Recruitment of a Double Bond Isomerase To Serve as a Reductive Dehalogenase during Biodegradation of Pentachlorophenol†

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ABSTRACT: Tetrachlorohydroquinone dehalogenase catalyzes the replacement of chlorine atoms on tetrachlorohydroquinone and trichlorohydroquinone with hydrogen atoms during the biodegradation of pentachlorophenol by *Sphingomonas chlorophenolica*. The sequence of the active site region of tetrachlorohydroquinone dehalogenase is very similar to those of the corresponding regions of maleylacetoacetate isomerases, enzymes that catalyze the glutathione-dependent isomerization of a *cis* double bond in maleylacetoacetate to the *trans* configuration during the catabolism of phenylalanine and tyrosine. Furthermore, tetrachlorohydroquinone dehalogenase catalyzes the isomerization of maleylacetone (an analogue of maleylacetoacetate) at a rate nearly comparable to that of a *bona fide* bacterial maleylacetoacetate isomerase. Since maleylacetoacetate isomerase is involved in a common and presumably ancient pathway for catabolism of tyrosine, while tetrachlorohydroquinone dehalogenase catalyzes a more specialized reaction, it is likely that tetrachlorohydroquinone dehalogenase arose from a maleylacetoacetate isomerase. The substrates and overall transformations involved in the dehalogenation and isomerization reactions are strikingly different. This enzyme provides a remarkable example of Nature's ability to recruit an enzyme with a useful structural scaffold and elaborate upon its basic catalytic capabilities to generate a catalyst for a newly needed reaction.

Many xenobiotic pesticides, polymers, textile dyes, and munitions are resistant to biodegradation because microorganisms lack metabolic pathways to accomplish their breakdown. However, some xenobiotics can be biodegraded. In such cases, microorganisms have assembled new metabolic pathways, probably primarily by recruiting existing enzymes to perform new roles (*1*−*3*). Subsequent mutations may improve the fitness of these recruited enzymes for their new roles. Here we report a dramatic example of an enzyme that has apparently been recruited from an unexpected source to provide a reductive dehalogenase required for biodegradation of pentachlorophenol (PCP)[1] by *Sphingomonas chlorophenolica*.

PCP was first introduced as a wood preservative in 1936 (*4*), and has been used in large quantities since that time. PCP would be expected to be recalcitrant to biodegradation for two reasons. First, it is highly chlorinated, and the resistance of aromatic xenobiotics to biodegradation generally increases with the number of chlorine substituents. Second, it is very toxic because it uncouples oxidative phosphorylation and perturbs membrane properties. Surprisingly, however, PCP can be degraded by some microorganisms. In the Gram-negative soil bacterium *S. chlorophenolica*, PCP degradation begins with an initial hydroxylation reaction which forms tetrachlorohydroquinone (TCHQ) (see Figure 1) (*5*). Subsequently, TCHQ is converted to trichlorohydroquinone (TriCHQ) and then 2,6-dichlorohydroquinone (DCHQ) by two successive reductive dehalogenation reactions catalyzed by TCHQ dehalogenase (*6*). Each of these reactions results in the conversion of 2 equiv of glutathione to glutathione disulfide. The final known step is the ring cleavage of DCHQ (*7*).

TCHQ dehalogenase has been the focus of several years of study in our laboratory (*8*−*10*). Our working model for its mechanism is shown in Figure 2. TCHQ dehalogenase has low but significant sequence identity to members of the theta and zeta classes (*8*, *11*) of the glutathione *S*-transferase (GST) superfamily. Although most members of the GST superfamily catalyze a simple nucleophilic attack of glutathione upon an electrophilic substrate to form a glutathione conjugate, in several cases this basic chemical strategy has been incorporated into a more complex transformation (*12*−*14*) (see Figure 3). TCHQ dehalogenase appears to be related to one of these enzymes, a maleylacetoacetate (MAA) isomerase that catalyzes the glutathione-dependent conversion of a *cis* double bond in MAA to the *trans* configuration during the catabolism of phenylalanine and tyrosine in mammals and some bacteria and fungi (see Figure 4). We show here that the sequence of TCHQ dehalogenase is quite similar to those of human, mouse, and fungal MAA

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[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; DCA, dichloroacetic acid; DCHMS, 2,4-dichloro-3-hydroxy-*cis*,*cis*-muconic semialdehyde; DCHQ, 2,6-dichlorohydroquinone; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; MA, maleylacetone; MAA, maleylacetoacetate; PCP, pentachlorophenol; TCHQ, tetrachlorohydroquinone; THF, tetrahydrofuran; TriCHQ, trichlorohydroquinone.

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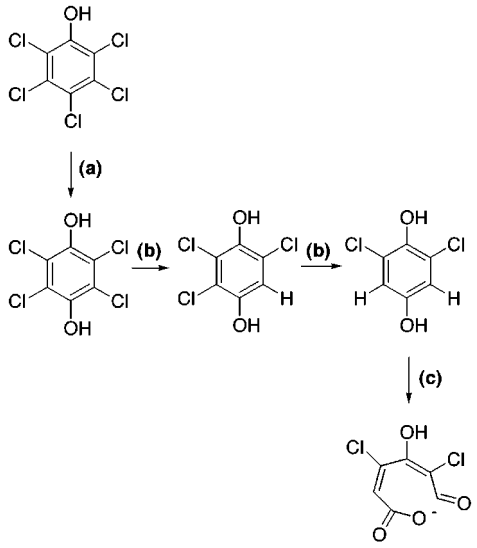

FIGURE 1: Pathway for degradation of PCP to 2,4-dichloro-3-hydroxy-*cis*,*cis*-muconic semialdehyde (DCHMS) in *S. chlorophenolica* sp. ATCC 39723. (a) Pentachlorophenol hydroxylase, O_2, 2 NADPH (*5*); (b) tetrachlorohydroquinone dehalogenase, 2 glutathione (*6*); (c) 2,6-dichlorohydroquinone dioxygenase, O_2 (*7*).

