A Mechanistic Investigation of the Thiol−Disulfide Exchange Step in the Reductive Dehalogenation Catalyzed by Tetrachlorohydroquinone Dehalogenase†

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ABSTRACT: Tetrachlorohydroquinone dehalogenase catalyzes the reductive dehalogenation of tetrachloro- and trichlorohydroquinone to give 2,6-dichlorohydroquinone in the pathway for degradation of pentachlorophenol by *Sphingobium chlorophenolicum*. Previous work has suggested that this enzyme may have originated from a glutathione-dependent double bond isomerase such as maleylacetoacetate isomerase or maleylpyruvate isomerase. While some of the elementary steps in these two reactions may be similar, the final step in the dehalogenation reaction, a thiol−disulfide exchange reaction that removes glutathione covalently bound to Cys13, certainly has no counterpart in the isomerization reaction. The thiol−disulfide exchange reaction does not appear to have been evolutionarily optimized. There is little specificity for the thiol; many thiols react at high rates. TCHQ dehalogenase binds the glutathione involved in the thiol−disulfide exchange reaction very poorly and does not alter its pK_a in order to improve its nucleophilicity. Remarkably, single-turnover kinetic studies show that the enzyme catalyzes this step by approximately 10000-fold. This high reactivity requires an as yet unidentified protonated group in the active site.

Tetrachlorohydroquinone (TCHQ)[1] dehalogenase is the third enzyme in the pathway for biodegradation of pentachlorophenol (PCP) by the soil bacterium *Sphingobium chlorophenolicum* (*1*, *2*). This enzyme catalyzes two successive reductive dehalogenations that convert TCHQ first to trichlorohydroquinone (TriCHQ) and then to 2,6-dichlorohydroquinone (DCHQ). The proposed mechanism for this unusual reaction is shown in Figure 1a (*3*−*5*). TCHQ dehalogenase is a member of the zeta class of the glutathione *S*-transferase (GST) superfamily (*6*, *7*). TCHQ dehalogenase may have evolved from another member of this superfamily, a maleylacetoacetate (MAA) or maleylpyruvate (MP) isomerase (*8*), that catalyzes the glutathione-dependent isomerization of a double bond from the *cis* to the *trans* configuration (*9*, *10*) (see Figure 1b). This suggestion was based on conservation of sequence in the active site region and the enzyme's robust isomerization activity with the substrate analogue maleylacetone.

The ancestral chemical reaction catalyzed by members of the GST superfamily is nucleophilic attack of glutathione upon an electrophilic substrate to form a glutathione conjugate. In both TCHQ dehalogenase and MAA/MP isomerases, this ancestral step (see boxes in Figure 1) is part of a more complex transformation. The thiol−disulfide exchange reaction that regenerates the free enzyme and forms glu-

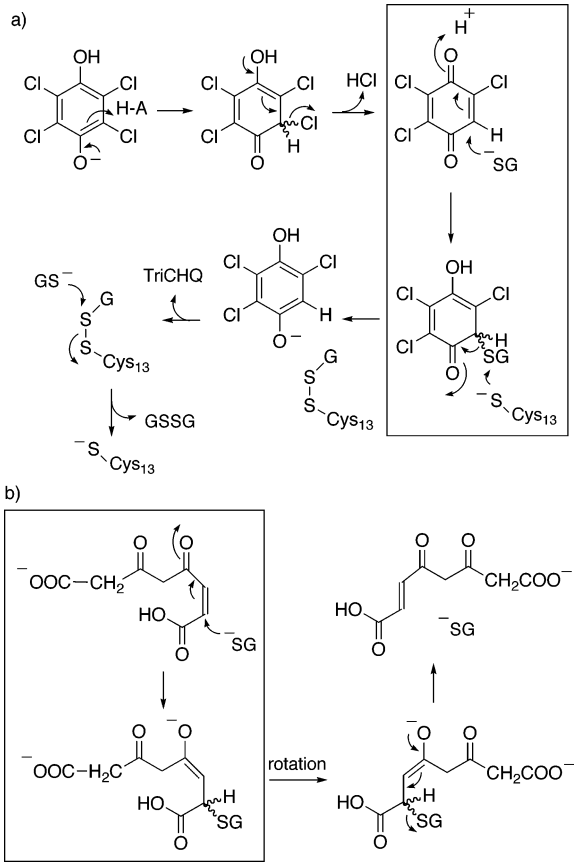

FIGURE 1: Postulated mechanisms for (a) TCHQ dehalogenase and (b) MAA isomerase with the step typical of the GST superfamily indicated by a box in each case.

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[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; DCHQ, 2,6-dichlorohydroquinone; DNPS-SG, 2,4-dinitrophenylglutathionyl disulfide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESSG, TCHQ dehalogenase Cys13-glutathionyl disulfide; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; IPTG, isopropyl *β*-D-galactopyranoside; MAA, maleylacetoacetate; MP, maleylpyruvate; PCP, pentachlorophenol; TCHQ, tetrachlorohydroquinone; TriCHQ, trichlorohydroquinone.

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tathione disulfide in the last step of the dehalogenation reaction is of particular interest. Most GST superfamily members, including MAA and MP isomerases, utilize only one glutathione and consequently have a single glutathione binding site (*11*−*13*). TCHQ dehalogenase, however, uses two molecules of glutathione to provide the reducing equivalents for each step of reductive dehalogenation. The first glutathione is involved in nucleophilic attack on an electrophilic intermediate, a reaction typical of those catalyzed by GSTs. The second glutathione is involved in the thiol−disulfide exchange reaction. During this step it is likely that the canonical glutathione binding site is occupied by the glutathionyl moiety attached to Cys13. Thus, we were particularly interested in whether the enzyme binds the second glutathione and to what extent the enzyme catalyzes this step that appears to have been "added on" to enable the novel reductive dehalogenation reaction to occur.

