Exploration of the Relationship between Tetrachlorohydroquinone Dehalogenase and the Glutathione *S*-Transferase Superfamily†

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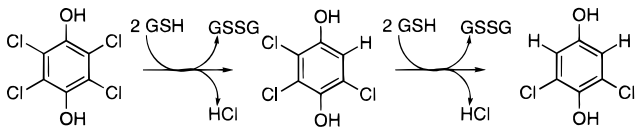
ABSTRACT: Tetrachlorohydroquinone dehalogenase is found in *Sphingomonas chlorophenolica*, a soil bacterium that degrades pentachlorophenol, a widely used wood preservative. This enzyme converts tetrachlorohydroquinone (TCHQ) to trichlorohydroquinone (TriCHQ) and TriCHQ to dichlorohydroquinone (DCHQ) (Xun et al. (1992) *J. Bacteriol. 174*, 8003−8007). The reducing equivalents for each step are provided by two molecules of glutathione (Xun et al. (1992) *Biochem. Biophys. Res. Commun. 182*, 361−366). In addition to the expected TriCHQ and DCHQ products, the enzyme also produces substantial amounts of 2,3,5-trichloro-6-*S*-glutathionylhydroquinone (GS-TriCHQ) and an unidentified isomer of dichloro-*S*-glutathionylhydroquinone (GS-DCHQ). Treatment of the purified enzyme with dithiothreitol dramatically decreases the formation of GS-TriCHQ and GS-DCHQ. Furthermore, enzyme in freshly-prepared crude extracts forms only very small amounts of GS-TriCHQ and GS-DCHQ. We conclude that GS-TriCHQ and GS-DCHQ are produced by enzyme that has undergone some type of oxidative damage and are therefore not physiologically relevant products. The fact that the oxidative damage can be repaired by DTT suggests that a cysteine or methionine residue may be involved. We have created the C13S and C156S mutants of the enzyme. The C13S mutant converts TCHQ to GS-TriCHQ and GS-DCHQ, rather than to DCHQ. Thus, Cys13 is required for the reductive dehalogenation of TCHQ. A mechanism for the reaction which involves Cys13 is proposed.

About 15 000 chlorinated organic compounds are currently in use as pesticides, solvents, pharmaceuticals, and polymers (Hileman, 1993). The large-scale manufacture, use, and disposal of these compounds has resulted in significant environmental contamination. Biological degradation is one of the most important mechanisms for breakdown of chlorinated organic compounds in the environment. Microorganisms that degrade even recalcitrant compounds can in many cases be isolated from contaminated sites. For example, microorganisms that degrade 4-chlorobenzoate (Adriaens et al., 1989; Löffler et al., 1991; Marks et al., 1984; van den Tweel et al., 1986), 3-chlorobenzoate (Dorn & Knackmuss, 1978; Haller & Finn, 1979), some congeners of polychlorinated biphenyls (PCBs) (Abramowicz, 1990), and many other xenobiotic compounds have been isolated.

Highly halogenated aromatic compounds such as PCBs[1] and pentachlorophenol are particularly resistant to biodegradation by aerobic microorganisms because chlorine substituents interfere with the action of dioxygenase enzymes that oxidatively cleave aromatic rings. Therefore, biodegradation of these compounds requires initial removal of halogen substituents from the aromatic rings. Removal of halogen substituents can take place in three fundamentally different ways. The first is a hydrolytic reaction in which the halogen substituent is replaced by a hydroxyl group derived from H_2O. The second is an O_2-dependent reaction in which the halogen is replaced by a hydroxyl group in which the oxygen is derived from O_2. The third is a reductive dehalogenation in which the halogen is replaced by a hydrogen atom. Of these three types of reactions, reductive dehalogenation is the least well understood.

Scheme 1



We have recently undertaken studies of tetrachlorohydroquinone (TCHQ) dehalogenase, an enzyme that catalyzes a reductive dehalogenation reaction. TCHQ dehalogenase is found in the Gram-negative soil bacterium *Sphingomonas chlorophenolica* sp. nov. (Xun et al., 1992a; McCarthy and Copley, unpublished results; Nohynek et al., 1995) (previously known as *Flavobacterium* sp. ATCC 39723), which degrades pentachlorophenol to CO_2, H_2O, and HCl. This enzyme catalyzes the reductive dehalogenation of TCHQ to trichlorohydroquinone (TriCHQ) and subsequently to 2,6-dichlorohydroquinone (DCHQ) (see Scheme 1). The reducing equivalents for the reaction are provided by glutathione (GSH) (Xun et al., 1992b).

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[1] Abbreviations: PCBs, polychlorinated biphenyls; TCHQ, tetrachlorohydroquinone; TriCHQ, trichlorohydroquinone; DCHQ, dichlorohydroquinone; MCHQ, chlorohydroquinone; GSH, glutathione; GSSG, glutathione disulfide; GS-TriCHQ, 2,3,5-trichloro-6-*S*-glutathionyl hydroquinone; GS-DCHQ, dichloro-*S*-glutathionylhydroquinone (isomer not specified); DTT, dithiothreitol; *β*-ME, *β*-mercaptoethanol; NADPH, nicotinamide adenine dinucleotide phosphate, reduced.

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TCHQ dehalogenase has limited sequence similarity with proteins in the theta class of the glutathione *S*-transferase superfamily (Orser et al., 1993). The sequence identity between TCHQ dehalogenase from *S. chlorophenolica* sp. nov. strain ATCC 39723 and the most similar glutathione *S*-transferases (those from *Proteus mirabilis*, *Escherichia coli*, and *Pseudomonas sp*. LB400, see Figure 7) ranges from ~26% to 30%. The sequence identity is somewhat greater in the N-terminal region (P. Babbitt, unpublished results). Most of the determinants for the glutathione binding site are located in the N-terminal domain of the glutathione *S*-transferases. Whether the similarity between TCHQ dehalogenase and the glutathione *S*-transferases reflects just a conserved glutathione binding site or fundamental similarities between the structures and mechanisms of these enzymes is of great interest with respect to the evolutionary origin of TCHQ dehalogenase. We describe here findings that suggest that the initial stages of the reaction catalyzed by TCHQ dehalogenase do indeed resemble reactions catalyzed by glutathione *S*-transferases. However, TCHQ dehalogenase has additional catalytic capabilities that allow it to convert glutathione conjugates into reduced products. By site-directed mutagenesis, we have shown that Cys13 is required for this process.

