Diverse mechanistic approaches to difficult chemical transformations: microbial dehalogenation of chlorinated aromatic compounds

Shelley D Copley

Chlorinated aromatic compounds represent an important class of environmental pollutants. Microbial dehalogenases play a crucial role in the biodegradation of these compounds. The three major classes of aromatic dehalogenases are discussed in this minireview.

Address: Department of Chemistry and Biochemistry, Campus Box 215, University of Colorado, Boulder, CO 80309, USA.

Electronic identifier: **1074-5521-004-00169**

Chemistry & Biology March 1997, 4:169–174

Chlorinated aromatic compounds are particularly important environmental pollutants because they are resistant to biodegradation and therefore tend to persist in the environment. Examples of these compounds include polychlorinated biphenyls (PCBs), dioxins and numerous pesticides, such as dichlorodiphenyltrichloroethane (DDT), atrazine, pentachlorophenol and 2,4-dichlorophenoxyacetic acid (2,4-D) (Fig. 1).

Under aerobic conditions, chlorinated aromatic compounds persist in the environment because chlorine atoms interfere with the action of many dioxygenase enzymes that normally initiate the degradation of aromatic rings. The interference is likely to be due to both steric and electronic effects, because chlorine atoms are both larger and more electron-withdrawing than hydrogen atoms. Despite these problems, microorganisms that can degrade chlorinated aromatic compounds can often be found at highly contaminated sites. Many of these microorganisms contain dehalogenase enzymes that catalyze the removal of chlorine atoms from the aromatic ring. Once the chlorine atoms are removed, the aromatic compound is quite susceptible to biodegradation.

Chlorinated aromatic compounds are also degraded under anaerobic conditions. For example, the PCBs in the sediments of the Hudson River are gradually being converted to the lesser chlorinated congeners [1]. Our knowledge of the pathways of anaerobic degradation of chlorinated aromatic compounds is less extensive than that of pathways for aerobic degradation, however. Furthermore, mechanistic studies of an aromatic dehalogenase from an anaerobic microorganism have never been carried out. This review will therefore focus on enzymes involved in aerobic degradation of chlorinated aromatic compounds.

Chlorine substituents can be removed from aromatic rings in three fundamentally different ways (Fig. 2): hydrolytic dehalogenases replace chlorine substituents with hydroxyl groups that are derived from water; reductive dehalogenases replace chlorine substituents with hydrogen atoms; and oxygen-dependent dehalogenases replace chlorine substituents with hydroxyl groups whose oxygen atoms are derived from O_2. Although only a limited number of dehalogenases have been studied, it is already evident that there is more than one way to accomplish each of these three classes of reactions. Here I discuss mechanisms used by enzymes in each of these three classes, with emphasis on the diversity of mechanisms found in nature and the relationships between dehalogenase enzymes and other known enzymes.

Figure 1

Examples of chlorinated aromatic compounds.

