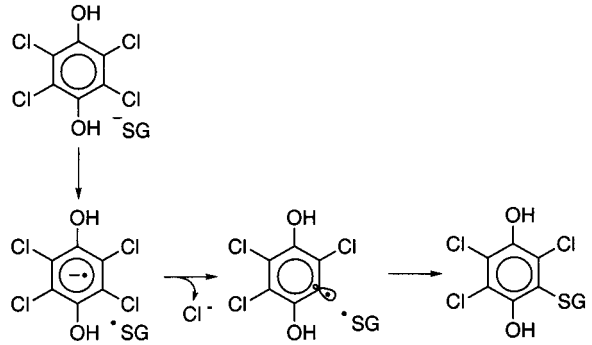


FIGURE 2: Initial mechanism proposed for TCHQ dehalogenase.

Scheme 2



(*7*), and we have shown using rapid flow quench and electrospray LC/MS that a mixed disulfide between Cys13 and glutathione forms and decays during substrate turnover (*8*). We now turn our attention to the initial part of the reaction.

The proposal of a nucleophilic aromatic substitution reaction was based upon the precedent for catalysis of S_NAr reactions by GSTs (*9*). Rates of S_NAr reactions are strongly dependent upon the electron-withdrawing abilities of the substituents on the ring (*10*), and reactants typically have one or two nitro substituents. The four chlorine substituents of TCHQ would not be expected to provide as much stabilization as two nitro groups. The substituent constants for chlorine are 0.37 and 0.24 for the meta and para positions, respectively (*11*), and the effect of a chlorine at the ortho position would be expected to be similar to that for the para position. For comparison, σ_p^- for a nitro group is 1.23 (*11*). Furthermore, TCHQ has two hydroxyl substituents whose influence will depend on both their protonation state and their position relative to an attacking nucleophile. However, GST-catalyzed S_NAr reactions are considerably less dependent upon the nature of substituents than are nonenzymatic reactions. The effect of substituents on S_NAr reactions can be described by the Hammett equation $\log k = \log k_0 + \rho\sigma^-$. For the nonenzymatic reaction of 4-substituted 1-halo-2-nitrobenzenes with glutathione, $\rho = 3.4$, while ρ for the corresponding reaction catalyzed by GST isozyme 4-4 is only 1.2 for k_{cat} and 2.4 for k_{cat}/K_M (*12*). Since it is difficult to estimate a priori how much stabilization of the Meisenheimer complex might be offered by charge and shape complementarity in the active site of TCHQ dehalogenase, experimental investigation of this mechanism was warranted. We have also considered the possibility that a nucleophilic aromatic substitution reaction might take place at the active site by an $S_{RN}1$-like reaction (see Scheme 2), since $S_{RN}1$ reactions are less dependent upon the nature of the substituents than are S_NAr reactions (*13*). Here we provide experimental evidence from investigations of the kinetic competence of the product of a nucleophilic aromatic substitution reaction, the pH dependence of the enzyme, and solvent kinetic isotope effects, that a nucleophilic aromatic substitution by either mechanism is unlikely to occur at the active site of TCHQ dehalogenase. In the following paper in this issue, we will present evidence supporting a new model for the mechanism of this unusual enzymatic reaction.

MATERIALS AND METHODS

Synthesis of TriCHQ and GS-TriCHQ. TriCHQ was prepared from trichlorobenzoquinone as previously described

(*5*). [U-^{14}C]TCHQ was prepared by incubating an extract from *Escherichia coli* strain CCL3 (40% v/v), which overexpresses PCP hydroxylase, with 2 mM NADPH, 5 mM ascorbate, and 200 μM [U-^{14}C]PCP (10.6 μCi/μmol, Sigma) in 25 mM potassium phosphate, pH 7.2, for 1 h. The reaction mixture was quenched by adding 0.1 vol of 6 N HCl, and precipitated proteins were removed by centrifugation. The supernatant was applied to a Vydac semipreparative C18 reverse phase HPLC column equilibrated with 30% acetonitrile in 0.1% acetic acid. [U-^{14}C]PCP and [U-^{14}C]TCHQ were eluted using a gradient of 30−90% acetonitrile in 0.1% acetic acid. GS-[U-^{14}C]TriCHQ was prepared by incubating 100 μM [U-^{14}C]TCHQ with 1 mM GSH, 5 mM ascorbate, and C13S TCHQ dehalogenase in 25 mM potassium phosphate, pH 7.0, for 30 min at 30 °C. The reaction was quenched with an equal volume of 1 N HCl, and GS-[U-^{14}C]TriCHQ was purified by reverse phase HPLC on a Rainin C18 column as described above.