MATERIALS AND METHODS

Materials. TCHQ was obtained from Kodak. 2,6-Dichlorobenzoquinone was obtained from Chem Service Chemicals. *E. coli* strains JDO1 and CCL3 were generous gifts from Dr. Cindy Orser. [U-^{14}C]TCHQ was prepared from [U-^{14}C]pentachlorophenol (Sigma) using an extract from *E. coli* strain CCL3, which overexpresses pentachlorophenol monooxygenase, plus NADPH. GS-TriCHQ was prepared by the method of van Ommen et al. (1988). GSSG reductase was obtained from Sigma.

Enzyme Assay. Enzyme activity was measured using a discontinuous HPLC assay. Reaction mixtures included 100 μM TCHQ, 1 mM GSH, 1−5 mM ascorbate (to protect hydroquinone substrates and products from oxidation), 1 mM EDTA, and enzyme in 25 mM potassium phosphate buffer, pH 7.2. In some cases, other components such as DTT, NADPH, and glutathione reductase were also added. At intervals after the addition of enzyme, aliquots were removed and quenched by the addition of an equal volume of 1 N HCl. Reaction mixtures were analyzed by reverse-phase HPLC on a Rainin C18 column. For analysis of TCHQ disappearance, an isocratic elution with 25% acetonitrile was performed, and the detector was set at 210 nm. For analysis of products, a gradient of 0.1% acetic acid and acetonitrile (0−50% between 0 and 10 min and 50−55% between 10 and 40 min) was employed. The detector was set at 285 nm. Reactants and products were quantitated either by comparison of peak areas with a standard curve generated on the same day or, in the case of experiments using [U-^{14}C]TCHQ, by scintillation counting of fractions.

Purification of TCHQ Dehalogenase. TCHQ dehalogenase was purified from *E. coli* strain JDO1, a derivative of JM105 which contains the *S. chlorophenolica* sp. nov. strain ATCC 39723 TCHQ dehalogenase gene (*pcp C*) under control of the *tac* promoter in pKK233-3. Bacteria were grown in 2YT medium containing potassium phosphate buffer (30 mM, pH 7.8), glucose (5 mM), and ampicillin (50 μg/mL). Enzyme expression was induced with IPTG (0.5 mM) when the cells had reached an optical density at 600 nm of 1.3. Three hours after induction, the cells were harvested by centrifugation at 9000*g* for 10 min. The cells were washed with 140 mM KCl and then resuspended in lysis buffer (25 mM potassium phosphate, pH 8.0, containing 200 mM KCl, 5 mM dithiothreitol (DTT), 1 mM EDTA, 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) protease inhibitor, 10 μg/mL DNase I, and 200 μg/mL lysozyme) (5 mL/g of cells) and incubated with stirring at 4 °C for 30 min. The suspension was then passed three times through a French pressure cell at 12000 psi. Cell debris was removed by centrifugation at 15000*g* for 30 min.

The supernatant was applied to a column of Reactive Blue 72 dye equilibrated in 100 mM potassium phosphate, pH 8.0, containing 1 mM EDTA and 2 mM DTT. The column was washed with 4 volumes of the same buffer. Proteins were eluted with 25 mM potassium phosphate buffer, pH 8.0, containing 2 M NaCl, 1 mM EDTA, and 2 mM DTT. Fractions containing TCHQ dehalogenase activity were pooled and concentrated by precipitation with ammonium sulfate (final concentration 70% of saturation). The pellet was collected by centrifugation, redissolved, and dialyzed against 20 mM MOPS buffer, pH 7.2, containing 1 mM EDTA and 2 mM DTT. The protein was then applied to a Pharmacia MonoQ anion exchange column equilibrated with 20 mM MOPS buffer, pH 7.2, containing 1 mM EDTA and 2 mM DTT. Proteins were eluted with a gradient of 0−0.25 M KCl in the same buffer. The final purification step was carried out on a Pharmacia Superdex 200 column equilibrated with 25 mM potassium phosphate, pH 7.2, containing 200 mM KCl, 1 mM EDTA, and 2 mM DTT.

Analysis of Metal Content. The metal ion content of 3 mg of pure, active enzyme was determined by inductively coupled plasma emission spectroscopy at the Soil, Water and Plant Testing Laboratory at Colorado State University.

Electrospray LC/MS Analyses. Positive ion electrospray mass spectrometry was carried out on a Perkin Elmer SCIEX API III LC/MS/MS system. Samples of the glutathione conjugates were purified by reverse-phase HPLC prior to analysis.

Construction of C13S and C156S Mutant Enzymes. The *pcpC* gene was excised from pJDO1 by digestion with *Hin*dIII and *Bam*HI and then ligated into the p-ALTER-1 vector provided by Promega. Mutagenesis reactions were carried out as described by Promega using the following mutagenic oligonucleotides: (1) for the C13S mutant, 5′-ATG TCG ATC AGC TCG ATG AAG-3′; and (2) for the C156S mutant, 5′-CAT GGC AAT AGC GCC TAT GAC-3′ (the underlined bases indicate the substitutions required to change the codon). The products of the mutagenesis reactions were initially transformed into *E. coli* mutS-ES1301. Subsequently, plasmids were transformed into *E. coli* JM105 for stable maintenance. Mutations were confirmed by DNA sequencing. The absence of undesired secondary mutations was demonstrated by electrospray mass spectral analysis of the purified proteins which showed that the masses of both mutant enzymes were 28 100 daltons, exactly as expected for the appropriate amino acid sequences lacking the N-terminal methionine.

Sequence Analysis. TCHQ dehalogenase was used as a query sequence to search all available sequence databases (including Genbank, PIR, PDB, SwissProt, EMBL) with the

blast programs in the GCG sequence analysis package and the blast suite of programs available at the NCBI (through the internet). Approximately 75 of the highest scoring nonredundant sequences were examined and used to generate a set of 25 glutathione *S*-transferase or glutathione *S*-transferase-like sequences representing a divergent range of primary structures, none of which was more than 60% identical to any other sequence in the set. These sequences were aligned using the PILEUP function in the GCG sequence analysis package (gapweight = 3.0; GapLengthWeight = 0.100). From this alignment, the 11 sequences most similar to TCHQ dehalogenase, overall, were used to generate Figure 7. Homology is shown by shading as determined from the SeqVu sequence analysis program (Kyte-Doolittle scale at a 65% setting as provided by the SeqVu package) (SeqVu, James Gardner, The Garvan Institute of Medical Research, Sydney, Australia (1995)).