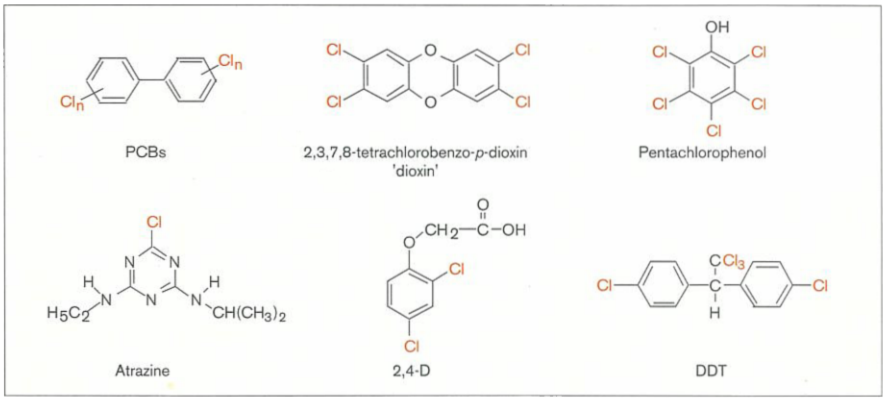
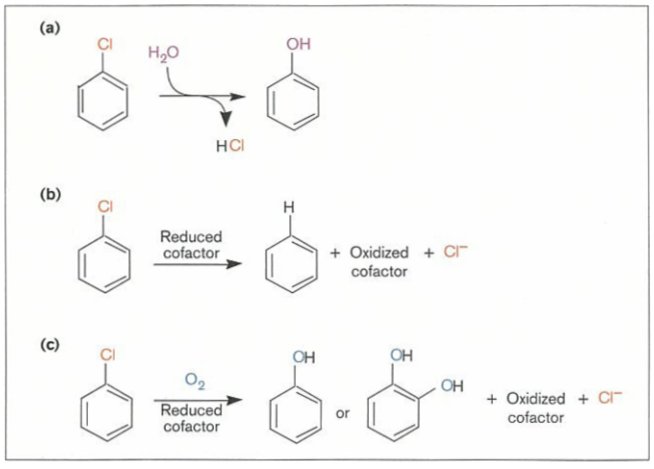


Figure 2



Removal of chlorine substituents from aromatic compounds. This can be achieved in three fundamentally different ways in which the dehalogenases replace chlorine substituents with **(a)** hydroxyl groups derived from water, **(b)** hydrogen atoms, and **(c)** hydroxyl groups whose oxygen atoms are derived from O_2.

Hydrolytic aromatic dehalogenases

The most thoroughly studied hydrolytic aromatic dehalogenase is 4-chlorobenzoyl coenzyme A (CoA) dehalogenase. This enzyme is found in several strains of bacteria that degrade 4-chlorobenzoate, a breakdown product of some PCBs. The mechanism of the enzymes from *Pseudomonas* species CBS3 [2] and *Arthrobacter* species 4-CB1 [3] has been studied in detail, and the crystal structure of the *Pseudomonas* sp. CBS3 enzyme was recently reported [4]. The reaction (Fig. 3) involves an initial attack of Asp145 (numbering based upon the sequence of the *Pseudomonas* enzyme) at the active site on 4-chlorobenzoyl CoA to form a Meisenheimer complex intermediate, which then expels chloride to form an aryl-enzyme intermediate. General-base catalyzed attack of water on the aryl-enzyme intermediate results in a tetrahedral intermediate which then collapses to release 4-hydroxybenzoyl CoA, regenerating the free enzyme.

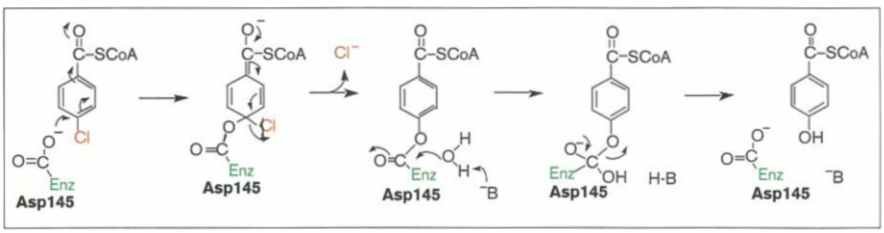
The reaction catalyzed by 4-chlorobenzoyl CoA dehalogenase is remarkable — aromatic substitution reactions on unactivated aromatic rings are notoriously difficult. The catalytic effectiveness of 4-chlorobenzoyl CoA dehalogenase is due in part to its ability to polarize the thioester substituent and thereby enhance its electron-withdrawing ability. Spectroscopic studies are consistent with a significant shift of electron density from the aromatic ring into the thioester when substrate analogs such as 4-methoxybenzoyl CoA are bound to the active site [5]. The crystal structure of the *Pseudomonas* enzyme suggests that the positive end of a helix dipole provides the polarization that is reflected in the spectroscopic studies [4].