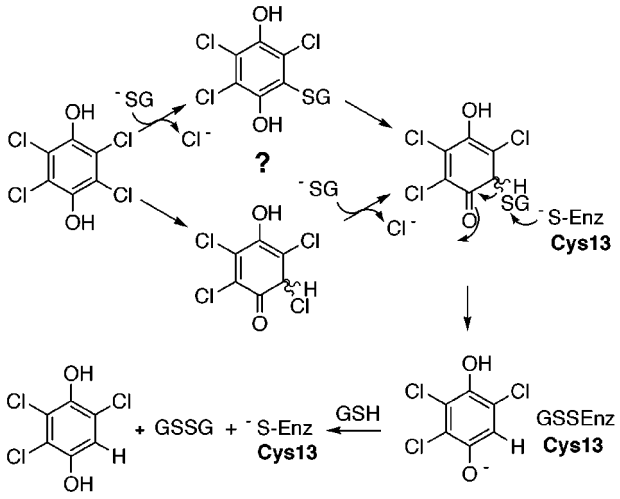

FIGURE 2: Working model for the mechanism of TCHQ dehalogenase. There is still some uncertainty about the order of the initial steps, so two possible sequences of events are shown.

isomerases in a region that we know contains active site residues. Furthermore, TCHQ dehalogenase has isomerase activity similar to that of a bacterial MAA isomerase, and Cys13 is required for both the dehalogenase and isomerase activities. The functional and evolutionary implications of these findings will be discussed below.

MATERIALS AND METHODS

Sequence Analysis. A BLAST (*15*) search of the NCBI nonredundant database was performed using the sequence of the human MAA isomerase (g2228731) as a query sequence. Zeta class GSTs were selected from the BLAST output based upon the presence of a sequence similar to the consensus sequence reported by Board et al. (SSCX-WRVIAL) (*11*). A subset of these proteins in which the pairwise sequence identity is no more than 50% was aligned using ClustalW (*16*). Motif analysis was performed using the MEME algorithm (*17*) at the San Diego Supercomputing

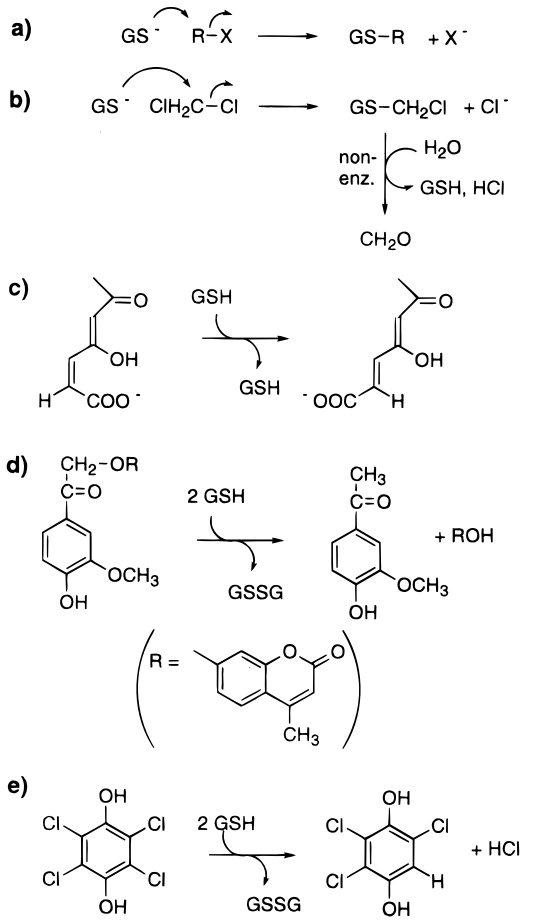

FIGURE 3: Reactions catalyzed by enzymes in the GST superfamily. (a) Nucleophilic attack of glutathione on an electrophilic substrate to form a glutathione conjugate, the step characteristic of GSTs; (b) dehalogenation of dichloromethane by dichloromethane dehalogenase; (c) isomerization of MAA by MAA isomerase; (d) reductive cleavage of an ether linkage in a lignin breakdown product by β-etherase; (e) reductive dehalogenation of TCHQ by TCHQ dehalogenase.

Facility. Control analyses were carried out in which the sequences were scrambled in order to determine the number and scores of motifs that might arise by chance in a set of this size and amino acid composition. The set of 21 GSTs analyzed by Board et al. (*11*), which includes GSTs of the zeta, alpha, mu, pi, phi, delta, theta, sigma, and kappa classes, as well as a yeast GST, was also subjected to motif analysis using the MEME algorithm.

Preparation of MA and TriCHQ. MA was synthesized by a modification of the method of Fowler and Seltzer (*18*). After opening of the lactone ring of 4-acetonylidenebut-2-ene-4-olide in 1 N sodium hydroxide on ice for 10 min, the solution was diluted 5-fold with 50 mM potassium phosphate, pH 7.4. The pH of the reaction mixture was adjusted to 7.4 with HCl, and the solution was used for kinetic assays within 3 h. This procedure results in a mixture of keto and enol tautomers. Assignment of the structures of these two forms requires collection of NMR data in H_2O so that the exchangeable enolic and methylene protons in the enol and keto tautomers, respectively, can be seen. Enol tautomer: ^{1}H NMR (500 MHz, H_2O) δ 6.28 (d, J = 12.5 Hz, 1 H), 6.08 (d, J = 12.5 Hz, 1 H), 5.64 (s, 1 H), 2.20 (s, 3 H). Keto tautomer: ^{1}H NMR (500 MHz, H_2O) δ 6.49 (d, J = 12.0

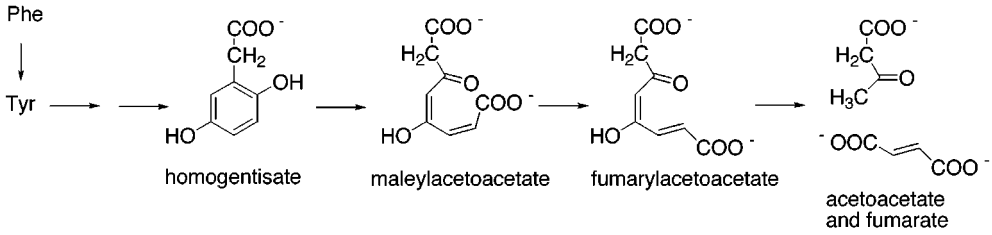

FIGURE 4: The homogentisate pathway for catabolism of phenylalanine and tyrosine in humans, mice, and some bacteria and fungi. MAA isomerase catalyzes the glutathione-dependent conversion of MAA to fumarylacetoacetate.