Single-Turnover Experiment with GS-[U-^{14}C]TriCHQ. All reaction mixtures contained 40 μM GS-[U-^{14}C]TriCHQ and 0.1% ascorbate in 25 mM potassium phosphate, pH 7.0. In addition, reaction 1 contained 25 mM DTT, reaction 2 contained 25 mM DTT and 40 μM TCHQ dehalogenase, reaction 3 contained 1 mM GSH and 40 μM TCHQ dehalogenase, and reaction 4 contained 25 mM DTT, 1 mM GSH, and 40 μM TCHQ dehalogenase. Reaction mixtures were incubated at 30 °C for 4 h. The reactions were quenched with an equal volume of 1 N HCl and injected onto a Vydac C18 reverse phase HPLC column. The column was eluted with a gradient of 50−53.5% acetonitrile in 0.1% acetic acid. Fractions were collected and radioactivity determined by scintillation counting.

Enzyme Purification. Wild type and C13S TCHQ dehalogenase were purified as previously described (*7*).

Assay for C13S Dehalogenase Activity. A continuous UV/vis assay using TriCHQ as a substrate was developed. Reactions were carried out at 25 °C in a tribuffer system consisting of 200 mM ethanolamine, 100 mM Tris-HCl, and 100 mM MES, containing 25 mM DTT. The pH was varied from pH 5.0 to 11.0. (High buffer concentrations were necessary because the concentration of glutathione in some reaction mixtures was as high as 10 mM.) The ionic strength in this buffer system is not completely constant across the pH range. However, control experiments in which KCl was added to adjust the ionic strength to a higher level indicated that the kinetic parameters were not sensitive to ionic strength. The TriCHQ concentration was varied between 10 μM and 1 mM while the GSH concentration was fixed at 10 mM. The GSH concentration was varied between 10 μM and 20 mM while the TriCHQ concentration was fixed at 700 μM. Reactions were initiated by addition of enzyme, and conversion of TriCHQ to products measured by following the OD_{346} as a function of time. Initial velocities were used for kinetic analyses. The C13S enzyme converts TriCHQ to a mixture of GS-DCHQ and DCHQ. Since the absorbance spectrum of the substrate and the distribution of products are both pH-dependent (see the following paper in this issue), and the distribution of products is also buffer-dependent, the value of $\Delta\epsilon_{346}$ depends on both pH and buffer. For this buffer system, values of $\Delta\epsilon_{346}$ for pH values from 6.0 to 10.0 are: 0.4 mM^{-1} cm^{-1} (pH 6.0); 1.0 mM^{-1} cm^{-1} (pH 6.5); 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); −1.5 mM^{-1} cm^{-1} (pH 9.5); −2.3 mM^{-1} cm^{-1} (pH 10.0). (Uncertainties in all $\Delta\epsilon_{346}$ values are approximately 0.1 mM^{-1} cm^{-1}.)

Determination of pK_a Values for TriCHQ. pK_a values of TriCHQ were determined by measuring the absorbance at 290, 296, 356, and 360 nm of solutions of TriCHQ (500 μM) in buffers ranging in pH from 4.0 to 10.0. The following buffers were used to cover the indicated pH ranges at intervals of 0.5 pH units: 200 mM sodium citrate, pH 3.0−5.5; 200 mM potassium phosphate, pH 5.0−9.0; 200 mM glycine, pH 8.0−12.0; 200 mM sodium borate, pH 8.0−11.0; and a tribuffer system consisting of 200 mM ethanolamine, 100 mM Tris-HCl, and 100 mM MES, pH 5.0−11.0. Spectra taken in the overlapping regions of pH in different buffers were identical. The data were fit to eq 1, in which A_1 and A_2 are the absorbances of the two species (the fully protonated and deprotonated species for the first pK_a, and the deprotonated and doubly deprotonated species for the second pK_a). The data at 290 and 296 nm were fit to obtain the first pK_a, and the data at 356 and 360 nm were fit to obtain the second pK_a.