Another important factor in catalysis is undoubtedly the optimization of nucleophilic attack. In non-enzymatic systems, the attack by water on an aromatic compound is facilitated by general-base catalysis by acetate ions. 4-Chlorobenzoyl CoA dehalogenase, however, uses the carboxylate of Asp145 as a nucleophile rather than as a general base. This has the dual advantages of enhancing the strength of the nucleophile (a desolvated carboxylate would be expected to be a better nucleophile than water activated by a general base) and decreasing the entropy of activation.

4-Chlorobenzoyl CoA dehalogenase uses an interesting combination of two catalytic strategies, each of which is seen in other enzymes. One strategy is to polarize the thioester carbonyl, facilitating attack of a nucleophile on a carbon conjugated to the thioester. This is also seen in enoyl CoA hydratase (crotonase) [6]. 4-Chlorobenzoyl CoA dehalogenase has 30% sequence identity with crotonase, and the recently published structure of crotonase [7] is strikingly similar to that of 4-chlorobenzoyl CoA dehalogenase [4]. The second strategy is to use an active site carboxylate to form an aryl-enzyme intermediate which is then hydrolyzed by attack of water at the carbonyl carbon. Comparable mechanisms involving alkyl-enzyme intermediates are used by epoxide hydrolase [8] and haloalkane dehalogenase [9,10]. In this case there is no sequence homology between these enzymes and 4-chlorobenzoyl CoA dehalogenase; furthermore, the steric and electronic requirements of S_N2 and S_NAr reactions are very different. It is therefore intriguing that the same catalytic strategy is used by all three enzymes.

The catalytic strategy used by 4-chlorobenzoyl CoA dehalogenase is not the only one available for hydrolytic

Figure 3



The mechanism of 4-chlorobenzoyl CoA dehalogenase. This enzyme (Enz) replaces the chlorine substituent with a hydroxyl group derived from water (as in Fig. 2a).

aromatic dehalogenation reactions. A 2,6-dichlorohydroquinone chlorohydrolase that requires ferrous ions for activity has recently been purified from *Sphingomonas chlorophenolica* ATCC 39723 (previously known as *Flavobacterium* sp. ATCC 39723) [11]. One can imagine several possible roles for a ferrous cofactor, including polarization of substrate functional groups, provision of hydroxide as a nucleophile, and/or electron transfer to initiate the dehalogenation reaction. Mechanistic studies of this enzyme are being initiated in our laboratory.

Hydrolytic dehalogenation of heteroaromatic compounds is also possible. De Souza *et al.* [12] have characterized an atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP, and a hydrolytic enzyme that catalyzes both dechlorination and deamination reactions on *s*-triazine substrates has been purified from *Rhodococcus corallinus* NRRL B-15444R [13].

Reductive aromatic dehalogenases

Reductive dehalogenation is particularly important for the degradation of highly chlorinated aromatic compounds, because replacing several chlorine atoms with hydroxyl groups would create a product that would be extremely prone to oxidation. Furthermore, reductive dehalogenation is thermodynamically more favorable when there are multiple electron-withdrawing chlorine substituents on the aromatic ring. Reductive dehalogenation is not restricted to highly chlorinated compounds, however; a reductive 3-chlorobenzoate dehalogenase has recently been purified [14].