Hz, 1 H), 6.16 (d, $J = 12.0$ Hz, 1 H), 3.92 (s, 2 H), 2.28 (s, 3 H).

TriCHQ was prepared by reduction of trichlorobenzoquinone (Aldrich) with sodium dithionite in a mixture of methylene chloride/H_2O (1:2). The organic phase containing trichlorobenzoquinone was treated 3 times with excess aqueous sodium dithionite, extracted with a saturated NaCl solution, and dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation.

Growth of Bacteria and Preparation of Extracts. Strains of *Pseudomonas cepacia*, *P. fluorescens*, and *P. putida* were obtained from Presque Isle Cultures (Erie, PA). These bacteria and *S. chlorophenolica* were grown in 1 L of M9 minimal medium containing tyrosine, benzoate, *o*-hydroxybenzoate, or *m*-hydroxybenzoate (0.1% w/v) at 25 °C until an OD_{600} of 1.0 was reached. The cells were harvested by centrifugation at 2600*g* at 4 °C for 12 min. The cell paste was suspended in 15 mL of 25 mM potassium phosphate, pH 7.4, containing 0.1 mM AEBSF and passed through a French pressure cell at 12 000 psi 4 times. Debris was removed by centrifugation at 18000*g* at 4 °C for 12 min. The clarified extract was concentrated using a Centricon 10 ultrafiltration unit and used immediately.

Purification of Enzymes. TCHQ dehalogenase was purified as described previously (*8*). MAA isomerase was partially purified from extracts of *S. chlorophenolica* prepared as described above using an affinity column constructed to mimic certain structural features of MA. *ω*-Aminodecylagarose (Sigma) was washed with water and THF, and then resuspended in THF. 4-Cyclopentene-1,3-dione (Aldrich) was dissolved in THF and added to the aminodecylagarose suspension to give a final ratio of 1:10 (w/w); the suspension was stirred on a shaker for 12 h at 25 °C. The agarose was collected by filtration and washed with water, 1 N HCl, 1 N NaOH, 1 M NaCl, and 25 mM potassium phosphate buffer, pH 7.4, to obtain a brown colored resin. The nature of the adduct formed is uncertain, but MAA isomerase binds tightly to the resin, as do some other proteins in the crude extract. A crude extract of *S. chlorophenolica* cells grown on tyrosine as a sole carbon source was loaded onto the affinity column, which had been preequilibrated with 25 mM potassium phosphate, pH 7.4. The column was washed with 10 column volumes of 25 mM potassium phosphate, pH 7.4, and then eluted with a gradient of KCl (0−0.5 M) in 25 mM potassium phosphate, pH 7.4. This procedure resulted in a 4-fold purification of the protein.

Assays. Dehalogenase assays were carried out as previously described (*8*). For the determination of $K_{M,TriCHQ}$, the glutathione concentration was 20 mM. For the determination of $K_{M,GSH}$, the TriCHQ concentration was 250 μM.

MA isomerase activity was assayed in reaction mixtures containing variable concentrations of MA and glutathione in 50 mM potassium phosphate buffer, pH 7.4, at 30 °C. At intervals, reaction mixtures were quenched with an equal volume of 1 N HCl, centrifuged to remove particulates, and analyzed by reverse phase HPLC to determine the concentration of MA. The substantial rate of the nonenzymic reaction was measured in the absence of the enzyme. For the determination of $K_{M,MA}$, the glutathione concentration was 2 mM. For the determination of $K_{M,GSH}$, the MA concentration was 10 mM.

Conversion of DCA to glyoxylate was measured by following the reduction of glyoxylate in the presence of NADH and lactic dehydrogenase (*19*−*21*) in 100 mM potassium phosphate, pH 7.0. For determination of $K_{M,DCA}$, the glutathione concentration was 5 mM, and for determination of $K_{M,GSH}$, the DCA concentration was 7 mM.

For comparison of the relative amounts of MA isomerase and TriCHQ dehalogenase activities in crude extracts, partially purified MAA isomerase, or purified TCHQ dehalogenase, assays were carried out as described above in reaction mixtures containing 10 mM MA and 1 mM GSH for isomerase activity and 0.1 mM TriCHQ and 3 mM GSH for dehalogenase activity.

The rate of the nonenzymatic isomerization of MA was measured at 30 °C in 10 mM potassium phosphate buffer at pH values between 7 and 9. (Control experiments showed that the reaction was accelerated by phosphate, but that the contribution of phosphate catalysis to the overall rate was negligible at 10 mM potassium phosphate.) At intervals, the reaction was quenched with HCl (final concentration 0.5 N), and the amount of MA remaining was analyzed by reverse phase HPLC using a Rainin C18 column equilibrated with 0.1% acetic acid/10% methanol. The nonenzymatic reaction of GSH with TriCHQ in 50 mM potassium phosphate buffer, pH 7.4, at 30 °C was monitored using the procedures used for the enzymatic reaction (*8*).

Homogentisate dioxygenase and glutathione-dependent MAA isomerase activities in crude extracts were detected using the method of Crawford and Frick (*22*). The crude extract was diluted 10-fold into potassium phosphate buffer (0.1 M, pH 7.4) containing 1% ascorbate (to protect homogentisate from oxidation) and treated with 300 μM *N*-ethylmaleimide in order to alkylate GSH present in the extract. A baseline absorbance at 320 nm was obtained before addition of 100 μM homogentisate. Cleavage of homogentisate to give MAA results in the formation of a characteristic absorbance at 320 nm. When this reaction was completed, 300 μM GSH was added to permit the conversion of MAA

to fumarylacetoacetate (FAA) and the subsequent hydrolysis of FAA to give products that do not absorb at 320 nm.