$$OD = \frac{A_1 + A_2 + 10^{(pH-pKa)}}{1 + 10^{(pH-pKa)}} \quad (1)$$

UV/Vis Spectra of TriCHQ in Solution and at the Active Site of C13S TCHQ Dehalogenase. UV/vis spectra of TriCHQ (65 μM) were obtained in a 1-cm quartz cuvette in the tribuffer system described above at pH values of 6.0, 8.0, and 10.0, and in 200 mM sodium acetate, pH 4.0, and 200 mM sodium borate, pH 12.0. All samples contained 25 mM DTT. Spectra were also obtained in the presence of 300 μM C13S TCHQ dehalogenase in the tribuffer system at pH values of 6.0, 8.0, and 10.0.

Solvent Kinetic Isotope Effect Studies. Solutions of Tris buffer were made up in either H_2O or D_2O by mixing 200 mM Tris-HCl and 200 mM Tris base to give pH or pD = 8.0. [pD was determined using the relationship "pD = meter reading + 0.4", which applies to a glass electrode (*14*).] Kinetic measurements were carried out as described above. The $\Delta\epsilon_{346}$ values for this buffer system are 2.6 mM^{-1} cm^{-1} for H_2O and 2.4 mM^{-1} cm^{-1} for D_2O.

RESULTS

GS-TriCHQ is not a Kinetically Competent Intermediate. GS-[U^{14}C]TriCHQ (40 μM) was incubated with various combinations of TCHQ dehalogenase (40 μM), ascorbate, DTT, and GSH for 4 h at 30 °C. The reaction mixture was injected onto a reverse-phase HPLC column and fractions collected for scintillation counting. The distribution of reaction components is shown in Figure 3. GS-TriCHQ is stable under the reaction conditions in the absence of the enzyme. However, in the presence of the enzyme, significant turnover of GS-TriCHQ to a mixture of GS-DCHQ and DCHQ is observed over the 4-h reaction period. The appearance of DCHQ suggests that GS-TriCHQ is a catalytically competent intermediate—that is, it can be converted to the normal product of the reaction. However, it is clearly not a kinetically competent intermediate. Under these single-

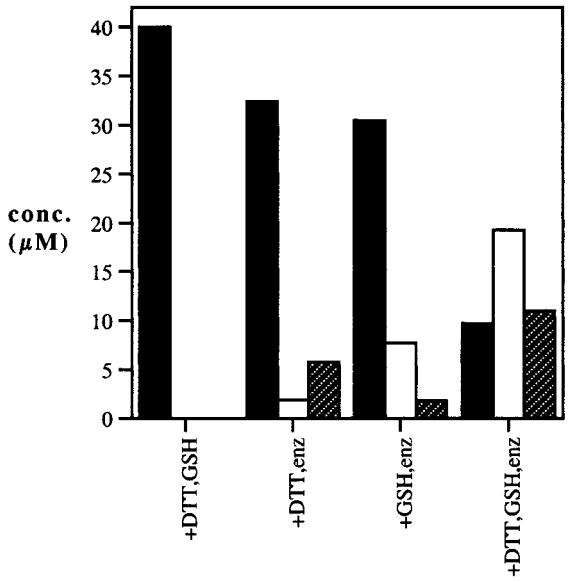


FIGURE 3: Products formed from GS-[U-^{14}C]TriCHQ by TCHQ dehalogenase under single-turnover conditions. Reaction mixtures contained GS-[U-^{14}C]TriCHQ, 5 mM ascorbate, and, as indicated, 40 μM TCHQ dehalogenase, 25 mM DTT, and/or 1 mM GSH. Solid bars, GS-TriCHQ; open bars, GS-DCHQ; hatched bars, DCHQ.

turnover conditions, TCHQ would be converted to TriCHQ and then to DCHQ in a few seconds.

