Identification of a Covalent Intermediate between Glutathione and Cysteine13 Formed during Catalysis by Tetrachlorohydroquinone Dehalogenase

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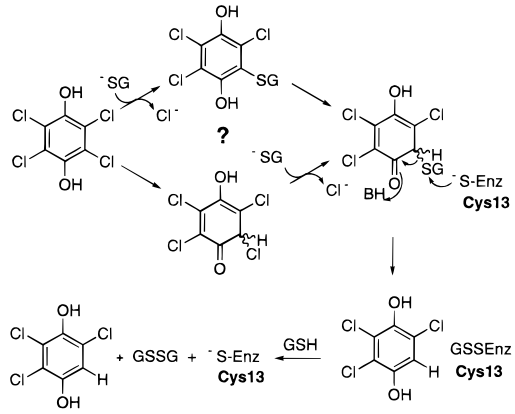
Tetrachlorohydroquinone (TCHQ) dehalogenase is found in strains of *Sphingomonas chlorophenolica*,[1,2] a Gram-negative soil bacterium isolated from soil highly contaminated with pentachlorophenol.[3−5] This bacterium degrades pentachlorophenol to CO_2, H_2O, and Cl^-. After conversion of pentachlorophenol to TCHQ by pentachlorophenol hydroxylase,[6] TCHQ dehalogenase catalyzes two successive reductive dehalogenations to convert TCHQ first to trichlorohydroquinone (TriCHQ) and then to 2,6-dichlorohydroquinone (DCHQ). The reducing equivalents for each step are provided by two molecules of glutathione (GSH).[7]

Although it seemed likely that a reductive dehalogenase would utilize either transition metal or organic cofactors, we have shown by electrospray ionization mass spectrometry coupled with reverse phase HPLC (LC/MS) and inductively coupled plasma emission spectrometry that TCHQ dehalogenase has no cofactors.[8] Furthermore, the protein has low but significant sequence identity (26−31%) with some microbial enzymes in the theta class of the glutathione *S*-transferase superfamily. The characteristic activity of these enzymes is the formation of glutathione conjugates rather than reduction. Thus, the mechanism of this reductive transformation has posed an intriguing puzzle.

We have previously found that Cys13 plays an important role in the reductive dehalogenation reaction. A mutant enzyme in which Cys13 has been replaced with serine catalyzes the disappearance of TCHQ at a rate comparable to that of wild-type enzyme but converts it to products that are consistent with the formation of 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone (a tautomer of 2,3,5-trichloro-6-*S*-glutathionylhydroquinone, GS-TriCHQ) at the active site.[8] This reactive intermediate decomposes, either in solution or at the active site, to give primarily GS-TriCHQ and an unknown isomer of dichloro-*S*-glutathionyl-hydroquinone. Based upon these findings, we propose the mechanism shown in Scheme 1. The mechanism of the initial stage of the reaction, in which the

Scheme 1



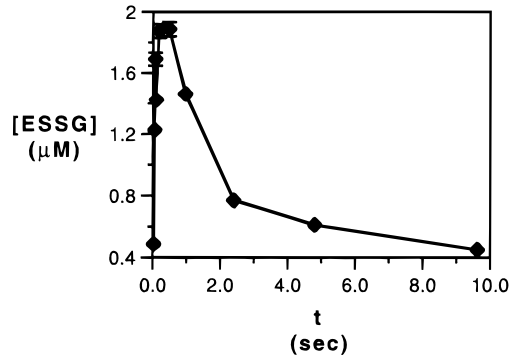


Figure 1. Accumulation and disappearance of a covalent adduct between TCHQ dehalogenase and [glycine-2-^{3}H]glutathione during turnover of TriCHQ: [TCHQ dehalogenase], 13.8 μM; [TriCHQ], 5 μM; and [glutathione], 200 μM. Samples were quenched with 1.0 N HCl and analyzed as described in the text.

tautomer of GS-TriCHQ is formed, is not yet defined. Possibilities include either a nucleophilic aromatic substitution reaction (via either an S_NAr or $S_{RN}1$mechanism), followed by tautomerization of GS-TriCHQ, or initial tautomerization of TCHQ, followed by an S_N2 displacement of chloride by glutathione. Subsequently, Cys13 attacks the sulfur of the glutathionyl substituent, liberating TriCHQ and forming a mixed disulfide. A second molecule of glutathione attacks the mixed disulfide to form glutathione disulfide and regenerate the free enzyme.