RESULTS AND DISCUSSION

Sequence Similarities between TCHQ Dehalogenase and Glutathione-Dependent MAA Isomerases. The possibility that TCHQ dehalogenase might have MAA isomerase activity was first suggested by the striking similarities between the active site region of TCHQ dehalogenase and the corresponding regions in human and fungal MAA isomerases, all of which are members of the zeta class of the GST superfamily (*11*, *23*). Figure 5 shows a multiple sequence alignment of a set of zeta class GSTs that have no more than 50% sequence identity with any other member of the set, along with the sequence of a structurally characterized theta class GST from *Arabidopsis thaliana*. This set includes the known MAA isomerases from human and *Emericella nidulans*. The mouse MAA isomerase (*24*) is not included because it is 85% identical to the human MAA isomerase. In addition to TCHQ dehalogenase, the set includes another reductive dehalogenase (LinD), a 2,5-dichlorohydroquinone dehalogenase found in a strain of *S. paucimobilis* that is capable of degrading lindane (*25*). Although TCHQ dehalogenase and 2,5-dichlorohydroquinone dehalogenase catalyze similar reactions and both come from Sphingomonads, they have only 23% sequence identity. Also included are the only two putative bacterial isomerases in the database. One (pMAAI.Sm) is encoded by an open reading frame downstream of a homogentisate dioxygenase gene in *Sinorhizobium meliloti*. This protein is 45% and 39% identical to the human and fungal MAA isomerases, respectively, and is therefore quite likely to be a MAA isomerase. [Surprisingly, this sequence lacks the cysteine that is conserved in all of the remaining proteins and is essential for TCHQ dehalogenase and MA isomerase activity (see below). The significance of this change is not clear, since this protein has not been characterized.] The second bacterial sequence, ORF3.Ss, is an open reading frame downstream of a gentisate dioxygenase gene in *Sphingomonas* sp. strain RW5, and is therefore likely to be a maleylpyruvate isomerase. (Maleylpyruvate is formed by ring-cleavage of gentisate, and differs from MAA in the lack of a single methylene group.) Curiously, TCHQ dehalogenase is only 27% identical to ORF3.Ss and 18% identical to pMAAI.Rm. ORF3.Ss and pMAAI.Rm are only 28% identical. There is apparently considerable diversity among bacterial zeta class GSTs.

Motifs identified in the zeta class GSTs shown in Figure 5 using the MEME algorithm (*17*) at the San Diego Supercomputing Center are mapped onto the multiple sequence alignment in Figure 5. These motifs were compared to the motifs found in a set of GSTs assembled by Board et al. (*11*), which includes representatives of several different classes of GSTs (zeta, alpha, mu, pi, phi, delta, theta, sigma, and kappa classes, and an unclassified yeast GST) (data not shown), to identify motifs which are specific to the zeta class. Motifs that correspond to or overlap motifs found in other classes of GSTs are shown in black, while those that are specific to the zeta class are shown in yellow. The most distinctive motif is the first yellow motif, which can be considered the signature motif for the zeta class. The consensus sequence for this motif (LYSYWR/LSSCSXR/KVRIAL) differs slightly from that originally described by Board et al. (SSCXWRVIAL) for a smaller set containing the human, *C. elegans*, and carnation proteins (*11*), but is a better representation of the motif because it is based upon a larger set of proteins. This motif includes two residues that we know to be present in the active site of TCHQ dehalogenase: Cys13, which acts as a nucleophile (see Figure 2) (*8*, *9*), and Ser11, which we suspect stabilizes the thiolate of glutathione at the active site in a manner analogous to the active site serine in many theta class GSTs (Navarette, Kiefer, and Copley, unpublished observations; *26*−*28*). It is rather puzzling that the reported sequence of the putative MAA isomerase from *Sinorhizobium meliloti* lacks the otherwise completely conserved cysteine in this motif. However, this protein has not been expressed and characterized, and it is possible that the reported sequence is in error, or that the protein lacks activity. Alternatively, there may indeed be a way to catalyze the isomerization reaction in the absence of an active site cysteine.

The positions of the regions corresponding to the remaining yellow motifs can be approximated based upon the alignment with the structurally characterized theta class GST from *Arabidopsis thaliana*. This enzyme has 40% identity to the putative GST from *Naegleria fowleri* and lower but still significant identity (>23%) to three other members of the set. The theta class structure should serve as a reasonable model of the zeta class enzymes because GSTs of every class for which structural information is available (alpha, mu, pi, theta, and sigma) share a similar fold (*29*). The secondary structural elements present in the theta class enzyme are indicated in Figure 5. Based upon this information, we predict that the second yellow motif will be found at the C-terminal end of helix α_3', which is the first segment of a long helix (α3) running the length of the protein and which forms part of the active site cleft in the C-terminal domain of the enzyme. In GSTs, the C-terminal domain is generally responsible for binding the electrophilic substrate, while the N-terminal domain is primarily responsible for binding and activating glutathione. Thus, we predict that this region is involved in binding the electrophilic substrate: MAA or maleylpyruvate in MAA or maleylpyruvate isomerases, respectively, and TCHQ or 2,5-dichlorohydroquinone in the case of the reductive dehalogenases. Notably, the similarity between the zeta class GSTs is particularly low in this region. Three of the sequences (the two reductive dehalogenases and the putative maleylpyruvate isomerase) have significant insertions that interrupt a motif that is highly conserved in the MAA isomerases and three proteins of unknown function. Furthermore, the correspondence between the zeta class enzymes and the theta class GST is also poor in this region. Since this region is expected to be involved in binding different substrates, it is not surprising that this should be a highly plastic area of the structure.

Based upon the comparison with the theta class structure, the third and fourth yellow motifs appear to be distant from the active site. The third motif is found only in the proteins that contain the second yellow motif. Thus, these two motifs seem to be most closely correlated with true MAA isomerases. The fourth yellow motif is found in the proteins that lack the second yellow motif, and may be correlated with zeta class GSTs with alternate substrate specificities. The function of these regions is not yet clear.