GS-TriCHQ Binds Poorly to the Active Site. TCHQ dehalogenase activity was assayed in the presence of 100 μM TCHQ, 5 mM ascorbate, 200 μM GSH, 25 mM DTT, and varying concentrations of GS-TriCHQ. A concentration of 1 mM GS-TriCHQ caused only about 40% inhibition of dehalogenase activity under these conditions (data not shown), indicating that TCHQ dehalogenase has a very low affinity for GS-TriCHQ.

pH Profile for C13S TCHQ Dehalogenase. The kinetic parameters for turnover of TriCHQ by C13S TCHQ dehalogenase were determined at a range of pH values between 6.0 and 10.0. Control experiments indicated that the enzyme was stable over this pH range. Figure 4 shows the dependence of k_{cat}, $k_{cat}/K_{M,TriCHQ}$, and $k_{cat}/K_{M,GSH}$ on pH. The activity is nearly constant over a wide range of pH values. k_{cat} decreases at low and high pH, but the slope of the line is less than one at low pH and less than negative one at high pH. Thus, the decrease in activity cannot be attributed to a single ionizable group. $k_{cat}/K_{M,TriCHQ}$ falls off only slightly above pH 9.5, but decreases below pH 7 with a slope that is equal to one between pH 6.5 and 6.0. This decrease may reflect the protonation of either the substrate or of an active site group required for substrate binding or catalysis. Unfortunately, we cannot confirm the trend at lower pH values because the low value of k_{cat} and the high value of $K_{M,TriCHQ}$ below pH 6.0 make it difficult to obtain reliable data. $k_{cat}/K_{M,GSH}$ also falls off slightly at low and high pH values, but the trends are not readily interpretable because the slope of the line is less than one at low pH and less than negative one at high pH.

Determination of the Protonation State of TriCHQ in Solution and at the Active Site of C13S TCHQ Dehalogenase. The various protonation states of TriCHQ can be distinguished by their UV/vis spectra. By observing the absorbance of TriCHQ as a function of pH at several wavelengths, we

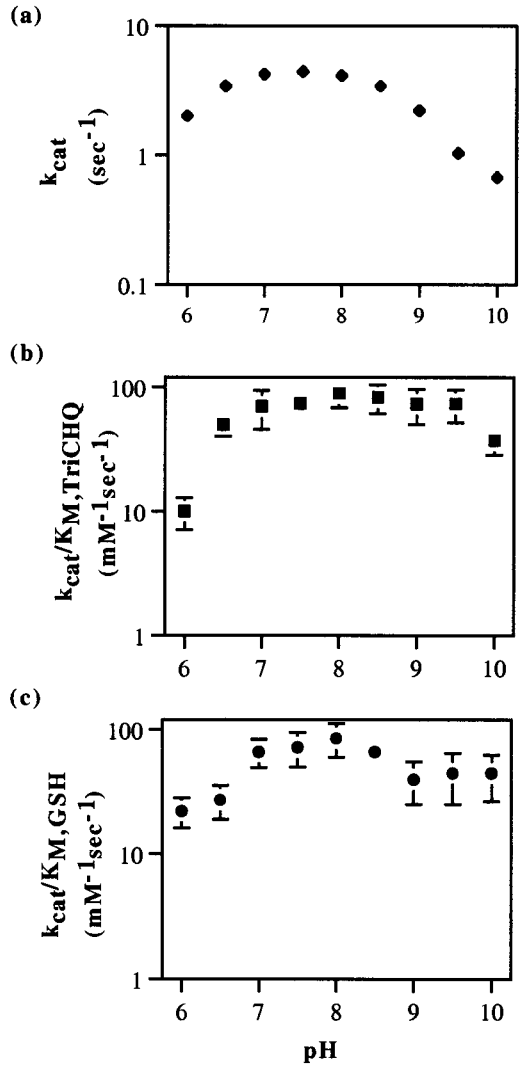


FIGURE 4: Dependence of k_{cat} (a), $k_{cat}/K_{M,TriCQH}$ (b), and $k_{cat}/K_{M,GSH}$ (c) on pH for the turnover of TriCHQ by C13S TCHQ dehalogenase. Where error bars are not visible, they are smaller than the data point.