RESULTS AND DISCUSSION

Development of Improved Enzyme Assay. The assay originally used by Xun et al. was an end-point assay in which reactions were quenched after 3 min by addition of acetonitrile and then analyzed by reverse-phase HPLC (Xun et al., 1992a). Using [U-^{14}C]TCHQ as a substrate, we discovered that this procedure results in elution of approximately 80% of the substrate in the void volume instead of at the expected retention time for TCHQ. We suspect that addition of acetonitrile denatures the protein and exposes hydrophobic areas of the protein. TCHQ apparently associates with the denatured protein and elutes in the void volume. This problem was avoided by quenching the reaction with acid. Under these conditions, TCHQ and its various reaction products elute at the expected retention times upon HPLC, and no radioactivity can be detected in the void volume. We have also modified the assay to include analysis of multiple time points, rather than a single end point, to allow for more accurate quantitation of the enzyme activity and determination of kinetic parameters.

TCHQ Dehalogenase Contains No Transition Metal or Organic Cofactors. The mechanism of a reductive dehalogenase such as TCHQ dehalogenase has never before been studied. Our initial efforts were directed at the question of whether reducing equivalents from glutathione might be shuttled to the substrate via a transition metal ion (or cluster) or an organic cofactor on the enzyme. The possibility that a transition metal catalyst accomplishes the reductive dehalogenation by an $S_{RN}1$-type reaction is appealing. The only other reductive dehalogenase that has been purified is a reductive 3-chlorobenzoate dehalogenase from *Desulfomonile tiedjei* DCB-1. The spectral characteristics of this enzyme suggest that it is a heme protein (Ni et al., 1995). 4-Hydroxybenzoyl CoA reductase is an iron−sulfur protein that catalyzes a reductive dehydroxylation that could be similar to reductive dehalogenation (Brackmann & Fuchs, 1993). In nonenzymatic systems, reductive dehalogenation of aryl halides has been accomplished with low-valent nickel compounds (Fox et al., 1991), as well as with cofactors containing transition metals. For example, vitamin B_{12}, coenzyme F_{430}, and hematin catalyze the reductive dechlorination of hexachlorobenzene. Vitamin B_{12} also catalyzes the reductive dechlorination of pentachlorobenzene and pentachlorophenol (Gantzer & Wackett, 1991).

An interesting model for the TCHQ dehalogenase reaction which involves transfer of reducing equivalents through an organic cofactor is the reaction catalyzed by glutathione reductase. This enzyme catalyzes the transfer of reducing equivalents from NADPH to oxidized glutathione (GSSG). The reducing equivalents are passed first from NADPH to a covalently bound flavin and then to a disulfide bond on the enzyme. A disulfide exchange reaction then accomplishes the reduction of GSSG (Douglas, 1987). A similar process in the reverse direction could be utilized to transfer reducing equivalents from two molecules of GSH to TCHQ.

To evaluate the possibility of indirect delivery of reducing equivalents from GSH to TCHQ, we have examined the cofactor content of the purified TCHQ dehalogenase. Inductively coupled plasma emission spectroscopy of a sample of purified, active enzyme indicated that the enzyme does not contain Fe, Mn, Cu, Zn, Ni, Mo, or Pb. The UV/vis spectrum of a concentrated sample of enzyme showed no evidence for an absorbance band due to an organic cofactor (data not shown). Extraction of the enzyme with chloroform did not release any chromophoric material. Finally, electrospray LC/MS analysis of the purified enzyme showed no evidence for cofactors of any kind, as the measured mass of the protein was exactly that predicted by the amino acid sequence.

Our finding that TCHQ dehalogenase contains neither metal ion nor organic cofactors rules out mechanisms involving indirect transfer of reducing equivalents between TCHQ and GSH and suggests that the reducing electrons must be transferred by some type of covalent interaction with either glutathione or an enzymic thiol.

Analysis of Products Formed in Reaction Mixtures Containing TCHQ, GSH, and TCHQ Dehalogenase. Figure 1 shows chromatograms of aliquots of a reaction mixture containing [U-^{14}C]TCHQ, TCHQ dehalogenase, GSH, and ascorbic acid (to protect TCHQ and GSH from oxidation) quenched early in the reaction and at the completion of the reaction. As expected, peaks due to TriCHQ and DCHQ are seen. Notably, two additional products also accumulate during the reaction. Positive ion electrospray mass spectral analysis of these products demonstrates that the peaks appearing in the HPLC chromatogram at 22.5 and 25.2 min are an unidentified isomer of 2-*S*-glutathionyldichlorohydroquinone (GS-DCHQ), and 2-*S*-glutathionyl-3,5,6-trichlorohydroquinone (GS-TriCHQ), respectively. (The peak assigned as GS-TriCHQ gives a molecular ion at m/z = 518, in agreement with that given by authentic GS-TriCHQ, and the peak assigned as GS-DCHQ gives a molecular ion at m/z = 484. Each molecular ion shows the appropriate chlorine isotope pattern.) The formation of glutathione conjugates during the dehalogenation reaction was also proposed by Xun et al., based upon the observation of peaks in HPLC chromatograms that contained both an organic chromophore and ^{3}H originating from [^{3}H]GSH (Xun et al., 1992a).