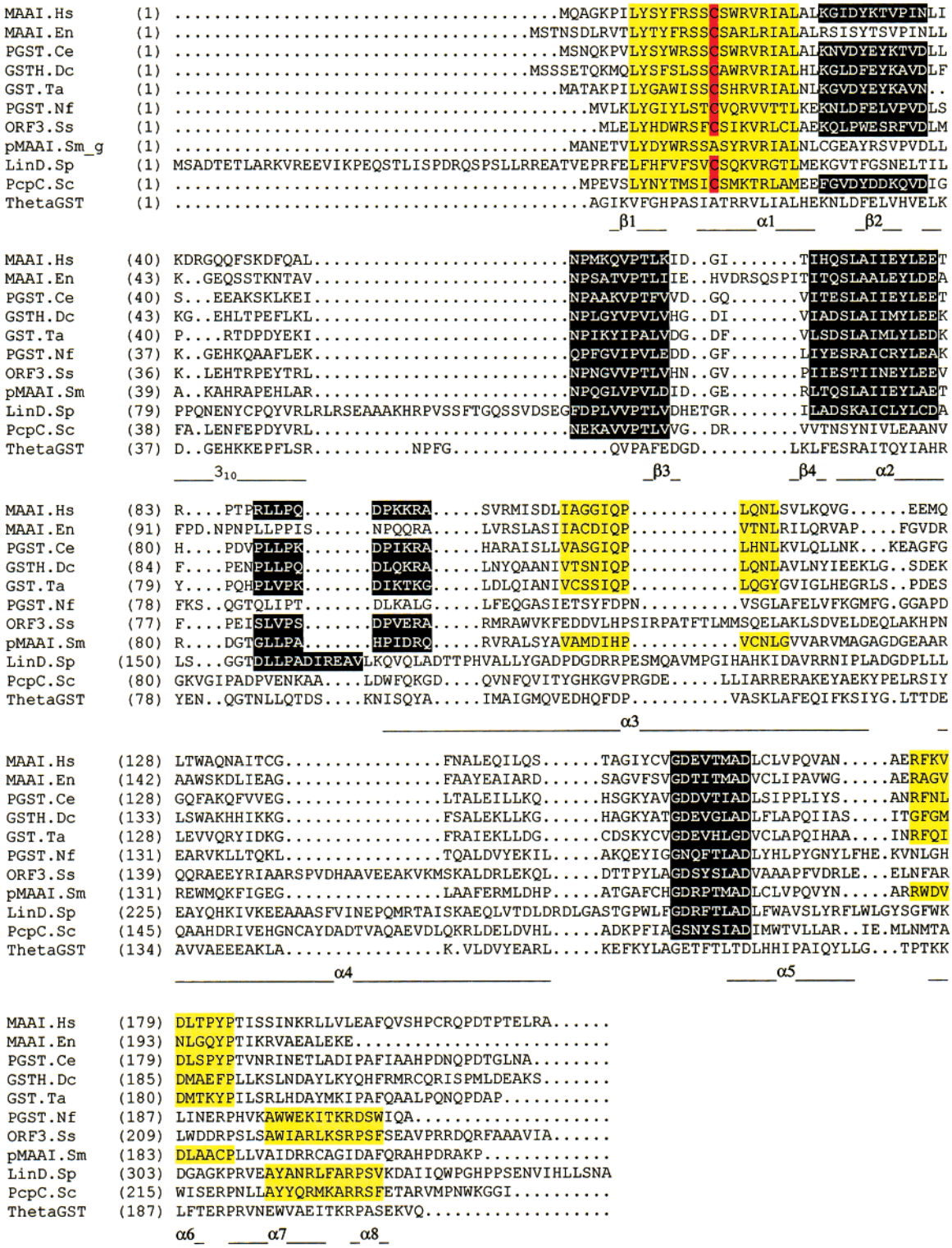

FIGURE 5: Multiple sequence alignment of members of the zeta class of the GST superfamily and a structurally characterized theta class GST from *Arabidopsis thaliana*. Motifs that are shared with other classes of GSTs are highlighted in black, and motifs specific to the zeta class are highlighted in yellow. The conserved cysteine corresponding to Cys13 in TCHQ dehalogenase is highlighted in red. MAAI.Hs, MAA isomerase, *Homo sapiens* (g2228731); MAAI.Mm, MAA isomerase, *Mus musculus* (g5478316); MAAI.En, MAA isomerase, *Emericella nidulans* (g3914021); GST.Dc, GST homologue, *Dianthus caryophyllus* (g18330); PGST.At, putative GST, *Arabidopsis thaliana* (g3894171); GST.Ta, GST, *Triticum aestivum* (g2183249); PGST.Ce, putative GST, *Caenorhabditis elegans* (g1051300); PGST.Nf, putative GST, *Naegleria fowleri* (g1353751); ORF3.Ss, ORF 3 downstream of gentisate dioxygenase gene, *Sphingomonas* sp. strain RW5 (g3550669); PMAAI.Sm, putative MAA isomerase, *Sinorhizobium meliloti* (g4808640); LinD.Sp, 2,5-dichlorohydroquinone reductive dehalogenase, *Sphingomonas paucimobilis* (g1731852); PcpC.Sc, TCHQ dehalogenase, *Sphingomonas chlorophenolica* (g148690); ThetaGST, GST, *Arabidopsis thaliana* (g2554770).

TCHQ Dehalogenase Has MA Isomerase Activity. The presence of the zeta class motif in TCHQ dehalogenase prompted us to determine whether TCHQ dehalogenase can isomerize MAA. Because of the instability of MAA, we used MA (see Figure 6b), a good alternative substrate for MAA isomerase (*30*), in our assays. The synthesis of MA results

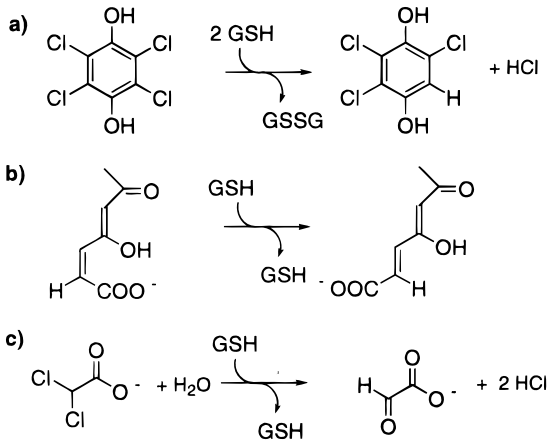

FIGURE 6: Reactions catalyzed by TCHQ dehalogenase. (a) Reductive dehalogenation of TCHQ and TriCHQ (only reaction of TCHQ is shown); (b) isomerization of MA to fumarylacetone; (c) conversion of DCA to glyoxylate.

in the initial formation of the *cis,cis* isomer, which rapidly equilibrates to a mixture of keto and enol tautomers. (Whether the enol tautomer is *cis,cis* or *cis,trans* is uncertain.) The unavoidable presence of both tautomers in the preparation of MA complicates the kinetic analysis, and the kinetic parameters reported here must be regarded as apparent parameters. TCHQ dehalogenase does indeed have MA isomerase activity (see Table 1). Kinetic parameters calculated from the data reported for the *Vibrio* MAA isomerase (which was assayed with MA prepared using a similar method which also generates a mixture of forms) (*13*, *30*) are given for comparison. The apparent k_{cat} for the isomerase activity of TCHQ dehalogenase is comparable to that of the *bona fide* bacterial MAA isomerase. The apparent $k_{cat}/K_{M,MA}$ is about 4-fold lower. The decreased $k_{cat}/K_{M,MA}$ may reflect some loss of efficiency in the isomerase reaction as a consequence of changes in the enzyme which have improved the dehalogenase reaction, but kinetic parameters for other MAA isomerases must be measured before definite conclusions can be made.