determined that the hydroxyl groups of TriCHQ ionize with pK_a values of 6.6 and 9.1 in solution (data not shown). Figure 5a shows UV/vis spectra of protonated, singly deprotonated, and doubly deprotonated TriCHQ obtained at pH values of 4, 8, and 12. The λ_{max} shifts from 304 to 322 nm and then to 346 nm as protons are successively removed. Figure 5b shows spectra of TriCHQ in solution over the range of pH values used in our kinetic experiments. As expected, the spectrum obtained at pH 6.0 indicates a mixture of protonated and singly deprotonated forms, while that at pH 10 indicates a mixture of singly and doubly deprotonated forms. Figure 5c shows that spectra of TriCHQ obtained in the presence of 300 μM C13S TCHQ dehalogenase are very similar to those obtained in the absence of the enzyme. (Note that the high absorbance due to the protein limits the interpretable data to wavelengths above 300 nm.) If we assume that $K_{M,TriCHQ}$ is equal to $K_{D,TriCHQ}$ at each pH value (a valid assumption for the rapid equilibrium random kinetic mechanism which will be discussed in the following paper), then we calculate that the percentage of TriCHQ bound to the enzyme was 57, 83, and 92% at pH 6, 8, and 10, respectively. These data suggest that the enzyme does not significantly perturb the pK_a of TriCHQ at the active site.

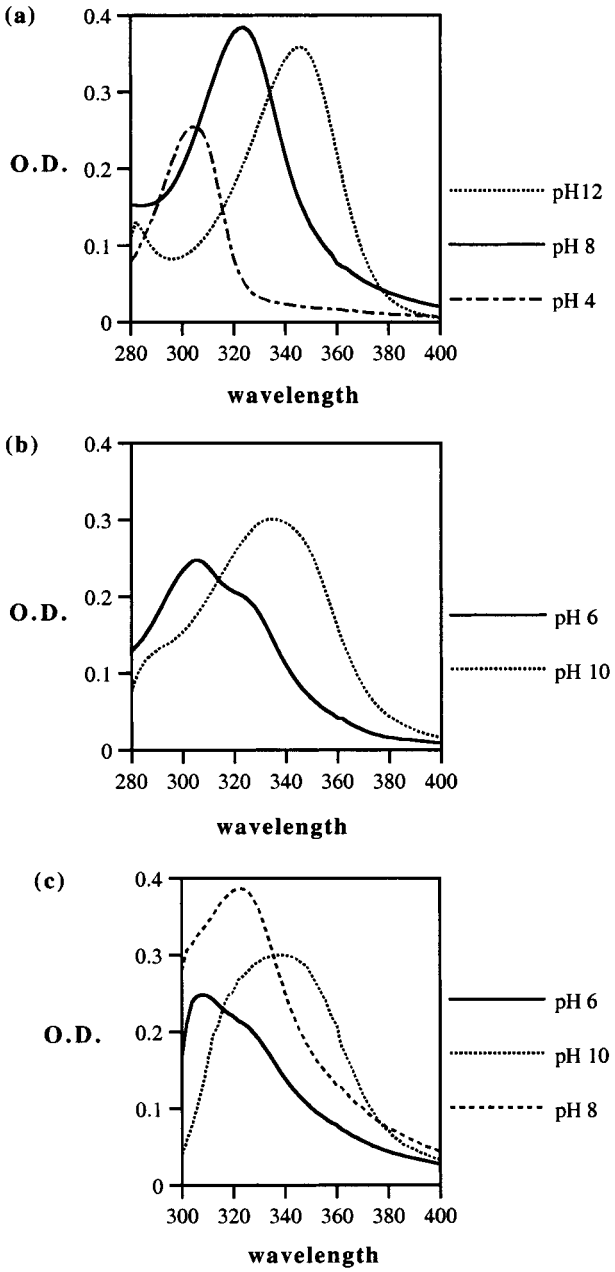


FIGURE 5: UV/vis spectra of (a) TriCHQ (65 μM) in aqueous solution at pH 4.0 (dashed line), 8.0 (solid line), and 12.0 (dotted line); (b) TriCHQ (65 μM) in aqueous solution at pH 6.0 (solid line), and 10.0 (dotted line); and (c) TriCHQ (65 μM) in the presence of C13S TCHQ dehalogenase (300 μM) at pH 6.0 (solid line), 8.0 (dashed line), and 10.0 (dotted line).