Figure 2 shows the composition of a reaction mixture containing [U-^{14}C]TCHQ, TCHQ dehalogenase, GSH, and ascorbic acid as a function of time. The results demonstrate that TriCHQ appears early in the reaction and that its disappearance is correlated with the appearance of DCHQ. GS-TriCHQ and GS-DCHQ accumulate to significant and stable levels. The amounts of GS-TriCHQ and GS-DCHQ formed from TCHQ vary between preparations of the

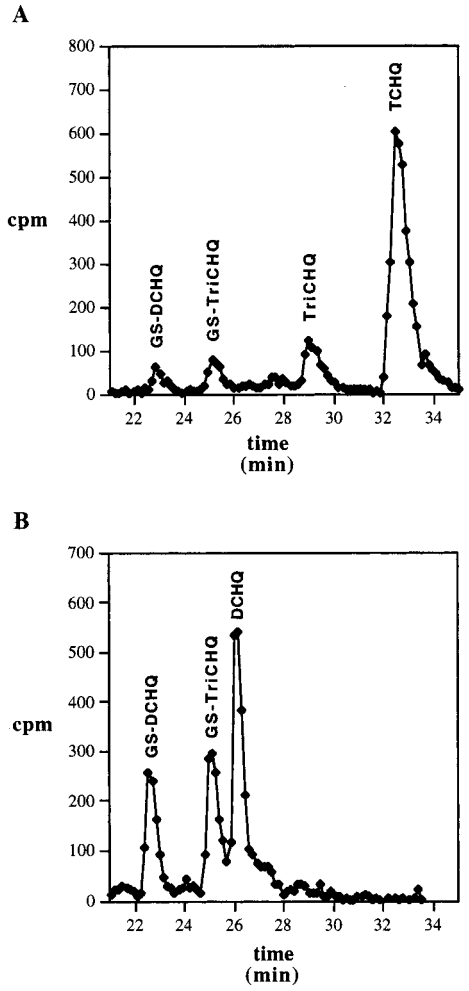

FIGURE 1: Reverse-phase HPLC chromatograms of quenched aliquots of reaction mixtures containing [U-^{14}C]TCHQ (100 μM), GSH (1 mM), ascorbate (5 mM), and TCHQ dehalogenase. Fractions were collected and radioactivity was determined by scintillation counting. (A) Quenched at 0.5 min; (B) quenched at 3.5 min.

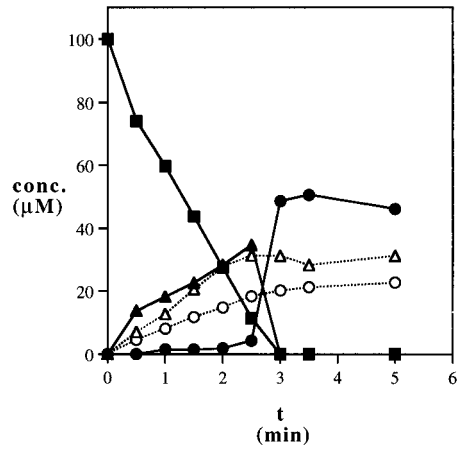

FIGURE 2: Time course of dehalogenation reaction containing [U-^{14}C]TCHQ (100 μM), GSH (1 mM), ascorbate (5 mM), and TCHQ dehalogenase. Aliquots were quenched with 1 N HCl at various times and analyzed by reverse-phase HPLC. Fractions were collected and radioactivity was determined by scintillation counting. (■) TCHQ; (▲) TriCHQ; (●) DCHQ; (△) GS-TriCHQ; (○) GS-DCHQ.

enzyme. In the experiment shown in Figure 2, GS-TriCHQ and GS-DCHQ ultimately account for 31% and 23% of the initial TCHQ substrate, respectively. No GS-TriCHQ or GS-DCHQ is formed when enzyme is omitted from the reaction mixture or when the enzyme is inactivated by heat treatment prior to addition of substrates. Furthermore, the amounts of GS-TriCHQ and GS-DCHQ are the same regardless of whether the reaction is run in the presence of room air or under an inert N_2 atmosphere (data not shown).

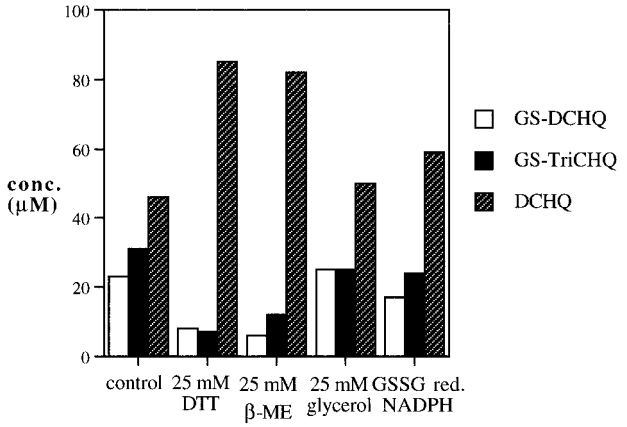

FIGURE 3: Effect of various reaction components upon the distribution of products formed from TCHQ by purified TCHQ dehalogenase. Reaction mixtures contained [U-^{14}C]TCHQ (100 μM), GSH (1 mM), ascorbate (5 mM), TCHQ dehalogenase, and additions as indicated. In the final experiment shown, the concentrations of GSSG and NADPH were 1 U/mL and 1 mM, respectively. Aliquots were quenched with 1 N HCl after 10 min and analyzed by reverse-phase HPLC. Fractions were collected and radioactivity was determined by scintillation counting.

The kinetics of the appearance of GS-TriCHQ and GS-DCHQ are surprising. We initially expected that GS-TriCHQ would be formed from TCHQ and GS-DCHQ would be formed from TriCHQ. However, GS-DCHQ clearly is not formed solely from TriCHQ, since it appears before a significant amount of TriCHQ accumulates. An explanation for these puzzling data is provided by experiments with the C13S mutant enzyme which will be described below.

Xun et al. suggested that glutathione conjugates are intermediates in the conversion of TCHQ to TriCHQ and of TriCHQ to DCHQ (Xun et al., 1992a). The data shown in Figure 2 do not support this hypothesis, since GS-TriCHQ and GS-DCHQ are not converted to their respective reduced products. Furthermore, there is no lag between the formation of GS-TriCHQ and the formation of TriCHQ, which would be expected if TriCHQ were formed from GS-TriCHQ (by analogy with the lag observed for the formation of DCHQ from TriCHQ). We can certainly conclude that TCHQ dehalogenase is not a bifunctional enzyme with one active site for conversion of TCHQ to GS-TriCHQ (and TriCHQ to GS-DCHQ) and a second active site for conversion of GS-TriCHQ to TriCHQ (and GS-DCHQ to DCHQ). However, from these data, we cannot make any conclusions about the intermediacy of the glutathione conjugates in the reductive dehalogenation reaction at the active site of the enzyme. If the glutathione conjugates are intermediates, however, they must be unable to rebind and complete the catalytic cycle once they are released from the surface of the enzyme.