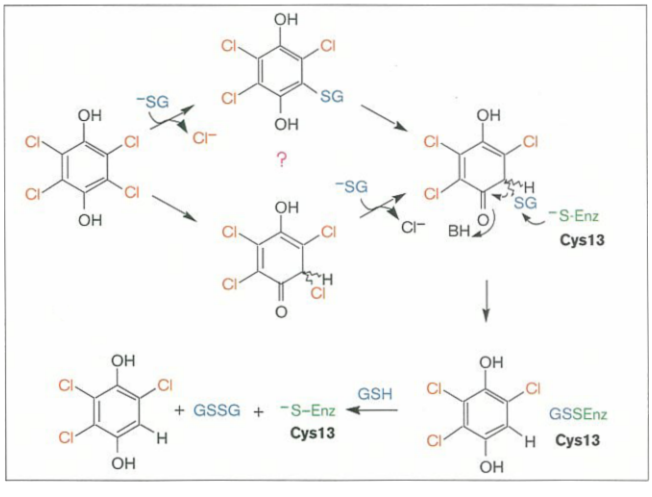
The only reductive aromatic dehalogenase that has been studied in detail is tetrachlorohydroquinone dehalogenase from *S. chlorophenolica* ATCC 39723 [15]. This enzyme catalyzes the conversion of tetrachlorohydroquinone to trichlorohydroquinone and then to 2,6-dichlorohydroquinone. The reducing equivalents are provided by two molecules of glutathione for each chlorine removed. Tetrachlorohydroquinone dehalogenase is not closely related to any known proteins, but it does have 26–30% identity with some microbial enzymes that belong to the theta class of the glutathione *S*-transferase (GST) superfamily. Although this level of sequence identity is rather low, there are some important similarities between tetrachlorohydroquinone dehalogenase and the GSTs. First, consensus residues characteristic of the theta class enzymes are conserved in tetrachlorohydroquinone dehalogenase. Second, tetrachlorohydroquinone dehalogenase appears to optimize the nucleophilicity of glutathione in the active site in a manner similar to the theta class of GSTs by stabilizing the thiolate of glutathione via a hydrogen bond with a nearby serine. Finally, mechanistic studies of the enzyme suggest that the initial stage of the reductive dehalogenation involves the nucleophilic attack of glutathione on an electrophilic form of the substrate (not yet conclusively identified) to form a glutathione conjugate.

The current working model for the mechanism of tetrachlorohydroquinone dehalogenase is shown in Figure 4. In the first stage of the reaction, tetrachlorohydroquinone undergoes either nucleophilic attack by glutathione followed by tautomerization or initial tautomerization followed by nucleophilic attack by glutathione, resulting in the formation of the tautomer of 2,3,5-trichloro-6-*S*-glutathionylhydroquinone. Cys13 at the active site then attacks the sulfur of the glutathionyl substituent, releasing trichlorohydroquinone and forming a mixed disulfide between Cys13 and glutathione. In the final stage, a second molecule of glutathione attacks the mixed disulfide to regenerate the free enzyme.

The mechanistic and structural characteristics of tetrachlorohydroquinone dehalogenase suggest that it may have evolved from a protein with the general architecture and glutathione binding properties of a GST. However, this enzyme clearly has a number of catalytic capabilities that GSTs do not have, including the ability to tautomerize a hydroquinone substrate, to catalyze the attack of Cys13 on the tautomer of the glutathione conjugate, to bind a second molecule of glutathione, and to catalyze a thiol–disulfide exchange reaction.

There are undoubtedly other mechanisms for reductive dehalogenation. Romanov and Hausinger [16] recently reported a reductive 2,4-dichlorobenzoyl CoA dehalogenase in *Corynebacterium sepedonicum* KZ-4 and *Coryneform* bacterium strain NTB-1 that uses NADPH as a reductant. In addition, a reductive 3-chlorobenzoate dehalogenase has

Figure 4



Postulated mechanism of tetrachlorohydroquinone dehalogenase. GSH, glutathione sulfide; GSSG, a dimer of glutathione sulfide; S-Enz, the thiol enzyme.

been purified from the anaerobic *Desulfomonile tiedjei* DCB-1 [17]. This membrane-bound enzyme appears to be a heme protein and uses methyl viologen as a reductant *in vitro*. Although mechanistic studies have not yet been reported for either of these enzymes, the differing reductants clearly imply significant differences between the mechanisms of these enzymes and tetrachlorohydroquinone dehalogenase.