Solvent Kinetic Isotope Effect Studies. The solvent kinetic isotope effect on k_{cat} and $k_{cat}/K_{M,TriCHQ}$ was determined at pL = 8.0. There is a substantial normal solvent kinetic isotope effect on both k_{cat} and $k_{cat}/K_{M,TriCHQ}$ (see Table 1).

DISCUSSION

GS-TriCHQ Is not a Kinetically Competent Intermediate. A classical approach to investigating enzyme mechanisms is to determine whether postulated intermediates can be turned over to product by the enzyme in a kinetically competent fashion. We synthesized GS-TriCHQ, the product that would be formed by a nucleophilic aromatic substitution reaction between TCHQ and GSH, and determined that it can be turned over to DCHQ, but at an extremely slow rate.

Table 1: Solvent Kinetic Isotope Effects for Nonenzymatic and Enzymatic Reactions of Glutathione with Electrophilic Substrates

enzyme	reaction	pH	$^{DOD}V_{max}$	$^{DOD}V_{max}/K_{M,sub}$[a]	$^{DOD}k_s$[e]
GST 1-1[b]	GSH + CDNB	8.0	0.971[c] 1.032[d]	0.793	NA
GST 2-2	GSH + CDNB	8.0		0.813	NA
none	GS^- + CDNB	NA[e]			0.837
C13S TCHQ dehalogenase	GSH + TriCHQ	8.0	2.87	4.42	NA

[a] Refers to the non-GSH substrate. [b] From Huskey et al., ref 13. [c] Varied CDNB, 1 mM GSH. [d] Varied GSH, 1.5 mM CDNB. [e] k_s is a pH-independent parameter describing the observed rate constant for reaction of GS^- with CDNB.

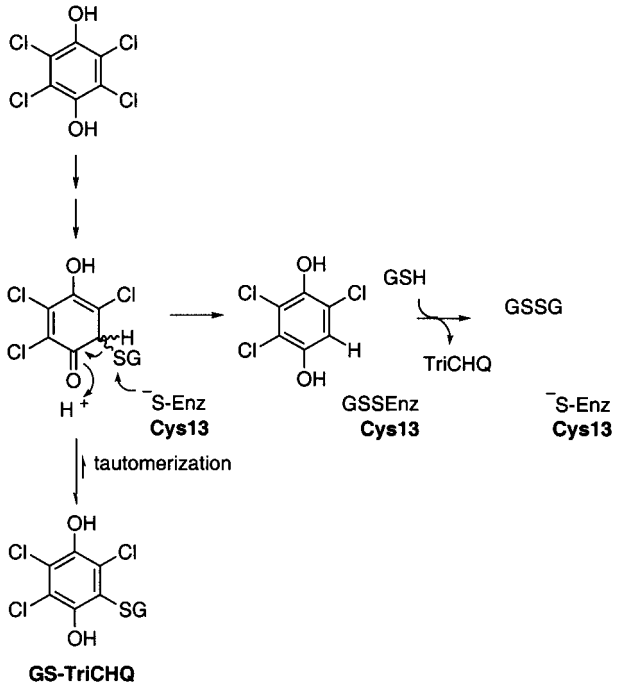


FIGURE 6: Possible explanation for the conversion of GS-TriCHQ to products by C13S TCHQ dehalogenase.

Thus, we conclude that GS-TriCHQ is not a kinetically competent intermediate.

The formation of GS-DCHQ and DCHQ from GS-TriCHQ at low rates under single-turnover conditions can be rationalized based upon our current understanding of the reaction mechanism. We have previously established the intermediacy of 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone in the dehalogenation reaction (*7*). GS-TriCHQ can form this intermediate by tautomerization (see Figure 6). Thus, a rare tautomerization event may result in the formation of 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone, which can be converted to TriCHQ at the active site. If GSH is present, the TriCHQ will be converted to DCHQ, as usual. In the absence of added GSH, DTT can carry out the thiol-disulfide exchange reaction needed to regenerate the enzyme, and the liberated GSH subsequently used to convert TriCHQ to DCHQ. Indeed, DCHQ is formed both in the absence and presence of GSH.