Effect of Reaction Conditions on the Rate of TCHQ Disappearance and Product Distribution. Addition of 25 mM DTT to reaction mixtures has a dramatic effect on the distribution of products formed from TCHQ by purified TCHQ dehalogenase (see Figure 3). The glutathione con-

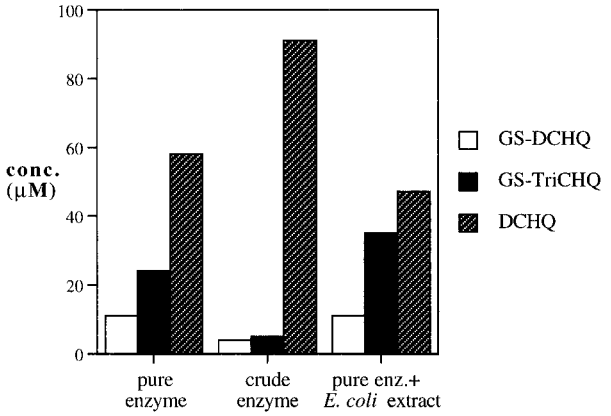

FIGURE 4: Distribution of products formed by TCHQ dehalogenase in crude extracts and by purified enzyme in the absence and presence of crude extract from a strain of *E. coli* that does not produce TCHQ dehalogenase. Reaction mixtures contained [U-^{14}C]TCHQ (100 μM), GSH (1 mM), ascorbate (5 mM), TCHQ dehalogenase, and additions as indicated. Aliquots were quenched with 1 N HCl after 30 min and analyzed by reverse-phase HPLC. Fractions were collected and radioactivity was determined by scintillation counting.

jugates GS-TriCHQ and GS-DCHQ account for 54% of the products formed from TCHQ by purified enzyme. The amount of these glutathione conjugates was significantly reduced (to 15%) by inclusion of 25 mM DTT in the reaction mixture. Addition of β-mercaptoethanol had similar effects, while addition of glycerol had no effect, demonstrating that the effect is due to the thiol moiety and not to some nonspecific effect of the sugar. Addition of glutathione reductase and NADPH to reduce the glutathione disulfide produced during the reaction had little effect. Therefore, the effect of DTT is not simply to maintain glutathione in the reduced state. Finally, the change in product distribution was not due to nonenzymatic reactions between the thiol reagents and the glutathione conjugates (data not shown).

The data shown in Figure 3 suggest that the glutathione conjugates are produced by enzyme that has undergone some type of oxidative damage that can be repaired by thiol reagents. To explore this hypothesis, we determined the distribution of products formed by TCHQ dehalogenase in freshly-prepared crude extracts. Figure 4 shows that enzyme in crude extracts produces only very small amounts of the glutathione conjugates. Since a crude extract might contain some protein or small molecule that could affect the product distribution, a control experiment was performed in which crude extract from a strain of *E. coli* that does not overproduce TCHQ dehalogenase was added to the purified enzyme. The product distribution was not affected by any components of the crude extract. Further control experiments showed that GS-TriCHQ (and therefore presumably GS-DCHQ) was stable in the presence of the crude extract. These data are consistent with the hypothesis that TCHQ dehalogenase undergoes oxidative damage during purification and that the glutathione conjugates are formed by the oxidatively damaged enzyme.

While the glutathione conjugates are apparently not physiologically relevant products, the fact that damaged enzyme catalyzes their production so effectively is intriguing. We have considered two possible explanations for the formation of these glutathione conjugates. First, the glutathione conjugates may represent true reaction intermediates that either cannot complete the normal catalytic cycle at or

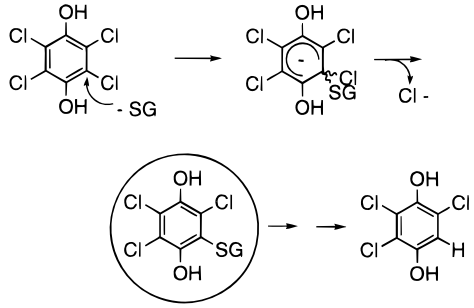

FIGURE 5: Possible mechanism for TCHQ dehalogenase in which GS-TriCHQ is an intermediate.

are inadvertantly released from a damaged active site. A mechanism consistent with this possibility is shown in Figure 5. The initial stage of this reaction is exactly analogous to the reactions catalyzed by glutathione *S*-transferases in that it involves nucleophilic attack of glutathione upon an electrophilic substrate to generate a glutathione conjugate. Subsequent steps (not specified at this point) would be required to convert the glutathione conjugate into the expected reduced product. Oxidative damage could impair the ability of catalytic groups at the active site to complete the reaction, either by direct damage to a catalytic group or by perturbation of the active site structure.

A second possible explanation for the formation of the glutathione conjugates is that the conjugates are products of catalysis at the active site of a damaged enzyme and are *not* true intermediates in the normal dehalogenation reaction. A mechanism consistent with this hypothesis is shown in Figure 6A. In this mechanism, glutathione attacks a chlorine substituent, rather than the aromatic ring. Nucleophilic displacements on halogen atoms are well documented, although displacements on chlorine are generally much slower than displacements on bromine or iodine (Zefirov & Makhon'kov, 1982; Jarvis & Marien, 1977; Montanari et al., 1993; Gentile & Mendarelli, 1988; Bard et al., 1979; Seshadri et al., 1981). Soft nucleophiles such as thiols are especially effective in this type of reaction. If the mechanism of the enzyme were as shown in Figure 6A, then oxidative damage to the active site might alter the position of the bound glutathione so that it would attack the carbon bearing the chlorine substituent, rather than the chlorine itself (see Figure 6B). The product would be GS-TriCHQ. In this case, the glutathione conjugates would *not* be intermediates in the normal dehalogenation reaction.

The data described so far do not allow us to determine whether the glutathione conjugates are actual intermediates in the normal catalytic process, or simply aberrant products produced at the active site of a damaged enzyme. We chose to investigate this issue further in the hope that understanding the origin of these glutathione conjugates would provide information about the structure and mechanism of the enzyme.