We have investigated the thiol−disulfide exchange reaction using rapid flow−quench kinetic studies of the reaction between thiols and ESSG, the form of the enzyme in which glutathione is covalently attached to Cys13. The results described here suggest that the second glutathione binds very weakly and that its pK_a is not significantly perturbed from that observed in solution. The solvent kinetic isotope effect and the dependence of the rate constant on the pK_a of the nucleophilic thiol suggest that the thiol−disulfide exchange step is partially or fully rate-limiting, depending on the pH. Finally, the data suggest that, despite the weak binding and the lack of perturbation of the pK_a of the glutathione, the enzyme manages to catalyze the reaction by approximately 10000-fold relative to the rate of a comparable thiol−disulfide exchange reaction in solution. An important part of this catalytic effect may be stabilization of the developing negative charge on Cys13 as the disulfide bond breaks by a protonated group either on the enzyme or on the glutathionyl moiety attached to Cys13.

MATERIALS AND METHODS

Chemicals. 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), dithiothreitol (DTT), and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Research Products International Corp. Glutathione (glycine-2-^{3}H-labeled) (0.05 mCi/ 0.05 mL) was from PerkinElmer Life and Analytical Sciences. β-Mercaptoethanol, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), glutathione (GSH), glutathione disulfide (GSSG), 2,4-dinitrobenzenesulfenyl chloride (DNPSCl), Sepharose 6B, 1,4-butanediol diglycidyl ether (95%), 3-mercaptopropionic acid, deuterium oxide (99.9%), L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin, pentachlorophenol (PCP), and ethylene glycol bis(thioglycolate) were purchased from Sigma-Aldrich. Ethylene glycol bis(thioglycolate) was purified by vacuum distillation. All other reagents were purchased from common commercial sources.

Preparation of N-Linked Glutathione−Sepharose Affinity Matrix. Sepharose 6B was activated with 1,4-butanediol diglycidyl ether as previously described (*14*). Glutathione disulfide (1.5 g) was dissolved in 25 mL of sodium carbonate (0.3 M, pH 10) and shaken with epoxy-activated agarose (13 g) at 37 °C for 40−50 h. β-Mercaptoethanol was added to a final concentration of 0.2 M to reduce glutathione disulfide and inactivate any remaining epoxide, and the suspension was shaken at 25 °C for 8 h. The N-linked glutathione−Sepharose was washed successively with 5 mM DTT, 1 M sodium chloride, and water. This procedure allowed linkage of 150 μmol of glutathione/g of dry Sepharose 6B as determined by Ellman's assay (*15*) using DTNB (150 μM) in 200 mM Tris-HCl, pH 8.0, containing 5 mM EDTA (ϵ_{412} = 13.6 mM^{-1} cm^{-1}) (*15*, *16*). The *N*-linked glutathione−Sepharose was stored in potassium phosphate buffer (25 mM, pH 7.5) containing 20 mM β-mercaptoethanol at 4 °C.

Enzyme Preparation. All experiments were carried out using C156S TCHQ dehalogenase, which has only one Cys residue at position 13 in the active site, to avoid complications arising from reactions with Cys156, which is located on the surface at a distance from the active site. The substitution of Ser for Cys at this position does not appear to affect any important properties of the enzyme (*6*). The gene encoding the C156S mutant of TCHQ dehalogenase from *S. chlorophenolicum* strain ATCC 39723 in pKK233-3 (Pharmacia) was recloned into pET-21b (Novagen) to increase expression. A stop codon was placed before the sequence encoding the C-terminal His tag in this vector because the tag appears to alter the kinetic properties of the enzyme. The C156S enzyme was expressed in a BL-21(DE3) *lacY*$^-$ strain of *Escherichia coli* (Novagen). Bacteria that had been freshly transformed with the expression plasmid were grown at 25 °C in 2×YT medium (1 L) containing potassium phosphate buffer (25 mM, pH 7.2), glucose (5 mM), and ampicillin (100 mg/L). Expression was induced with IPTG (0.5 mM) when the cells had reached an optical density at 600 nm of 0.6−1.0. After growth for 4 h, the cells were harvested by centrifugation at 10000*g* for 10 min at 4 °C. The cells were suspended in lysis buffer [25 mM potassium phosphate, pH 7.5, containing 5 mM EDTA, 5 mM DTT, and 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) at 4 °C], and the suspension was passed three times through a French pressure cell at 12000 psi. Cell debris was removed by centrifugation at 15000*g* for 35 min at 4 °C. The supernatant was dialyzed against buffer A (25 mM potassium phosphate, pH 7.5, containing 5 mM EDTA and 5 mM DTT) and then passed through a 0.22 μm syringe filter. The filtrate was loaded onto 60 mL of *N*-linked glutathione−Sepharose affinity matrix equilibrated in buffer A. The column was washed with buffer A containing sodium chloride (2 M). TCHQ dehalogenase was then eluted in buffer A containing sodium chloride (2 M) and GSH (5 mM). The protein was quantified by absorbance at 280 nm (ϵ_{280} = 37.6 mM^{-1}) (*17*), and the purity was ascertained by SDS−PAGE. This procedure routinely resulted in yields of 0.85 mg of pure TCHQ dehalogenase/mL of matrix or 50 mg of TCHQ dehalogenase from 3.5 g of cell paste harvested from a 1 L culture.

Preparation of 2,4-Dinitrophenyl-glutathionyl Disulfide (DNPS-SG). DNPS-SG was prepared as previously described (*18*). 2,4-Dinitrophenyl-(*glycine*-2-^{3}H)glutathionyl disulfide (DNPS-[^{3}H]SG) was prepared as described (*18*) with some modifications. Glutathione (0.06 μmol) was dissolved in 30 μL of water. (*glycine*-2-^{3}H)Glutathione (10 μCi, 0.24 nmol)

was added, and the pH was adjusted to pH 2 by addition of 1 M HCl. DTT present in the radiolabeled glutathione was extracted using four 140 μL portions of ethyl acetate, and the final aqueous solution was concentrated in vacuo (*19*). The resulting solid was dissolved in 10 μL of formic acid (95−97%), and 2,4-dinitrobenzenesulfenyl chloride (0.12 μmol) was added in an additional 10 μL of formic acid. The reaction was allowed to continue for 3 h at room temperature. The solution was added to 1.5 mL of cold diethyl ether, and the yellow precipitate was collected by centrifugation and washed with 1.5 mL of cold diethyl ether. The solid DNPS-[^{3}H]SG was then dried in vacuo and used immediately.