An important test of the validity of our mechanistic model is to determine whether a covalent adduct between Cys13 and glutathione is formed during turnover of TCHQ and TriCHQ. We have addressed this question using rapid flow-quench. TCHQ dehalogenase (27.5 μM) and ^{3}H-glutathione (400 μM, 6.1 μCi/μmol) in one syringe of an Applied Photophysics rapid flow-quench instrument were rapidly mixed with TriCHQ (10 μM) in a second syringe.[9] After variable delay times, the solutions were quenched with 1.0 N HCl to denature the enzyme and prevent further reaction of the putative mixed disulfide. The samples were then subjected to multiple cycles of concentration and dilution with 0.1 N HCl in a Filtron nanosep ultrafiltration

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(1) Xun, L.; Topp, E.; Orser, C. S. *J. Bacteriol.* **1992**, *174*, 8003−8007.
(2) McCarthy, D. L.; Claude, A.; Copley, S. D. *Appl. Env. Microbiol.* **1997**, *63*, 1883−1888.
(3) Radehaus, P. M.; Schmidt, S. K. *Appl. Env. Microbiol.* **1992**, *58*, 2879−2885.
(4) Saber, D. L.; Crawford, R. L. *Appl. Env. Microbiol.* **1985**, *50*, 1512−1518.
(5) Nohynek, L. J.; Suhonen, E. L.; Nurmiaho-Lassila, E.-L.; Hantula, J.; Salkinoja-Salonen, M. *System. Appl. Microbiol.* **1995**, *18*, 527−538.
(6) Xun, L.; Orser, C. S. *J. Bacteriol.* **1991**, *173*, 2920−2926.
(7) Xun, L.; Topp, E.; Orser, C. S. *Biochem. Biophys. Res. Commun.* **1992**, *182*, 361−366.
(8) McCarthy, D. L.; Navarrete, S.; Willett, W. S.; Babbitt, P. C.; Copley, S. D. *Biochemistry* **1996**, *35*, 14634−14642.

(9) TriCHQ rather than TCHQ was used to avoid complications arising from a second turnover. Solutions were degassed immediately before use. The final solution contained 416 mM Bis-Tris HCl, pH 7.0, 1 mM EDTA and 100 μM DTT.

(10) We have not attempted to obtain rate constants for formation and decay of the covalent adduct from these data because the enzyme is not completely saturated under these conditions and therefore these are not strictly single-turnover conditions. Furthermore, TriCHQ can, in theory, bind to the active site in three different orientations, only one of which produces 2,6-DCHQ. The uncertainty over the extent to which TriCHQ binds in the other two orientations complicates any kinetic analysis.

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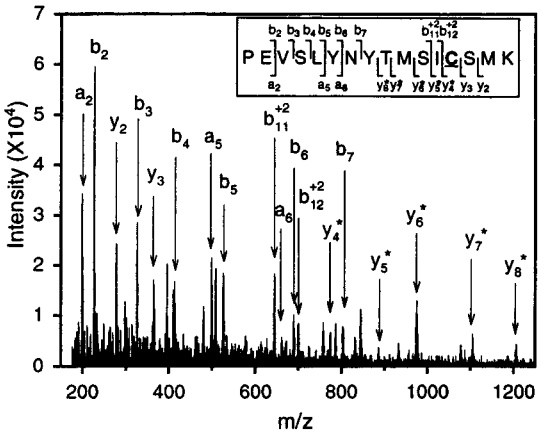