We began by considering the types of oxidative damage to proteins that can be repaired by treatment with DTT. The most likely possibilities are an inappropriate disulfide bond, a cysteine sulfenic acid, or a methionine sulfoxide. TCHQ dehalogenase contains two cysteines and eight methionines which could be involved in oxidative damage. One of these cysteines is a particularly promising candidate. Figure 7 shows the first 27 residues of the N-terminal region of TCHQ dehalogenase aligned with the N-termini of several members

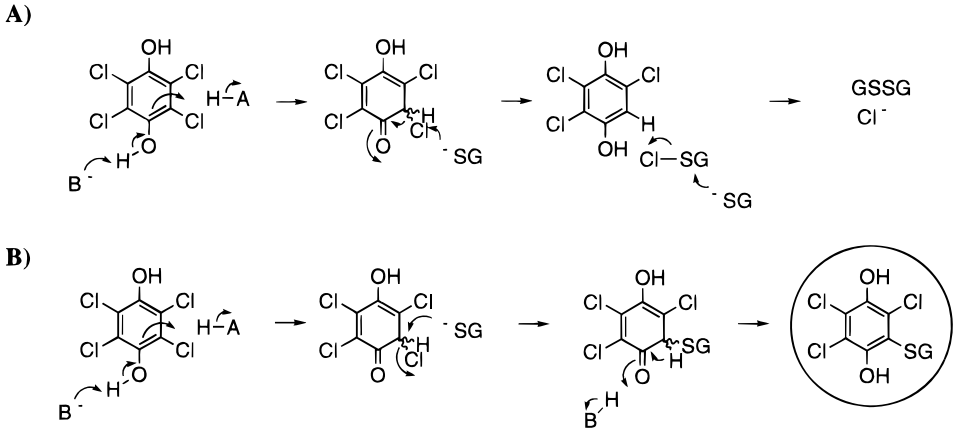

FIGURE 6: Possible mechanism for TCHQ dehalogenase in which GS-TriCHQ is not an intermediate. (A) At an undamaged active site; (B) at a damaged active site.

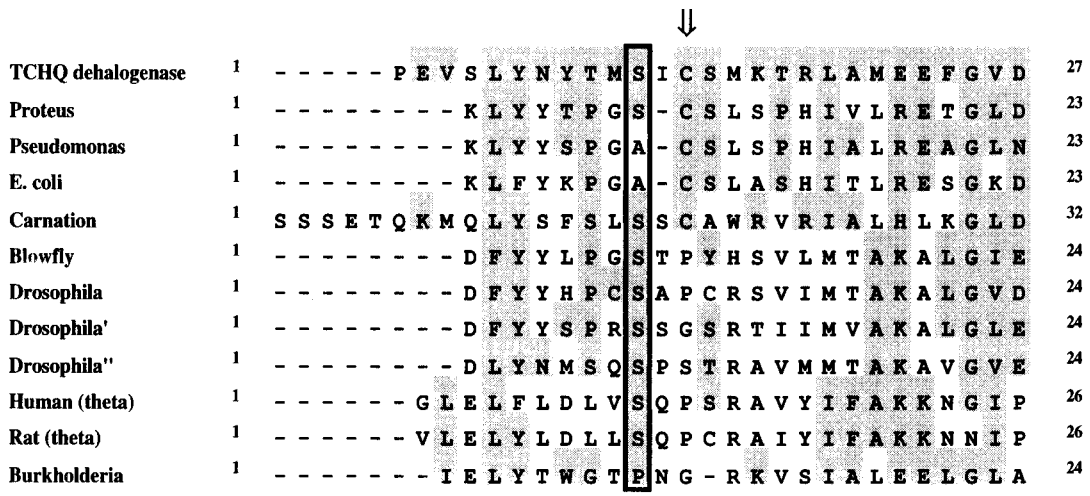

FIGURE 7: Alignment of the N-terminal region of TCHQ dehalogenase with the corresponding regions of some members of the glutathione *S*-transferase superfamily. The conserved serine residue is boxed, and the nearby Cys13 residue in TCHQ dehalogenase is marked with an arrow. Sequence homology in relation to TCHQ dehalogenase is designated by gray shading as described in the Materials and Methods. Database accession numbers for the sequences used in the alignment are taken from Genbank unless otherwise designated as PIR (Protein Identification Resource) or SP (SwissProt). TCHQ dehalogenase, M9855; *Proteus*, S29772 (SP); *Pseudomonas*, X76500; *E. coli*, D38497; carnation, X58390; blowfly, L23126; Drosophila, JQ1378 (PIR); Drosophila′, E46681 (PIR); Drosophila″, B46681 (PIR); human (theta), L38503; rat (theta), S27161 (PIR); Burkholderia, U19883, [Orf3].

of the theta class of the glutathione *S*-transferase superfamily (Rossjohn et al., 1996) as well as other superfamily members not yet definitely assigned to a glutathione *S*-transferase class. The generally conserved serine (boxed in Figure 7) has been proposed to be responsible for stabilizing the thiolate of glutathione at the active site in many of the theta class enzymes (Rossjohn et al., 1996). The recently published structure of the Australian sheep blowfly theta class glutathione *S*-transferase shows that the hydroxyl group of the serine at position 9 is 3.9 Å away from the sulfur atom of the glutathione (Wilce et al., 1995), and mutation of this serine to an alanine abolishes enzyme activity (Board et al., 1995). Notably, one of the two cysteine residues in TCHQ dehalogenase is only two residues away from the conserved serine (see arrow in Figure 7). Thus, Cys13 is likely to be in or near the active site, and oxidative damage to this residue could certainly affect the catalytic abilities of the enzyme.

In order to explore the roles of Cys13 and Cys156 in catalysis by and oxidative damage to TCHQ dehalogenase, we have created mutant enzymes in which these residues have been replaced with Ser. These replacements should perturb the structure of the enzyme only slightly. If one of these Cys residues is required for catalysis, the mutant enzyme should show altered reactivity. On the other hand, if a Cys residue is merely involved in the oxidative damage, the mutant enzyme might have wild-type activity, but no longer be susceptible to oxidative damage. The functional consequences of these replacements are revealing. In the presence of DTT, the overall rate of disappearance of TCHQ is similar for the mutant and wt enzymes (data not shown). However, the striking difference between the enzymes is that the C13S mutant converts TCHQ *only* to GS-TriCHQ and GS-DCHQ and does not produce any DCHQ (see Figure 8). Clearly, Cys13 is required for the reductive dehalogenation of TCHQ to TriCHQ and of TriCHQ to DCHQ. However, the observed product distribution poses an interesting mechanistic puzzle. Any mechanistic model we propose must account for why the C13S mutant, which appears to be incapable of catalyzing the normal reductive reaction, is nevertheless capable of producing GS-DCHQ, which is reduced with respect to TCHQ and GS-TriCHQ.