Comparison of the Abilities of TCHQ Dehalogenase To Carry Out Reductive Dehalogenation of TriCHQ and Isomerization of MA. The ability of this enzyme to effectively catalyze reactions as different as reductive dehalogenation of TCHQ and TriCHQ and glutathione-dependent isomerization of MA is unprecedented. Consequently, we undertook efforts to quantify these two activities. Unfortunately, this comparison is complicated by the fact that the dehalogenation reaction is subject to substrate inhibition by the aromatic substrate, which appears to arise from competition between the aromatic substrate and the second molecule of glutathione required to complete the reductive dehalogenation reaction (Kiefer, Anandarajah, and Copley, unpublished observations). The severe substrate inhibition makes determination of meaningful catalytic parameters for the reductive dehalogenation reaction difficult. Consequently, we will present the kinetic parameters for the simpler reaction catalyzed by the C13S mutant enzyme, which catalyzes the reaction through the formation of 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone (see top line, Figure 2) (*8*), and is not subject to substrate inhibition. It is likely that the mutant catalyzes the most difficult steps in the reaction: either the S_NAr reaction in the top pathway or the tautomerization reactions in either the top or the bottom pathway are expected to be energetically more demanding than either the nucleophilic attack of Cys13 on 2,3,5-trichloro-6-*S*-glutathionyl-4-hydroxycyclohexa-2,4-dienone or the subsequent thiol−disulfide exchange reaction. Furthermore, the C13S mutant enzyme uses only 1 equiv of glutathione, rather than 2, and is therefore more comparable to MA isomerase in this respect. Thus, the kinetic parameters for the mutant enzyme provide a reasonable, although not ideal, basis for comparison with those for the isomerization reaction catalyzed by the wild-type enzyme.

The values of k_{cat} for the partial reaction of TriCHQ catalyzed by the C13S mutant enzyme and for the isomerization of MA by the wild-type enzyme are comparable (see Table 1). The $k_{cat}/K_{M,TriCHQ}$ for the partial reaction with TriCHQ catalyzed by the mutant enzyme is 90-fold higher than the apparent $k_{cat}/K_{M,MA}$ for the isomerization reaction catalyzed by the wild-type enzyme. Since MA is a substrate analogue and lacks one of the carboxylates found in MAA, the actual $k_{cat}/K_{M,MAA}$ would be expected to be somewhat higher than the measured $k_{cat}/K_{M,MA}$. The specific activity of the *Vibrio* MAA isomerase under nonsaturating conditions was reported to be 2.7-fold higher for MAA than for MA (*30*), suggesting that the effect of the missing carboxylate is not large. Thus, the k_{cat}/K_M for the partial reaction of TriCHQ should be on the order of 30-fold higher than that for the isomerization of MAA. Although these parameters are not strictly comparable, the data suggest that the active site enzyme is somewhat specialized for catalyzing the initial steps of the reductive dehalogenation reaction.

The enzyme is quite effective at accelerating both the isomerization and dehalogenation reactions. The second-order rate constant for isomerization of MA via reaction with H_2O is 9.2×10^{-7} M^{-1} s^{-1}.[2] Comparison with the k_{cat}/K_M for the enzyme-catalyzed isomerization suggests that the enzyme accelerates the reaction by more than 8 orders of magnitude. The reaction of TriCHQ with glutathione is undetectable, and the second-order rate constant is therefore less than 10^{-3} M^{-1} s^{-1} (our limit of detection). The $k_{cat}/K_{M,TriCHQ}$ for the reaction catalyzed by the mutant enzyme is 3.6×10^4 M^{-1} s^{-1}. This reaction is accelerated by at least 7 orders of magnitude. Thus, the enzyme is actually quite effective at catalyzing both the isomerization reaction and the first part of the dehalogenation reaction. However, the overall catalytic abilities of the enzyme are considerably compromised by the substrate inhibition, which limits the ability of the enzyme to catalyze the entire dehalogenation reaction.

MAA Isomerases Generally Lack Significant Dehalogenase Activity. The ability to dehalogenate TCHQ is not a general property of MAA isomerases. We have found MAA isomerase activity in crude extracts of several Gram-negative soil bacteria that are capable of growth on tyrosine as a sole

[2] The nonenzymic isomerization in the absence of buffer catalysis can occur via reaction of MA with either H_2O or hydroxide. The pseudo-first-order rate constant under these circumstances is given by $k_{obs} = k_w[H_2O] + k_{OH}[OH^-]$. At pH 7, k_{obs} is 5.04 ($\pm$0.01) $\times$ 10^{-5} s^{-1}. Variation of the hydroxide concentration by 2 orders of magnitude from 10^{-7} to 10^{-5} M resulted in no significant change in k_{obs}, demonstrating that the primary reaction at pH values near neutral involves reaction of MA with H_2O. Division of k_{obs} by 55 M therefore gives the second-order rate constant for the nonenzymic isomerization of MA (9.2×10^{-7} M^{-1} s^{-1}).

Table 1: Kinetic Parameters for Activities of TCHQ Dehalogenase and Similar Enzymes

activity	enzyme	k_{cat} (s^{-1})	$K_{M,sub}$ (μM)	$k_{cat}/K_{M,sub}$ (M^{-1} s^{-1})	$K_{M,GSH}$ (μM)
MA isomerase	*S. chlorophenolica*				
	wt	0.82 (0.10)	2000 (69)	410 (52)	<500[a]
	C13S	ND[b]			
	Vibrio (*13*, *30*)	1	600	1670	140
TriCHQ dehalogenase	*S. chlorophenolica*				
	C13S	0.61 (0.01)	17 (5)	36000 (10600)	296 (17)
DCA dehalogenase	*S. chlorophenolica*				
	wt	0.013(0.0002)	560 (30)	23 (1)	32 (2)
	C13S	ND	NA	NA	NA
	rat zeta GST (*31*)	0.6	71	8500	59

[a] Only an estimate for this value can be obtained. We must measure the disappearance of MA, since the product undergoes further reactions and product appearance cannot be quantified. At the levels of MA required to saturate the enzyme, the small changes in peak area during reactions with glutathione at concentrations less than 1 mM are not detectable. [b] ND, not detectable.