The formation of GS-DCHQ from GS-TriCHQ may occur in the rare event of GS-TriCHQ binding in the active site in an orientation such that the sulfur atom of the glutathionyl substituent occupies a site normally occupied by a chlorine substituent, while the rest of the glutathionyl moiety protrudes

out of the active site. The reaction could then occur according to the usual mechanism. This reaction would require GSH. Figure 3 shows that GS-DCHQ is formed in substantial amounts only in the presence of 1 mM GSH. The small amount formed in the absence of added GSH can be attributed to reaction with the GSH liberated during the conversion of GS-TriCHQ to DCHQ (discussed above). Alternatively, GS-DCHQ could be formed from TriCHQ if the active site cysteine in some of the enzyme has been oxidatively damaged (*7*).

The inability of the enzyme to turn over GS-TriCHQ at a kinetically competent rate is consistent with the hypothesis that GS-TriCHQ is not actually an intermediate during the normal catalytic cycle. However, an alternative interpretation that must be considered is that GS-TriCHQ is truly an intermediate, but is formed at a point in the catalytic cycle at which the active site has a conformation different from that of the free enzyme. In such a case, the intermediate may not be able to bind to the free form of the enzyme. GS-TriCHQ does in fact bind very poorly to TCHQ dehalogenase. Addition of 1 mM GS-TriCHQ to a reaction mixture containing 100 μM TCHQ, 200 μM GSH, 25 mM DTT, and 5 mM ascorbate resulted in only about 40% inhibition of the reaction rate (data not shown).

The poor binding of GS-TriCHQ is not surprising if TCHQ dehalogenase has the typical architecture of the GST superfamily. The sulfur atom of the nucleophile in a nucleophilic aromatic substitution reaction would have to approach TCHQ from above the plane of the ring to interact with the antibonding orbitals of the ring. However, once the substitution reaction takes place, the sulfur atom must lie in the plane of the aromatic ring. Consequently, the shape of the glutathione conjugate is significantly different from that of the reactants. In a crystal structure of a GST with bound product, Ji et al. have shown that the glutathionyl moiety of the glutathione conjugate is bound as it is in the enzyme−glutathione complex, but the aromatic ring protrudes from the active site (*15*). The enzyme cannot simultaneously bind the glutathionyl and aromatic portions of the glutathione conjugate in optimal orientations. We do not yet have a crystal structure of TCHQ dehalogenase, so the possibility of a conformational change that allows the active site to accommodate the glutathione conjugate cannot be eliminated, but it seems unlikely based upon the architecture of the GST superfamily. Thus, the observation that GS-TriCHQ is not a kinetically competent intermediate and binds poorly to the enzyme suggests that it is not formed during the normal catalytic cycle. Further evidence to confirm this statement would be helpful.

C13S TCHQ Dehalogenase is Active at High pH when the Substrate is Deprotonated or Doubly Deprotonated. To further address the mechanistic details of the initial stage of the dehalogenation reaction, we chose to work with the C13S mutant enzyme. The C13S mutant enzyme catalyzes the reaction up to the point at which Cys13 is required. In the absence of Cys13, the intermediate 3,5-dichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone decomposes to a mixture of products in a pH-dependent fashion. This mutant thus allows us to investigate the initial steps of the reaction in the absence of the later steps. We recognize that the change from Cys to Ser at position 13 may have some effects on the initial steps in the mechanism, particularly if the Cys residue is charged while the Ser residue is not. However, we must use the C13S enzyme for kinetic studies because it is not subject to the profound substrate inhibition that makes kinetic studies of the wild-type enzyme impossible (Anandarajah, Kiefer, and Copley, unpublished results). We have designed a continuous UV assay using TriCHQ as a substrate that allows accurate determination of kinetic parameters under a variety of conditions.