A mechanism consistent with our findings is shown in Figure 9. The mechanism begins with the nucleophilic aromatic substitution of a chlorine substituent in a reaction similar to those catalyzed by glutathione *S*-transferases. To form the final product, the glutathionyl substituent must be

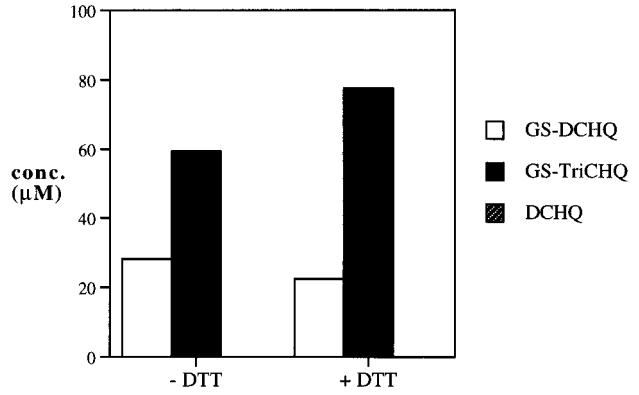

FIGURE 8: Comparison of products formed from TCHQ by purified C13S TCHQ dehalogenase enzymes in the absence and presence of DTT. Reaction mixtures contained [U-^{14}C]TCHQ (100 μM), GSH (1 mM), ascorbate (1 mM), C13S TCHQ dehalogenase, and additions as indicated. Aliquots were quenched with 1 N HCl after 30 min and analyzed by reverse-phase HPLC. Fractions were collected and radioactivity was determined by scintillation counting.

replaced by a hydrogen. This process is likely to require transfer of the glutathionyl moeity to Cys13. Direct attack of Cys13 on GS-TriCHQ would require departure of a phenyl anion, a poor leaving group. A more attractive alternative involves initial tautomerization of the ring. Subsequent attack of Cys13 on the sulfur atom of the glutathionyl moeity results in expulsion of the reduced aromatic product and the formation of a mixed disulfide between Cys13 and glutathione. In the final step, attack of GSH on the mixed disulfide regenerates the free enzyme.

We expect that the products formed from TCHQ by the C13S mutant enzyme result from diversion of a reactive intermediate at the active site to abnormal products in the absence of Cys13. According to the model shown in Figure 9, the C13S mutant enzyme should be able to convert TCHQ to intermediate III. This intermediate is very unstable and would be expected to undergo further reactions, either at the active site or after release into solution (see Figure 10). A major product expected would be GS-TriCHQ (resulting from rearomatization of the ring). Trichlorobenzoquinone (resulting from elimination of glutathione) should also be formed. Trichlorobenzoquinone would undergo further reactions with glutathione and ascorbate in the reaction mixture. Substitution of a chlorine substituent with glutathione, followed by reduction with ascorbate, would yield GS-DCHQ. Reduction of trichlorobenzoquinone with ascorbate would form TriCHQ, which is, of course, a substrate for the enzyme. TriCHQ should be converted to the dichlorinated intermediate analogous to intermediate III in Figure 9. Decomposition of this intermediate would result in formation of GS-DCHQ, along with smaller amounts of GS-MCHQ and DCHQ. Thus, based on the mechanistic model shown in Figure 9, the major products formed by the C13S enzyme should be GS-TriCHQ and GS-DCHQ, with smaller amounts of GS-MCHQ and DCHQ. The major products of the reaction are indeed GS-TriCHQ and GS-DCHQ. We were not able to detect any GS-MCHQ or DCHQ in our reaction mixtures.

The time course of products formed by the C13S enzyme provides further support for this hypothesis. As shown in Figure 11, a small amount of TriCHQ does accumulate and then disappear during the reaction. Furthermore, no lag is observed for formation of GS-DCHQ, suggesting that GS-DCHQ can be formed directly from TCHQ without prior formation of TriCHQ. GS-DCHQ can also be formed from TriCHQ. As TriCHQ disappears during the latter part of the reaction, a concomitant increase in the amount of GS-DCHQ is observed.

The formation of GS-DCHQ directly from TCHQ by the C13S mutant enzyme suggests an explanation for the early appearance of GS-DCHQ during turnover of TCHQ by the wild-type enzyme in the absence of DTT (see Figure 2). If the oxidative damage to the wild-type enzyme involves Cys13, then the oxidatively damaged enzyme would be functionally equivalent to the C13S mutant and would be expected to form intermediate III, which could decompose directly to GS-DCHQ without requiring prior formation of TriCHQ.

Conclusions. The kinetic data for the C13S mutant have provided us with two important pieces of the mechanistic puzzle. First, the importance of Cys13 for the reductive dehalogenation has been revealed. Second, the observed product distribution, the transient appearance of TriCHQ, and the evidence that GS-DCHQ can be formed both directly from TCHQ and from TriCHQ are all consistent with the intermediacy of the highly reactive tautomer of GS-TriCHQ (intermediate III). If the mechanism had involved direct attack of Cys13 on GS-TriCHQ to release a phenyl anion, the only product formed by the C13S mutant would have been GS-TriCHQ.

Although TCHQ dehalogenase appears to be one of the most divergent members of the glutathione *S*-transferase superfamily yet found, its global similarity to many other glutathione *S*-transferases (17−30% identity overall) (P. Babbitt, unpublished results), as well as conservation of "consensus" residues associated with the theta class in particular (Rossjohn et al., 1996), identify it as a glutathione *S*-transferase from primary structural considerations. Furthermore, our mechanistic data suggest that the sequence homology between TCHQ dehalogenase and the glutathione *S*-transferases reflects not only the existence of a glutathione binding site, but also the ability to catalyze the nucleophilic attack of glutathione on an electrophilic substrate to form a glutathione conjugate. Clearly, however, TCHQ dehalogenase has some additional catalytic capabilities that glutathione *S*-transferases do not have that allow it to convert glutathione conjugates to reduced products. Cys13 is obviously required for this process, but other acids and bases at the active site may be involved as well. A search for other catalytic residues is currently underway.