Oxygen-dependent aromatic dehalogenases

O_2-dependent aromatic dehalogenases fall into two classes: dioxygenases that catalyze the formation of a catechol with concomitant loss of chloride, and monooxygenases that replace the chlorine atom with a hydroxyl group. These enzymes are 'accidental' dehalogenases in that their catalytic machinery operates to form an unstable product that then decomposes spontaneously to release chloride. Examples of reactions catalyzed by these types of enzymes are shown in Figure 5.

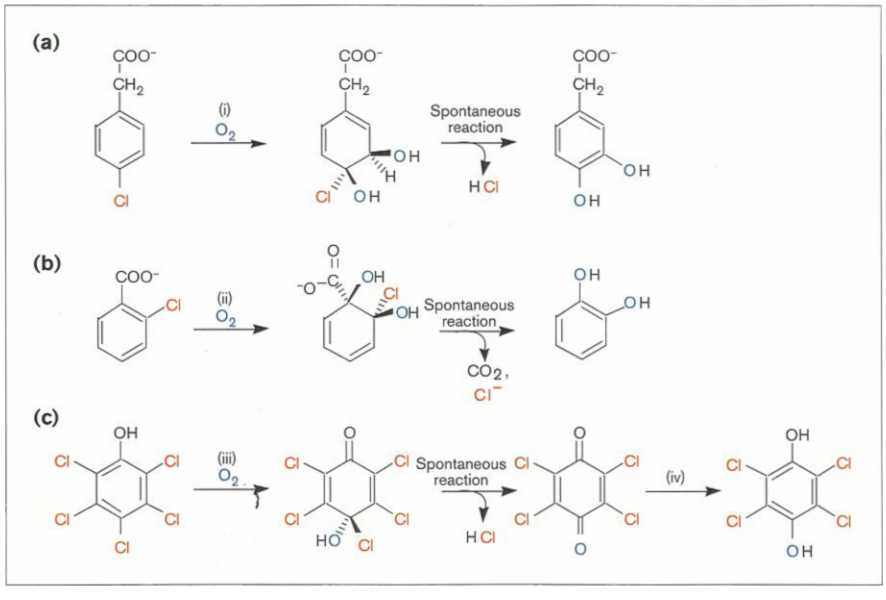
Dehalogenases of the dioxygenase type

Enzymes that catalyze the introduction of two hydroxyl groups onto aromatic substrates are widespread in nature, as this is generally the first step in the degradation of aromatic compounds by aerobic microorganisms. These enzymes are multi-component systems comprising an oxygenase component that catalyzes the addition of the hydroxyl groups to the aromatic substrate and either one or two components that carry electrons from NADPH through flavin and [2Fe–2S] cofactors to the oxygenase component.

Two-component dioxygenase enzymes that dehalogenate 4-chlorophenylacetate and 2-halobenzoates have been described in *Pseudomonas* sp. CBS3 [18] and in *Pseudomonas cepacia* 2CBS [19], respectively. A three-component dioxygenase system that dehalogenates both 2-chlorobenzoate and 2,4-dichlorobenzoate has been purified from *Pseudomonas aeruginosa* 142 [20]. In these cases, the dioxygenation reaction itself is believed to result in the formation of a *cis*-diol product which spontaneously undergoes either *syn* elimination of HCl or decarboxylation coupled with chloride release to give a catechol product (Fig. 5a,b).