Preparation of TCHQ Dehalogenase Cys13-(glycine-2-^{3}H)glutathionyl Disulfide (^{3}H-ESSG). C156S TCHQ dehalogenase (0.1 μmol) was subjected to gel filtration chromatography (Sephadex G-25, 40 mL; Sigma) in degassed 200 mM Tris-HCl, pH 7.5, containing 5 mM EDTA at 4 °C in order to remove DTT added during purification to protect the active site Cys from oxidation. The protein was concentrated to a volume of 1 mL in a Centriprep YM-10 centrifugal filter device (Millipore) and added to solid DNPS-[^{3}H]SG (0.6 equiv). The yellow solution was allowed to sit for 4 h at 4 °C. ^{3}H-ESSG was then purified by gel filtration on a Sephadex G-25 column using degassed reaction buffer. ^{3}H-ESSG was concentrated with a Centriprep YM-10 and used immediately. Nonradiolabeled ESSG was prepared in the same manner using DNPS-SG. In this case, because DNPS-SG was available in large quantities, a 50-fold excess of DNPS-SG over enzyme was used to drive the reaction to completion.

Confirmation of the Structure of ESSG. TCHQ dehalogenase and ESSG (1 nmol) were digested with TPCK-treated trypsin (100 nM) in 100 μL of 100 mM Tris-HCl, pH 7.0, containing 1 mM $CaCl_2$, for 3 h at 25 °C. Aliquots of the digested product (20 pmol) in a α-cyano-4-hydroxycinnamic acid matrix were analyzed by MALDI-TOF MS using an Applied Biosystems Voyager-DE STR in reflector mode. A peptide with a mass of 2172 amu (consistent with that of an adduct between GSH and the N-terminal peptide containing residues 1−16) was found in the digest of ESSG but not in the digest of TCHQ dehalogenase (data not shown). A sample of digested ESSG in 0.1% formic acid (10 μL, 20 pmol) was injected onto a Phenomenex Jupiter C18 capillary column connected to an ABI-Qstar Quadrapole/TOF LC/MS/MS. Peptides were eluted with a gradient from 0% to 50% acetonitrile over 50 min. The N-terminal peptide eluted at 40 min as a MH_3^{3+} ion (m/z = 724.3). This peptide was sequenced by MS/MS (see Figure 2) and found to contain glutathione covalently linked to Cys13 as previously observed for the corresponding peptide from ESSG formed during turnover of TCHQ dehalogenase (*5*).

Rapid Quench Experiments. Thiol−disulfide exchange reactions between ^{3}H-ESSG and various thiols were analyzed using an Applied Photophysics SQ.1E rapid flow−quench instrument. The concentration of thiol was in vast excess over that of ^{3}H-ESSG so that the thiol−disulfide exchange could be treated as a pseudo-first-order reaction. Experiments were carried out by mixing ^{3}H-ESSG (2 μM, 70 μL, 38000 cpm/ nmol) with glutathione or other thiols (70 μL) in reaction buffers as described below. Thiol concentrations were determined immediately prior to experiments using Ellman's assay (*15*). In all cases, the concentrations of

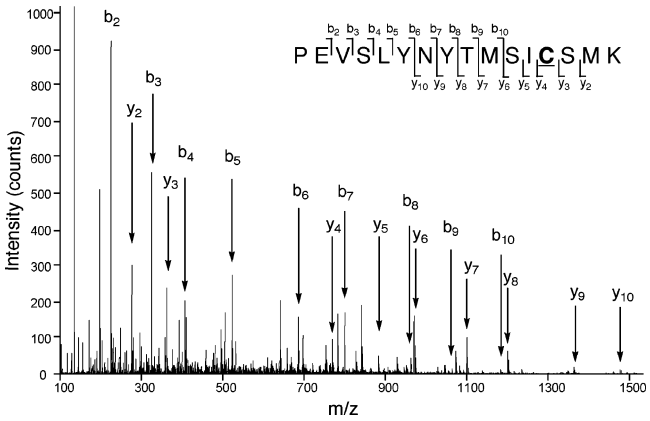


FIGURE 2: LC/MS/MS spectrum of the N-terminal peptide of TCHQ dehalogenase containing a disulfide bond between Cys13 (designated as "**C**") and glutathione. The covalent adduct, ESSG, was prepared and digested with trypsin. An ion of m/z = 724.3 corresponding to the N-terminal MH_3^{3+} peptide was selected for fragmentation. Cleavage of the amide bond results in N-terminal fragments designated as "b" and C-terminal fragments designated as "y". The masses of the y ions are consistent with glutathione covalently linked to Cys13. The m/z values for the labeled peaks are as follows: b_2, 227.1; b_3, 326.2; b_4, 413.2; b_5, 526.3; b_6, 689.4; b_7,803.4; b_8, 966.5; b_9, 1067.5; b_{10}, 1198.5; y_2, 278.2; y_3, 365.2; y_4, 773.3; y_5, 886.3; y_6, 973.4; y_7, 1104.4; y_8, 1205.5; y_9, 1368.5; y_{10}, 1482.6.

enzyme and substrates mentioned in the text are those obtained after mixing. After variable delay times, the reaction was quenched with 1 N HCl (70 μL). The protein was removed by centrifugal filtration in a Microcon YM10 (Millipore). ^{3}H-GSSG was quantified by scintillation counting of the filtrate. In general, 10 samples were collected with delay times between 10 ms and 30 s.