It is curious that the glutathione *S*-transferase superfamily members most similar in *overall* primary structure to TCHQ dehalogenase (those from *Proteus mirabilis*, *Pseudomonas sp*. LB400, *E. coli*, and carnation, see Figure 7) have a Cys residue that aligns with Cys13 of TCHQ dehalogenase. A reductive dehalogenation activity has not been reported for any of these proteins, although the possibility that the *Pseudomonas* sp. LB400 protein might carry out a reductive dehalogenation during the degradation of PCBs has been raised (Hofer et al., 1994). Three of these proteins (those from *P. mirabilis* (Di Ilio et al., 1988), *Pseudomonas sp*. LB400 (Hofer et al., 1994), and *E. coli* (Arca et al., 1990; Shishido, 1981) differ from TCHQ dehalogenase in that they have *bona fide* glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene, a substrate commonly used to assay glutathione *S*-transferases, whereas TCHQ dehalogenase has

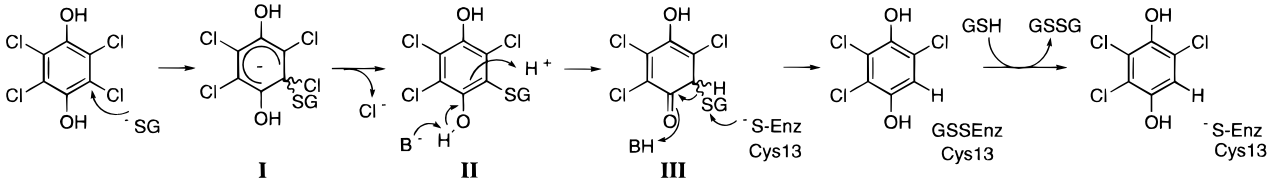

FIGURE 9: Postulated mechanism for TCHQ dehalogenase.

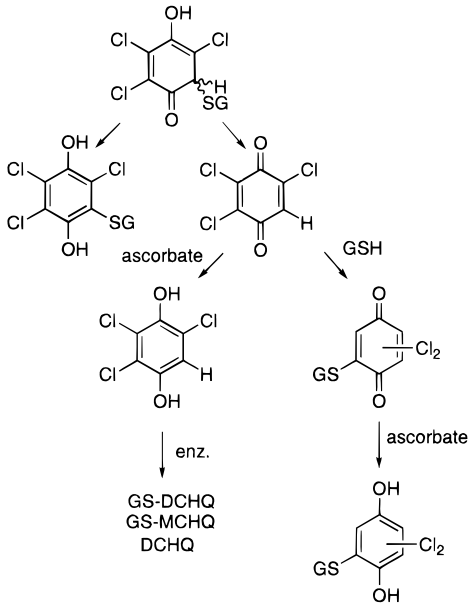

FIGURE 10: Expected routes for reaction of intermediate III from Figure 9.

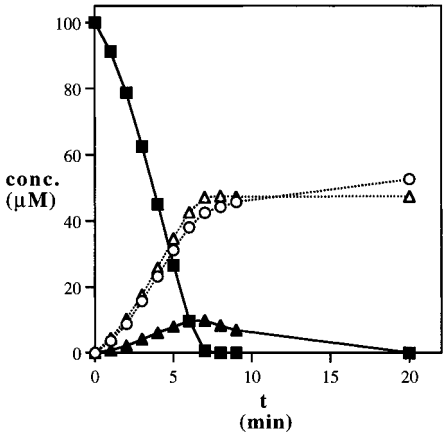

FIGURE 11: Time course of dehalogenation reaction containing [U-^{14}C]TCHQ (100 μM), GSH (1 mM), ascorbate (1 mM), and C13S TCHQ dehalogenase. Aliquots were quenched with 1 N HCl at various times and analyzed by reverse-phase HPLC. Fractions were collected and radioactivity was determined by scintillation counting. (■) TCHQ; (▲) TriCHQ; (△) GS-TriCHQ; (○) GS-DCHQ.

no activity with this substrate. The carnation protein, which is found in senescent petals and is induced by ethylene, has not been purified, and neither its physiological function nor its ability to catalyze a reaction with 1-chloro-2,4-dinitrobenzene is known (Meyer et al., 1991; Itzhaki & Woodson, 1993). Further mechanistic and structural characterization will be required to determine the role of the conserved Cys residues in these enzymes.

The overall catalytic strategy of TCHQ dehalogenase is reminiscent of the reactions catalyzed by proline reductase and glycine reductase. In proline reductase, the reduction reaction is accomplished by initial formation of a covalent adduct between the substrate and a thiol, followed by attack of a second thiol on the sulfur atom of the covalent adduct to release the reduced product and form a disulfide species (Arkowitz et al., 1994). The mechanism of glycine reductase appears to be similar except that the initial nucleophilic attack is carried out by a selenocysteine at the active site (Arkowitz & Abeles, 1991).

TCHQ dehalogenase provides an interesting case study of an enzyme that may be in the process of evolving a new function. This enzyme is found in soil microorganisms that degrade pentachlorophenol. While many halogenated aromatic natural products have been identified, only a few have more than one or two halogen atoms per ring (Gribble, 1994; Siuda & DeBernardis, 1973). Therefore, it is unlikely that microorganisms have been exposed to large quantities of highly chlorinated compounds prior to the introduction of compounds such as PCBs and pentachlorophenol during the last forty years. Furthermore, TCHQ dehalogenase is constitutively expressed in *Sphingomonas chlorophenolica* sp. ATCC 39723 (Orser et al., 1993; Xun et al., 1992a). This constitutive expression may indicate that the enzyme has some other essential function in the cell and that it is recruited to function as a TCHQ dehalogenase during the degradation of pentachlorophenol.

Petsko et al. and others have proposed that the evolution of new enzymes is likely to occur via conservation of an active site architecture suitable for the performance of a particular chemical reaction, with changes as necessary to alter the specificity of the enzyme (Petsko et al., 1993; Babbitt et al., 1995). This pattern is evident in the reaction catalyzed by TCHQ dehalogenase. The initial steps in the reaction appear to be exactly those expected for an enzyme that is related to the glutathione *S*-transferases. However, TCHQ dehalogenase has obviously acquired additional catalytic capabilities that glutathione *S*-transferases do not have.

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