Figure 2. LC/MS/MS sequencing of the N-terminal peptide of the covalent adduct between TCHQ dehalogenase and glutathione. The peptide digest was applied to a POROS R120 C4 capillary column and the ion corresponding to the MH_3^{3+} ($m/z = 725.1$) of the N-terminal peptide modified with glutathione was selected on-line for fragmentation by collisional activation with argon. Nomenclature for identification of fragments is as described in Biemann.[13] Cleavage of the amide bond results in N-terminal fragments (designated as "b") and C-terminal fragments (designated as "y"). Cleavage of the bond between the α carbon and the carbonyl carbon results in N-terminal fragments (designated as "a") and C-terminal fragments (designated as "x"). The subscript indicates the number of amino acid residues in the fragment, and the superscript indicates the charge of the fragment ions. The masses of the y ions indicated with an asterisk are consistent with the presence of glutathione covalently linked to Cys13, which is underlined in the sequence shown. The *m*/*z* values for the labeled peaks are a_2, 198.7; a_5, 498.7; a_6, 661.4; b_2, 226.9; b_3, 326.3; b_4, 413.0; b_5, 526.0; b_6, 689.0; b_7, 803.3; b_{11}^{+2}, 643.7; b_{12}^{+2}, 699.8; y_2, 278.1; y_3, 364.4; y_4*, 774.2; y_5*, 886.4; y_6*, 975.4; y_7*, 1104.8; y_8*, 1206.4. The b_8 (966.7) and y_9* (1369.2) ions are not labeled because their intensities were less than five times background.

tube (3000 dalton cutoff) to wash excess glutathione into the filtrate but retain the protein and any covalently bound glutathione. An aliquot of the final retentate was subjected to scintillation counting to quantitate the level of the covalent adduct. As shown in Figure 1, a covalent adduct between the enzyme and glutathione increased to a maximum concentration of 1.9 μM at 500 ms and then declined.[10]

The nature of the covalent adduct was explored further using LC/MS/MS. A sample of protein was manually quenched with 100 mM iodoacetamide during turnover of TCHQ under multiple turnover conditions to alkylate residual glutathione and free cysteine residues and digested with TPCK-treated trypsin (100 nM) in 100 mM Tris-HCl, pH 7.0, containing 1 mM $CaCl_2$ for 2 h at 25 °C. The tryptic digest was injected onto an Applied Biosystems HPLC coupled to a Perkin-Elmer Sciex API-III triple quadropole mass spectrometer equipped with a nebulization-assisted electrospray source and a high pressure collision cell. A peptide with a mass of 2173 amu (consistent with that of an adduct between glutathione and the N-terminal peptide, which contains Cys13) was found when the reaction mixture included both TCHQ and glutathione but not when TCHQ was omitted. The identity of this peptide was confirmed by LC/MS/MS analysis of the MH_3^{3+} ion (see Figure 2). This procedure allowed identification of eight residues from the N-terminal and nine residues from the C-terminal ends of the fragment. Thus, the sequence of the entire 16-residue peptide was clearly established. Notably, the masses of all of the y fragments from y_4 through y_9 are larger than those expected from the unmodified N-terminal peptide by 306.3 daltons, which corresponds to the mass of glutathione minus the two hydrogens that are lost upon disulfide bond formation. These results unambiguously establish that the covalent adduct involves a mixed disulfide formed between Cys13 and glutathione.

The results described here provide strong support for our previous hypothesis about the role of Cys13 in the reductive dehalogenation reaction and for the events postulated to take place in the latter part of the catalytic cycle. This transformation is rather convoluted, but it provides an effective means of transferring the reducing equivalents from a pair of thiols to the aromatic ring. The mechanism of TCHQ dehalogenase is reminiscent of the mechanisms of proline reductase[11] and glycine reductase,[12] which use two cysteines or a selenocysteine and a cysteine, respectively, at the active site to reduce their amino acid substrates. Since TCHQ dehalogenase uses two molecules of glutathione rather than two active site residues to provide reducing equivalents, it seems that Nature has invented this catalytic strategy multiple times.

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(11) Arkowitz, R. A.; Dhe-Paganon, S.; Abeles, R. H. *Arch. Biochem. Biophys.* **1994**, *311*, 457−459.
(12) Arkowitz, R. A.; Abeles, R. H. *Biochemistry* **1991**, *30*, 4090−4097.
(13) Biemann, K. *Methods Enzymol.* **1990**, *193*, 455−479.