To determine whether the ^{3}H originating in ^{3}H-ESSG was found in glutathione or GSSG in quenched reaction mixtures, the products were separated by reverse-phase HPLC using a Rainin C18 column (0.46 cm × 5 cm) equilibrated with 0.1% trifluoroacetic acid. The absorbance detector was set at λ = 210 nm. Under these conditions, glutathione and GSSG elute at 1.3 and 4.5 min, respectively. Fractions containing glutathione and GSSG were collected and analyzed by scintillation counting.

For measurement of the dependence of reaction rate on glutathione concentration, reactions were carried out in 200 mM potassium phosphate, pH 7.7, at 5 °C using ^{3}H-ESSG and concentrations of glutathione varying between 0.2 and 20 mM after mixing. The low reaction temperature was necessary to allow observance of the very fast reactions that occurred at high glutathione concentrations.

For measurement of reaction rates with various thiols, ^{3}H-ESSG was mixed with thiol (1 mM after mixing) in 66 mM potassium phosphate, pH 7.0, at 30 °C. Thiols used included glutathione, ethylene glycol bis(thioglycolate), 3-mercaptopropionic acid, DTT, and β-mercaptoethanol.

For measurement of the dependence of reaction rate on pH, concentrated glutathione in water (adjusted to pH 7.5 with NaOH) and concentrated ^{3}H-ESSG in 5 mM Tris-HCl, pH 7.5, were diluted separately in a constant ionic strength reaction buffer [100 mM morpholinoethanesulfonic acid (MES), 50 mM Tris-HCl, 50 mM ethanolamine, 200 mM sodium chloride] to give final concentrations after mixing in the rapid flow−quench instrument of 1 mM GSH and 1

μM ^{3}H-ESSG (*20*). Reaction rates were measured at 25 °C at pH values ranging from 6 to 10. (The pH was determined after mixing, and the glutathione concentration was determined both before and after each experiment using Ellman's assay.) TCHQ dehalogenase has previously been shown to be stable in this buffer system and over this pH range (*4*). Experiments in D_2O were conducted in the same manner. All solutions were prepared fresh in D_2O and stored under nitrogen. The pD was adjusted with DCl and NaOD and determined by the following relationship: pD = pH + 0.4 (*21*). The residual H_2O content was less than 2% as determined by NMR (*22*).

Stopped-Flow Experiments. Stopped-flow experiments to measure the rate of reaction between glutathione and DTNB were carried out using an Applied Photophysics stopped-flow instrument (SX17.MV) by mixing glutathione (20 μM, 60−65 μL) with DTNB (280 μM, 60−65 μL) in the constant ionic strength reaction buffer described above at 25 °C. Solutions of DTNB (100 mM) were prepared in ethanol and diluted in reaction buffer such that the concentration of ethanol after mixing was less than 0.2%. The concentration of glutathione was determined just prior to experiments by Ellman's assay. The absorbance at 412 nm was monitored and fit to a single exponential. Reaction rates were determined at pH values ranging from 6 to 10 (measured after mixing). Experiments in D_2O were conducted in the same manner, but the pD was varied from 6 to 11.

Data Analysis. Time courses were fit by an iterative process to a first-order exponential rate equation to obtain values for the pseudo-first-order rate constant k'_{obs} using Kaleida Graph 3.6 (Synergy Software) or software provided with the stopped-flow spectrophotometer. Rate constants determined from two to four individual time courses were then averaged. All other data were fit to equations as given in the text using Kaleida Graph.

Measurement of in Vivo Glutathione Concentration. In vivo concentrations of glutathione were measured using a modification of procedures described by Fahey and Newton (*23*) and Anderson (*24*). *S. chlorophenolicum* was grown in M9 minimal medium (*25*) containing glutamate (24 mM) and $FeSO_4$ (1 mM) at room temperature until an OD_{560} of 0.5 was reached. PCP was added to a final concentration of 188 μM, and the cells were grown for a further 1.5 h before being harvested by centrifugation at 8000*g* and 4 °C for 20 min. Cell pellets were stored at −70 °C for 8 h before analysis. The cell pellets were washed twice with cold water and resuspended in 5 mL of degassed 200 mM methanesulfonic acid at 4 °C. The cells were broken by passage through a French pressure cell twice at 12000 psi. An equal volume of 4 M sodium methanesulfonate was added, and the cell debris was removed by centrifugation at 8500*g* and 4 °C for 20 min. The sample was added to a solution of 188 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (HEPPS) and 5 mM diethylenetriaminepentaacetic acid (DTPA), pH 8.0, under N_2. Monobromobimane was added to a final concentration of 2.9 mM, and the reaction was allowed to proceed at room temperature for 12 min in the dark. The reaction was quenched by addition of methanesulfonic acid to a final concentration of 96 mM and a final pH of 5. Samples to allow for quantitation of recovery were prepared similarly except that a known amount of glutathione (1 μmol) was added to the cell pellet before disruption. The concentration of derivatized glutathione in the sample was determined by reverse-phase HPLC using a Rainin C18 column (0.46 cm × 5 cm) equilibrated with 0.25% acetic acid with elution with increasing amounts of methanol. The fluorescence detector was set at λ_{ex} = 232 nm and λ_{em} = 474 nm.

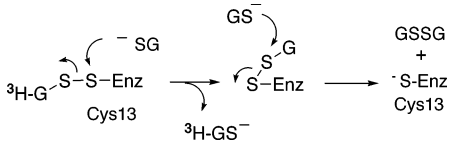


FIGURE 3: Possible alternative mechanism for the thiol−disulfide exchange reaction.