Table 2: Comparison of TriCHQ Dehalogenase and MA Isomerase Activities in TCHQ Dehalogenase, Crude Extracts from Soil Bacteria Grown on Tyrosine, and Partially Purified MAA Isomerase

source of enzyme	TriCHQ dehalogenase[a]/ MA isomerase[b]
purified TCHQ dehalogenase	1.8
Pseudomonas putida extract	0.001
Pseudomonas fluorescens extract	0.002
Pseudomonas cepacia extract	0.0002
S. chlorophenolica extract	0.0001
partially purified MA isomerase from *S. chlorophenolica*	0[c]

[a] Assayed using 100 μM TriCHQ and 3 mM GSH. [b] Assayed using 10 mM MA and 1 mM GSH. [c] Dehalogenase activity not detectable.

carbon source. As shown in Table 2, the ratio of dehalogenase activity to isomerase activity is very low in these extracts, in stark contrast to the ratio for purified TCHQ dehalogenase, which is 1.8 under the reaction conditions used. We do not yet know whether the small amount of dehalogenase activity in these extracts is due to a secondary activity of the MAA isomerase or to a separate protein; this question is under investigation. Notably, a crude extract of *S. chlorophenolica* grown on tyrosine also had a very low ratio of dehalogenase and isomerase activities, suggesting either that some unknown factor in the extract interfered with the dehalogenase activity but not the isomerase activity (an unlikely possibility since both activities are catalyzed at the same active site, see further below) or that *S. chlorophenolica* contains an additional MAA isomerase that has little or no dehalogenase activity and provides the bulk of the isomerase activity under these growth conditions. The latter possibility is correct, as we have partially purified a MA isomerase (by 4-fold) that has no detectable dehalogenase activity.

TCHQ Dehalogenase Also Has Dichloroacetate Dehalogenase Activity. Recently, the human and rat zeta class GSTs have been shown to catalyze the conversion of dichloroacetic acid (DCA), a carcinogenic drinking water disinfection byproduct, to glyoxylate (*31*) (see Figure 6c). TCHQ dehalogenase also catalyzes this transformation (see Table 1), but the k_{cat} is 50-fold lower than those for the other activities and for the comparable reaction catalyzed by the rat liver zeta class GST (*31*). Although the $k_{cat}/K_{M,DCA}$ is quite low, the enzyme still accelerates this reaction by several orders of magnitude compared to the undetectably slow nonenzymatic reaction.

The Unusual Catalytic Versatility of TCHQ Dehalogenase. It is quite astounding that the active site of this enzyme is capable of recognizing substrates as different as MA, TCHQ, and DCA, and of catalyzing overall transformations as different as isomerization of a double bond, reductive dehalogenation of a chlorinated aromatic compound, and conversion of DCA to glyoxylate (see Figure 6). Many enzymes are capable of catalyzing secondary reactions as a consequence of the peculiar assembly of catalytic residues in their active sites. (This ability is referred to as catalytic promiscuity.) The rate of the secondary reaction is generally quite low compared with that of the primary reaction. For example, alkaline phosphatase from *E. coli* catalyzes the hydrolysis of *p*-nitrophenyl sulfate with a k_{cat}/K_M that is 9 orders of magnitude lower than the k_{cat}/K_M for hydrolysis of *p*-nitrophenyl phosphate (*32*). *O*-Succinylbenzoate synthase from *Amycolaptosis* sp. is an unusually effective enzyme in terms of catalyzing a secondary reaction, the racemization of *N*-acyl amino acids (*33*). In this case, k_{cat}/K_M for the secondary reaction is 700-fold lower than that of the primary reaction, and k_{cat} is 10-fold lower. TCHQ dehalogenase, therefore, is the most dramatic example of a catalytically promiscuous enzyme of which we are aware.

The mechanisms of secondary reactions invariably involve the use of the active site catalytic machinery to carry out steps that are similar to those that occur during catalysis of the primary reaction. For example, the common mechanistic theme in the case of *O*-succinylbenzoate synthase is the removal of a proton alpha to a carboxylate (*33*), the prototypical step catalyzed by this and other members of the enolase superfamily (*34*, *35*). In the case of TCHQ dehalogenase, we can identify two factors that apparently contribute to a common mechanistic strategy employed in the catalysis of the three seemingly very different reactions shown in Figure 6. First, GSTs are specialized to promote the nucleophilic attack of glutathione upon an electrophilic substrate to form a glutathione conjugate (*29*). Our current mechanistic model for the dehalogenation reaction includes such a step (see Figure 2), and it is likely that the isomerization reaction and the dehalogenation of DCA also involve a comparable step. Second, Cys13 is absolutely required for isomerization of MA and dehalogenation of DCA (see Table 1), and for the completion of the reductive dehalogenation reaction (see Figure 2) (*8*, *9*), suggesting that nucleophilic attack of this active site cysteine upon the substrate or an intermediate may also be a common theme in catalysis of these three reactions. (The active site Cys is clearly not involved in glutathione binding or ionization, because the C13S mutant enzyme is not impaired in its ability

to catalyze the initial part of the dehalogenation reaction.) The currently accepted model for the mechanism of MAA isomerase involves nucleophilic attack of glutathione at C-2 of MAA, rotation about the resulting single bond, and then elimination of glutathione to generate fumarylacetoacetate (*13*). This perfectly logical mechanism includes no role for a catalytic Cys, and is therefore likely to be wrong. There are several mechanisms that can be written for both isomerization of MAA and dehalogenation of DCA that involve Cys13; efforts to differentiate between these are underway.