Figure 4 shows the pH profile for dehalogenation of TriCHQ by the C13S mutant enzyme. The C13S enzyme maintains activity over a broad range of pH values, even at pH values as high as 10. (The wild type enzyme is also active at pH 10, but kinetic parameters cannot be determined due to the substrate inhibition.) The UV/vis spectrum of TriCHQ at the active site of TCHQ dehalogenase suggests that the substrate is present as a mixture of deprotonated and doubly deprotonated forms at pH 10 (see Figure 5). Thus, the enzyme can dehalogenate substrate that is negatively charged and possibly, at high pH, even substrate that is doubly negatively charged. This observation makes a nucleophilic aromatic substitution reaction extremely unlikely. An S_NAr reaction that proceeds through an intrinsically unstable negatively charged intermediate could not occur with a negatively charged substituent on the ring. Likewise, it is unlikely that the initial electron transfer required for an $S_{RN}1$-like reaction would occur to a substrate bearing a negatively charged substituent.

Solvent Kinetic Isotope Effects on the Dehalogenation Reaction. To further probe the possibility of an S_NAr reaction during dehalogenation of TCHQ and TriCHQ, we investigated the solvent kinetic isotope effect on the conversion of TriCHQ to products by the C13S mutant enzyme. Solvent kinetic isotope effects for the reaction of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) have been reported by Huskey et al. (*16*). Their results for the enzymic reaction catalyzed by GST isozymes 1-1 and 2-2 and for the nonenzymic reaction are summarized in Table 1. Notably, the solvent kinetic isotope effects on $V/K_{M,CDNB}$ for both isozymes and on k_s for the uncatalyzed reaction are substantially inverse. (k_s is the apparent rate constant for the reaction of the thiolate form of glutathione with the substrate and is pH-independent.) The inverse kinetic isotope effect has been interpreted as being due to changes in solvation of the sulfur atom between the thiolate nucleophile and the transition state, in which the negative charge on the sulfur is diminished. In contrast to these results, the solvent kinetic isotope effects for both V and $V/K_{M,TriCHQ}$ for the dehalogenase reaction are large and normal. If the dehalogenase mechanism included an S_NAr reaction, then we would expect an inverse solvent kinetic isotope effect, since the attack of GS^- on TCHQ should be more difficult, and consequently, more kinetically significant, than the attack of GS^- on CDNB catalyzed by isozymes 1-1 and 2-2. The observed large normal solvent kinetic isotope effects will be discussed in the context of another mechanism for the enzyme in the following paper in this issue.

CONCLUSION

The mechanism shown in Figure 2 was proposed based upon the known ability of enzymes in the GST superfamily to catalyze S_NAr reactions. A nonenzymic S_NAr reaction

involving TCHQ would be expected to be difficult because of the relatively weak ability of chlorine substituents to stabilize the negative charge in the ring in the Meisenheimer complex intermediate. In fact, the nonenzymic reaction is undetectably slow. However, rates of enzymatic S_NAr reactions are considerably less sensitive to the nature of substituents (*12*). Furthermore, enzymes are capable of catalyzing reactions by factors of up to 10^{17} (*17*), and it is difficult to predict what level of catalysis might be achieved at an active site that is configured to accelerate the reaction by desolvating the nucleophile and providing shape and charge complementarity to the Meisenheimer complex intermediate. Consequently, we undertook experimental studies to test the validity of the mechanism shown in Figure 2. We have shown using three independent approaches that a nucleophilic aromatic substitution reaction is unlikely to occur during dehalogenation of TCHQ. First, the product of a nucleophilic aromatic substitution reaction, GS-TriCHQ, is not a kinetically competent substrate for TCHQ dehalogenase. Second, the enzyme is active when the substrate at the active site is negatively charged due to ionization of one or even both hydroxyls. Third, the solvent kinetic isotope effects on V and $V/K_{M,TriCHQ}$ are inconsistent with those expected for a reaction involving a kinetically significant attack of GS^- on the aromatic substrate. The combination of these three findings allows us to eliminate this mechanism from further consideration. The following paper will explore two other possible mechanisms for the reaction which begin with ketonization of the aromatic ring to form 2,3,5,6-tetrachloro-4-hydroxycyclohexa-2,4-dienone, rather than a nucleophilic aromatic substitution reaction.

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