To determine the cytoplasmic concentration of glutathione, it was necessary to determine the corrected wet weight of the cell pellet (by accounting for residual intercellular fluid in the pellet) (*26*) and the total dry weight of the pellet (*27*). The volume of intercellular fluid in a cell pellet was determined by first weighing the pellet and then resuspending the pellet (approximately 0.6 g) in 1 mL of water containing 10 mg/mL blue dextran 2000. The concentration of blue dextran 2000 in the supernatant after centrifugation at 12000*g* for 10 min at 4 °C was determined by measuring the absorbance at 618 nm (ϵ_{618} = 0.95 mg^{-1} mL cm^{-1}). This value was used to calculate the dilution of the stock solution of blue dextran due to the intercellular water in the cell pellet and, thus, the volume of the intercellular water. The total dry weight was determined by heating the pellet in an oven at 105 °C in a tared glass tube until a constant weight was achieved. Using these procedures, it was determined that the corrected wet weight of the cell pellet was typically 67% of the nominal wet weight and the total dry weight was typically 15% of the nominal wet weight. Calculation of the cytoplasmic concentration of glutathione was accomplished by dividing the measured amount of glutathione in the cell pellet by the estimated volume of the cytoplasm. The latter figure was obtained by subtracting the total dry weight from the corrected wet weight in order to obtain the weight of water in the pellet and assuming that 80% of the water inside the cell is located in the cytoplasm.

RESULTS

Confirmation That Reaction of Glutathione with ^{3}H-ESSG Produces ^{3}H-GSSG. Our model for the mechanism of the reaction (see Figure 1a) suggests that glutathione attacks the glutathionyl cysteine residue of ESSG to produce GSSG and release the free enzyme. We also considered the possibility that Cys13 might be accessible from the face opposite to that occupied by the attached glutathionyl moiety. Thus, an alternative possibility is that glutathione might attack Cys13 and form a different version of ESSG that is more accessible to subsequent attack by glutathione, which would then release GSSG and free enzyme (see Figure 3). To test this possibility, we carried out reactions of ^{3}H-ESSG (5 μM) with glutathione (1 mM), quenched the reactions after variable delay times from 10 to 500 ms, and collected the products for analysis by HPLC. The results (data not shown) indicated that radiolabel was found only in GSSG and not in glutathione. Under the conditions of the reaction, GSSG would be

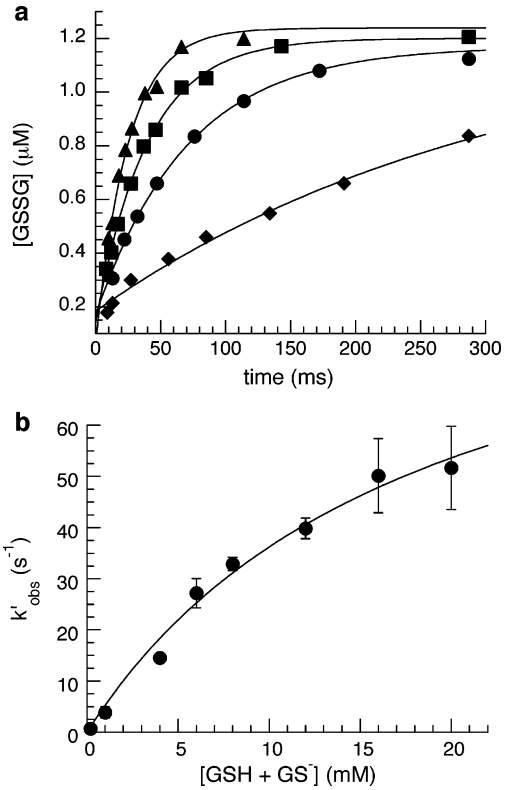


FIGURE 4: Rapid flow−quench kinetic data for (a) formation of ^{3}H-GSSG by reaction of ^{3}H-ESSG with glutathione at concentrations of 1 mM (◆), 4 mM (●), 6 mM (■), and 8 mM (▲) and (b) k'_{obs} as a function of glutathione concentration. Reactions were carried out with 1 μM ESSG in 200 mM potassium phosphate buffer, pH 7.7, at 5 °C. Points shown in panel b represent the average rate constants obtained from two to four separate experiments.

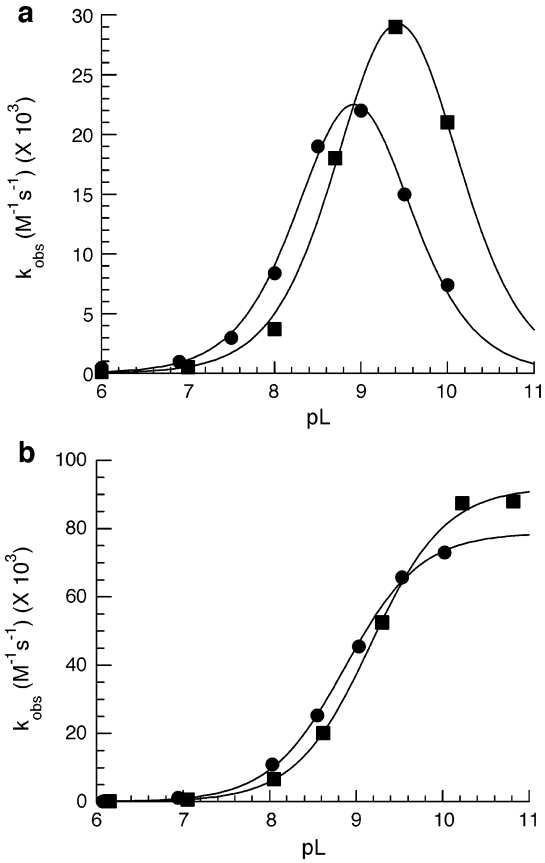


FIGURE 5: (a) Plot of k_{obs} vs pH (●) or pD (■) for the reaction of ^{3}H-ESSG (1 μM) with glutathione (1 mM) at 25 °C. (b) Plot of k_{obs} vs pH (●) or pD (■) for the nonenzymatic reaction of DTNB (140 μM) with glutathione (10 μM) at 25 °C. Points shown represent the averages obtained from two to four separate experiments. Error bars were omitted for clarity; standard deviations were less than 20% in all cases.

expected to undergo nonenzymatic thiol−disulfide exchange with a $t_{1/2}$ of about 2 min based on reported rates of thiol−disulfide exchange reactions (*28*). Furthermore, after the reaction was quenched, the rate of thiol−disulfide exchange would be drastically reduced and should occur with a $t_{1/2}$ of greater than 1 week. Thus, negligible exchange should occur between GSSG and glutathione during the reaction and the period between quenching of the reaction and analysis of the products. Therefore, we conclude that the initial product formed during reaction of ^{3}H-ESSG and glutathione is ^{3}H-GSSG and not ^{3}H-GSH and that, as shown in Figure 1a, glutathione attacks the glutathionyl cysteine rather than Cys13.