The extraordinary versatility of TCHQ dehalogenase can be attributed in part to the active site architecture typical of the GST superfamily, in which most of the residues that contribute to glutathione binding and ionization are provided by the N-terminal domain, while most of the residues that contribute to substrate specificity are provided by the C-terminal domain (*29*). Thus, the C-terminal domain is free to mutate to provide alternative substrate binding interactions and additional catalytic residues without compromising the basic catalytic machinery. In addition, the active site must contain catalytic amino acids capable of participating in one or more of these diverse reactions. Several highly conserved residues in the zeta class motif (see Figure 5) (which is found in the N-terminal domain) are likely to be required for the isomerization reaction, but apparently are also in positions appropriate for catalysis of dehalogenation of TCHQ and DCA. We are beginning studies of the mechanism of the MAA isomerase reaction in order to elucidate the roles of these residues.

Considerations of the Evolutionary Origin of TCHQ Dehalogenase. The ability of TCHQ dehalogenase to isomerize MA, the sequence conservation in the active site regions of TCHQ dehalogenase and the known MAA isomerases, and the fact that both are members of the zeta class of the GST superfamily suggest a close structural and evolutionary relationship between these proteins that catalyze seemingly very different reactions. We have considered three possible models for the evolutionary relationship between these proteins. TCHQ dehalogenase may have arisen from a MAA isomerase. Alternatively, MAA isomerase may have arisen from a reductive dehalogenase. Finally, both the dehalogenase and the isomerase may have arisen by divergent evolution from a common ancestor that had some other function. The data available are most consistent with the hypothesis that TCHQ dehalogenase arose from a MAA isomerase. MAA isomerase is part of a pathway for degradation of tyrosine, a compound which must be widespread in the environment due to decaying biomass. The tyrosine degradation pathway is likely to be quite ancient since it has been found in phylogenetically diverse organisms such as Gram-negative bacteria, fungi, and mammals. In contrast, TCHQ dehalogenase catalyzes reductive dehalogenation of an unusual substrate that must be less abundant in the environment. Its immediate precursor in *S. chlorophenolica*, PCP, has only been present in the environment for 60 years. Prior to this time, TCHQ might have been formed from a highly chlorinated natural product such as drosophilin A (*p*-methoxytetrachlorophenol) (*36*), but such natural products would certainly have been less abundant in the environment than tyrosine. Consequently, the possibility that MAA isomerase arose from a reductive dehalogenase appears remote. The third possibility, that both MAA isomerase and TCHQ dehalogenase arose from a common precursor with some other function, is also not satisfying, since it seems unlikely that an enzyme that was evolving to become a reductive dehalogenase would adventituously also develop a high level of MAA isomerase activity.

The most appealing hypothesis at this time is that the original role of TCHQ dehalogenase was the isomerization of MAA derived from ring-cleavage of homogentisate (see Figure 4). Isomerization of maleylpyruvate derived from ring-cleavage of gentisate should also be considered, since MAA and maleylpyruvate differ by only a methylene group, and MA is an analogue of both. *S. chlorophenolica* can grow on tyrosine, a precursor of homogentisate, but not on benzoate, *o*-hydroxybenzoate, or *m*-hydroxybenzoate, precursors of gentisate (data not shown). (Homogentisate and gentisate themselves were not tested as growth substrates because they are rapidly oxidized under aerobic conditions.) Furthermore, crude extracts of *S. chlorophenolica* grown on tyrosine were able to cleave homogentisate and catalyze the glutathione-dependent disappearance of the ring cleavage product (data not shown). Thus, *S. chlorophenolica* has a pathway for homogentisate degradation, but none for gentisate degradation, and therefore the original role of the enzyme must have been isomerization of MAA rather than maleylpyruvate.

LinD also catalyzes glutathione-dependent reductive dehalogenation of chlorinated hydroquinones, although with a substrate specificity different from that of TCHQ dehalogenase. LinD has the characteristic zeta class motif, and therefore is also likely to be descended from a glutathione-dependent isomerase. LinD is involved in degradation of lindane, which, like PCP, is a xenobiotic compound. Thus, LinD appears to be a second example of recruitment of an enzyme derived from a double bond isomerase to serve as a reductive dehalogenase during degradation of a xenobiotic compound. The intriguing question of whether LinD and TCHQ dehalogenase arose independently from isomerases, or whether a single reductive dehalogenase that arose from an isomerase was adapted to give LinD and TCHQ, cannot be answered at this time.

One of the most fascinating and controversial issues regarding the possible evolution of TCHQ dehalogenase from MAA isomerase is the question of when the dehalogenase evolved. Since PCP was only introduced early in the 20th century, TCHQ dehalogenase may have evolved over a period of only a few decades. Alternatively, MAA isomerase may have been recruited to serve as a reductive dehalogenase for TCHQ or a similar compound derived from a natural product long ago. Although we cannot definitively answer this question, the severe substrate inhibition of TCHQ dehalogenase by the aromatic substrate suggests that the enzyme is "immature". We suspect that the substrate inhibition is a consequence of the recruitment of an enzyme with a single glutathione binding site to catalyze a reaction requiring 2 equiv of glutathione. This "design flaw" has apparently not been remedied, either because of inadequate time or because of the absence of the necessary selective pressure. Indeed, there is currently no selective pressure to improve the performance of this enzyme, since the rate-limiting step for the pathway appears to be the initial conversion of PCP to TCHQ (*37*). This design flaw is consistent with, but does not prove, a recent divergence of MAA isomerase and TCHQ dehalogenase. The robust level

of MA isomerase activity found in TCHQ dehalogenase is also consistent with a recent divergence of the two enzymes. Since *S. chlorophenolica* contains another MAA isomerase, retention of the isomerase activity in TCHQ dehalogenase should not have been required. If the enzymes had diverged long ago, we would have expected the isomerase activity to have been greatly diminished as a consequence of changes in the active site that developed the dehalogenase activity.

The findings reported here suggest that the development of metabolic pathways for PCP degradation in certain soil bacteria has been possible due to the recruitment of a glutathione-dependent MAA isomerase to serve as a reductive dehalogenase. Such enzymes are common but not ubiquitous in soil bacteria (*22*, *38*), so only a limited number of organisms will have the potential to develop a TCHQ dehalogenase. The same factor is also likely to be important for the development of pathways for degradation of lindane. The recruitment of a double bond isomerase to serve as a reductive dehalogenase illustrates the creativity of bacteria when faced with an environmental toxin (either PCP, lindane, or a toxic natural product). The presence of the toxin provides a strong incentive for the bacterium to modify that toxin, and an enzyme may be recruited from a source that we would never suspect based upon a consideration of the overall transformations.

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