Determination of k_{cat} and K_D. ^{3}H-ESSG was mixed in a rapid flow−quench apparatus with varying concentrations of glutathione at pH 7.7 and 5 °C. Glutathione was present in large excess so that the reaction progress could be fit to a pseudo-first-order process with the pseudo-first-order rate constant $k'_{obs} = k_{obs}([GSH] + [GS^-])$. (Rate constants that are based upon the total concentration of the thiol and thiolate forms of glutathione in solution are designated as observed rate constants.) Figure 4a shows examples of time courses for formation of GSSG at various concentrations of glutathione. Figure 4b shows a plot of k'_{obs} vs $[GSH] + [GS^-]$. Because of the rapid rate of the reaction at high glutathione concentrations, it was not possible to measure rate constants for concentrations higher than 20 mM. Fitting the data to the equation $k'_{obs} = k_{cat}[S]/K_M + [S]$ gives $k_{cat} = 103 \pm 15$ s^{-1}, $K_M = 18.6 \pm 5$ mM, and $k_{cat}/K_M = 5540 \pm 1700$ M^{-1} s^{-1}. Experiments described below suggest that the chemical step is rate-limiting, and therefore rapid equilibrium binding of the thiol substrate is a reasonable assumption. Thus, K_M should be equal to K_D in this case. (Note that we cannot distinguish between bound and free GSSG because the quench conditions should result in release of any bound product. Thus, k_{cat} reflects simply the rate of formation of GSSG. The rate of dissociation of GSSG cannot be determined using this single-turnover approach.)

Dependence of the Reaction Rate on pH and Solvent Isotope. Figure 5a shows the pH dependence of the observed rate constant for the thiol−disulfide exchange reaction between ^{3}H-ESSG and glutathione for reactions carried out in both H_2O and D_2O. Figure 5b shows comparable data for the nonenzymatic thiol−disulfide exchange reaction between glutathione and DTNB. The pH profiles for the enzymatic reaction in H_2O and D_2O are bell-shaped. Fitting the data to an equation for two titrating groups (eq 1) (*29*) gives pK_a values of 8.6 ± 0.2 and 9.2 ± 0.2 for the reaction in H_2O and 9.0 ± 0.2 and 9.9 ± 0.2 for the reaction in D_2O. [Note that when the pK_a values for two successively titrating groups differ by less than two pH units, they do not correspond to the pH values at which half of the maximal activity is obtained but must be determined analytically by fitting the data to eq 1 (*29*).] In contrast, the pH profile for the nonenzymatic reaction between glutathione and DTNB can be fit with an equation dependent upon a single titrating

Table 1: Rate Constants for Reaction of Thiols with GSSG and ^{3}H-ESSG at pH 7.0 and 30 °C

thiol	pK_a	k_{obs} for reaction with GSSG[a] ($M^{-1}\,s^{-1}$)	k_{obs} for reaction with ^{3}H-ESSG ($M^{-1}\,s^{-1}$)	k for reaction with ^{3}H-ESSG ($M^{-1}\,s^{-1}$) ($\times 10^3$)
ethylene glycol bis(thioglycolate)	7.7	1.5	67000	400
glutathione	8.7		12500	640
dithiothreitol	9.2	0.24	7800	1200
2-mercaptoethanol	9.6	0.15	5300	2100
3-mercaptopropionic acid	10.6	0.05	6000	24000

[a] From Szajewski and Whitesides (*28*). Values for k are calculated from values of k_{obs} using eq 3.

group (eq 2) (*29*) to give a pK_a of 8.9 ± 0.12 in H_2O and 9.2 ± 0.15 in D_2O.

$$k_{obs} = \frac{k_{max}K_{a1}[H^+]}{K_{a1}[H^+] + [H^+]^2 + K_{a1}K_{a2}} \quad (1)$$

$$k_{obs} = \frac{k_{max}K_a}{K_a + [H^+]} \quad (2)$$

The pH profile for the enzymatic reaction in D_2O is similar to that for the reaction in H_2O but is shifted slightly to the right, as expected based upon the effect of solvent isotope on pK_a values (*22*, *30*). Notably, the solvent kinetic isotope effect is small and normal below a pH(D) of about 8.9 but inverse at higher pH. The solvent isotope effect for the nonenzymatic reaction is also small and normal below pH 9.5 but inverse at higher pH.

The Rate Constant for the Thiol−Disulfide Exchange Reaction Depends on the pK_a of the Nucleophile. ESSG reacts readily with a number of other thiols in addition to glutathione. We measured the observed rate constants for reactions between ^{3}H-ESSG and β-mercaptoethanol, dithiothreitol, 3-mercaptopropionic acid, glycol dimercaptoacetate, and glutathione at pH 7.0 and 30 °C. These reaction conditions were chosen to allow a direct comparison of our rate constants for thiol−disulfide exchange reactions between ^{3}H-ESSG and thiols with rate constants for nonenzymatic thiol−disulfide exchange reactions between GSSG and thiols reported by Szajewski and Whitesides (*28*). Because the reaction with glutathione would be too fast to measure if the enzyme were saturated with glutathione, and the concentrations required to saturate the enzyme with other thiols are likely as high as or higher than that for glutathione, these reactions were carried out using concentrations of 1 mM for each thiol. The observed rate constants for reactions between various thiols and ^{3}H-ESSG are summarized in Table 1. (Note that the second-order rate constant for reaction of glutathione with ^{3}H-ESSG differs from that obtained by fitting the data in Figure 4b because the temperature and pH were different in this experiment.)

Observed rate constants (which reflect the dependence of the rate on the total concentration of the thiol and thiolate forms) were converted to actual rate constants for reaction with the thiolate form using the equation (*28*):

$$k = k_{obs}(1 + 10^{pK_a,thiol-pH}) \quad (3)$$

A Bronsted plot of log k vs pK_a of the nucleophile was fit to the equation log k = 0.7 + 0.6 pK_a, with R^2 = 0.88 (see Figure 6).

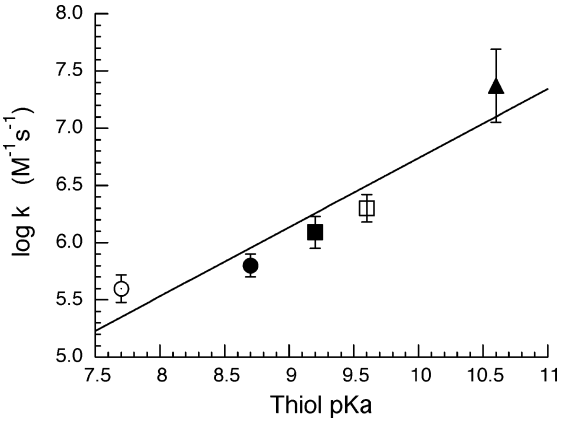


FIGURE 6: Bronsted plot for the thiol−disulfide exchange reaction between ^{3}H-ESSG (1 μM) and the following thiols (1 mM): (○) ethylene glycol bis(thioglycolate); (●) glutathione; (■) dithiothreitol; (□) 2-mercaptoethanol; (▲) 3-mercaptopropionic acid. Reactions were carried out at pH 7.0 and 30 °C. Points shown represent the averages obtained from two separate experiments. The equation used to generate the line that best fits the data is log k = 0.7 + 0.6 pK_a, with R^2 = 0.88 (*29*).

Measurement of the in Vivo Concentration of Glutathione. The amount of glutathione in the cytoplasm of *S. chlorophenolicum* grown in the presence of PCP was determined by analysis of cellular extracts derivatized with monobromobimane, which forms a fluorescent adduct with thiols, to be 4.4 μmol/g total dry weight, or approximately 1.6 mM, in three independent cultures.

DISCUSSION

Kinetic studies of the thiol−disulfide exchange reaction that forms glutathione disulfide and regenerates the free form of TCHQ dehalogenase were undertaken to determine whether the enzyme accelerates this step, which has no parallel in the reaction catalyzed by MAA and MP isomerases. Our findings reveal that the enzyme does indeed catalyze this reaction but that catalysis does not take advantage of specific binding of the nucleophile or alteration of its pK_a to enhance its nucleophilicity.

The data shown in Figure 4b indicate that glutathione binds very poorly to ESSG, with a K_D of approximately 19 mM. This finding is not unexpected, since enzymes in the GST superfamily, including MAA and MP isomerases, typically use only one glutathione and consequently have only one glutathione binding site in each monomer of the dimeric enzyme. (TCHQ dehalogenase is an unusual member of the superfamily in this respect, as it is a monomer.) The canonical glutathione binding site in the ESSG form of TCHQ dehalogenase is likely to be occupied by the glutathione covalently attached to Cys13. Thus, the second

glutathione must bind elsewhere, probably in a site that overlaps the binding site for the aromatic substrates, TCHQ and TriCHQ. Although the binding of glutathione is unusually weak, at a physiological concentration of 1.6 mM glutathione and pH 7.0, the thiol−disulfide exchange reaction would proceed with a rate of 15−20 s^{-1} at 30 °C. Under comparable conditions the initial steps in the dehalogenation reaction that are catalyzed by the C13S mutant enzyme proceed with a rate of 2.4 s^{-1} (*3*). However, ongoing experiments indicate that the thiol−disulfide exchange reaction is inhibited by the aromatic substrate (Warner and Copley, unpublished observations), so the rate of the thiol−disulfide exchange reaction under physiologically relevant conditions might be significantly lower, although not because of an intrinsically low rate of the thiol−disulfide exchange reaction.

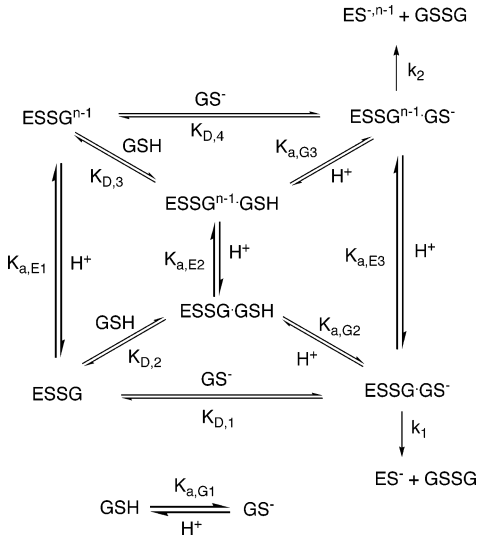
The data in Figure 5a demonstrate that the rate of the reaction depends on the protonation state of two groups with kinetically determined pK_a values of 8.6 and 9.2. We attribute the pK_a of 8.6 to ionization of glutathione based upon the observance of a very similar pK_a for the nonenzymatic thiol−disulfide exchange reaction (see Figure 5b). Since thiolates are orders of magnitude more reactive than the corresponding thiols (*31*), the increase in reaction rate as the pH increases can be attributed to an increase in the concentration of the reactive thiolate form of glutathione. The finding that the enzyme does not significantly perturb the pK_a of the second equivalent of glutathione is interesting in light of the body of literature suggesting that glutathione *S*-transferases typically enhance the nucleophilicity of glutathione by lowering its pK_a from 8.5 to below 7 (*32*). It appears that TCHQ dehalogenase does not take advantage of this strategy for enhancing the nucleophilicity of the glutathione involved in the thiol−disulfide exchange reaction, probably because the binding site for the second glutathione is rudimentary and not equipped to alter the pK_a of the thiol.

The decrease in reaction rate as the pH increases beyond pH 9 suggests that a group with a pK_a of 9.2 must be in its protonated state for reaction to occur. This group could be Arg, Lys, Tyr, or the glutathione covalently attached to Cys13. We can eliminate from consideration the amino group of the glutathione substrate because the enzyme is active with thiol substrates that lack an amino group (see Figure 6 and Table 1).

We can describe the thiol−disulfide exchange reaction as shown in Scheme 1, in which all of the possible routes to product are considered. (Note that this scheme does not assume that the pK_a values for glutathione and the enzyme are the same in all forms of the enzyme−substrate complex. It is not possible to obtain reliable estimates for each of the pK_a values in Scheme 1 from the data in Figure 5, which give us only apparent pK_as.) The decrease in reaction rate at high pH values suggests that either the complex $ESSG^{n-1}{\cdot}GS^-$ cannot form or, if it forms, it cannot react. The first scenario might occur if $K_{D,4}$ is infinite and $K_{a,G3}$ and $K_{a,E3}$ are 0 at high pH. The second scenario might occur if a protonated group in the active site is required to stabilize the negative charge on Cys13 as the disulfide bond breaks (i.e., k_2 is very low at high pH).

The solvent kinetic isotope effect data provide additional insights into the mechanism of the reaction. The solvent isotope effects for both the enzymatic reaction of ESSG and glutathione and the nonenzymatic reaction of glutathione and

Scheme 1



DTNB are small and normal at pH values below 9 but become inverse at high pH. At high pH values, the nonenzymatic reaction is expected to occur by attack of the thiolate form of glutathione on DTNB. Reactions involving nucleophilic attack of thiolates show inverse solvent kinetic isotope effects because of changes in the interaction of solvent molecules with the sulfur atom as its charge diminishes in the transition state (*33*, *34*). Thus, the inverse kinetic isotope effect observed at high pH values is consistent with the expected mechanism of the reaction. At low pH, this inverse solvent isotope effect should be obscured by the normal solvent isotope effect associated with deprotonation of the thiol to form the thiolate.

The pH profile of the enzymatic reaction is more complicated than that of the nonenzymatic reaction due to the involvement of an unidentified group with an apparent pK_a of 9.2. Interpretation of solvent kinetic isotope effects on reactions characterized by bell-shaped pH profiles is difficult because solvent isotope effects can be seen due simply to the effect of solvent isotope on pK_a values, which has the effect of shifting the entire pH profile to the right in D_2O (*22*, *30*). The analysis is further complicated for solvent isotope effect values on k_{cat}/K_D because of effects of solvent and pH on substrate binding. The inverse solvent isotope effect for the enzymatic reaction at high pH is consistent with rate-determining nucleophilic attack of the thiolate of glutathione at high pH values. However, this conclusion cannot be made with certainty because the identity of the group that titrates at high pH, and therefore the effect of solvent isotope on its pK_a, is unknown.

Further insight into the nature of the rate-limiting step was obtained from kinetic experiments using a range of structurally diverse thiols. The rate constants for these reactions are all quite high (see Table 1). The Bronsted plot in Figure 6 shows a linear dependence of log k upon the pK_a of the nucleophilic thiolate, with $\beta_{nuc} = 0.6$. This result is typical of thiol−disulfide exchange reactions; previous workers have reported linear Bronsted plots with slopes of 0.5 ± 0.15 for nonenzymatic thiol−disulfide exchange reactions involving aliphatic thiols (*28*, *35*). These data suggest that a chemical step is rate-limiting at pH 7.0. If the rate were limited by substrate binding, such a correlation would be unexpected,

as the rate of the reaction should be sensitive to the structure of the nucleophile, as well as its pK_a. Thus, both the solvent kinetic isotope effect data and the Bronsted plot are consistent with the chemical step being fully or partially rate-limiting, depending on the pH. Furthermore, the lack of substrate specificity is consistent with the weak binding of glutathione indicated by the observed K_D of nearly 19 mM.

The observed rate constant for the thiol−disulfide exchange reaction can be compared with the second-order rate constants for nonenzymatic thiol−disulfide exchange reactions in order to provide an assessment of the enzyme's ability to accelerate this reaction. The reduction of GSSG by various thiols provides a reasonable model for the thiol−disulfide exchange reaction at the active site of TCHQ dehalogenase. Observed rate constants for reduction of GSSG by various thiols at pH 7.0 and 30 °C range from 0.05 to 1.5 M^{-1} s^{-1}, depending on the pK_a of the thiol (*28*). (The pK_a values of the thiols tested ranged from 7.7 to 10.6.) The observed second-order rate constant for reaction of glutathione with ESSG under comparable conditions is 12500 M^{-1} s^{-1}. The rate constants for other thiols ranged between 5000 and 67000 M^{-1} s^{-1} (see Table 1). This analysis suggests that the thiol−disulfide exchange reaction is more than 10000-fold faster than expected for a similar sterically unhindered uncatalyzed reaction. This finding is remarkable in view of the previously discussed findings that the enzyme binds the second equivalent of glutathione very weakly and does not optimize its nucleophilicity by decreasing its pK_a. This catalytic effect may be due to a combination of factors, including stabilization of the developing negative charges on Cys13 and on the sulfur of the covalently attached glutathione in the transition state and desolvation of the reactants at the active site.

CONCLUSIONS

The data described here are consistent with the proposal that TCHQ dehalogenase has evolved from an enzyme that originally carried out a different function and that its catalytic abilities are not highly optimized. The K_D for the second glutathione required for the thiol−disulfide exchange reaction is very high, and the enzyme does not potentiate the nucleophilicity of this glutathione by the obvious mechanism of lowering its pK_a. Furthermore, there is little specificity for the thiol substrate; a number of structurally different thiols react with high rate constants. Nevertheless, a substantial amount of catalysis occurs. This situation is interesting in the context of evolution of new enzymatic functions by addition of new chemical steps. Thiol−disulfide exchange reactions are fairly facile in aqueous solution due to the high nucleophilicity of thiolates. Thus, incorporating a thiol−disulfide exchange reaction into an enzymatic reaction is a relatively easy way to expand the catalytic capabilities of an enzyme.

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