It seems reasonable to expect that the structures and mechanisms of these enzymes will be closely related to those of the family of arene dioxygenase enzymes, which includes more thoroughly studied enzymes such as phthalate dioxygenase, benzoate dioxygenase and benzene 1,2-dioxygenase. The oxygenase components of the arene dioxygenase family of enzymes are oligomeric proteins of variable composition ($\alpha_2\beta_2$, $\alpha_3\beta_3$, α_2, α_3 and α_4) that contain one [2Fe–2S] Rieske site and one mononuclear non-heme iron per α subunit [21]. The sequence of events at the non-heme iron center that results in the introduction of two hydroxyl groups on the aromatic substrate is not known. This dearth of information is due at least in part to the difficulty in studying events at the non-heme iron in the presence of the Rieke [2Fe–2S] center, whose spectroscopic properties obscure those of the non-heme iron. However, recent magnetic circular dichroism studies of the non-heme iron site in phthalate dioxygenase [22] suggest that this site contains a six-coordinate Fe^{2+} in the resting state. The substrate does not bind directly to the iron, but it induces a conformational change that results in the release of one of the six ligands to give a

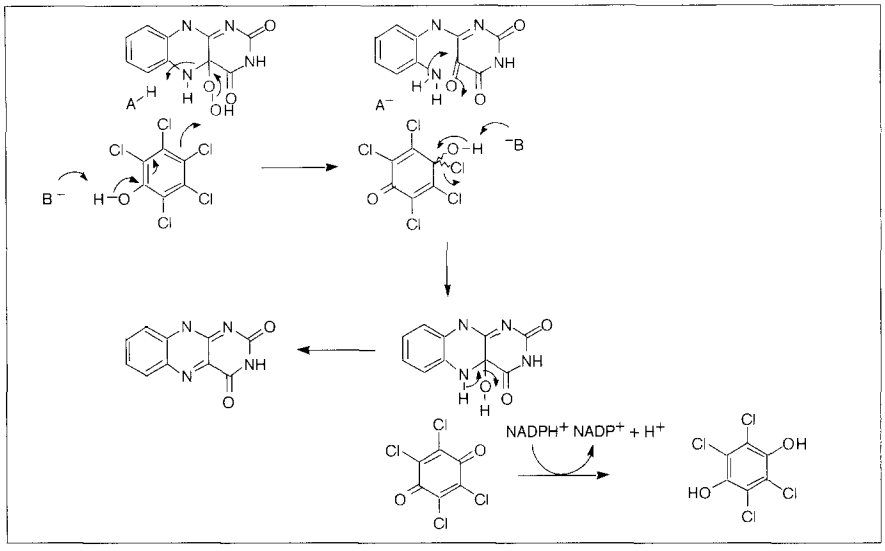
Figure 5



Examples of reactions catalyzed by O_2-dependent aromatic dehalogenases. **(i)** 4-Chlorophenylacetate dioxygenase; **(ii)** 2-chlorobenzoate dioxygenase; **(iii)** pentachlorophenol monooxygenase; and **(iv)** either quinone reductase or non-enzymatic reaction.

Figure 6

Proposed mechanism for the dechlorination of pentachlorophenol by pentachlorophenol monooxygenase. The flavin-4a-hydroperoxide shown is the product of prior reaction between a reduced flavin and O_2. Elimination of HCl to form tetrachlorobenzoquinone and reduction of the quinone to tetrachlorohydroquinone may be non-enzymatic.



five-coordinate Fe^+ with a vacant coordination position. This position has been suggested to provide the binding site for the O_2.

Dehalogenases of the monooxygenase type

Monooxygenase-type dehalogenases that convert pentachlorophenol to tetrachlorohydroquinone have been identified in a number of microorganisms, including several strains of the Gram-negative bacterium *S. chlorophenolica* (previously identified as strains of *Pseudomonas* and *Flavobacterium*) and the Gram-positive actinomycete *Mycobacterium* (previously identified as strains of *Rhodococcus*). Pentachlorophenol monooxygenase from *S. chlorophenolica* ATCC 39723 has been purified [23] and the gene cloned and sequenced [24]. The enzyme contains one flavin adenine dinucleotide cofactor and requires O_2 and two equivalents of NADPH for the turnover of each molecule of pentachlorophenol. Although mechanistic studies of this enzyme have not yet been carried out, it is likely that the mechanism is similar to that shown in Figure 6, which is based upon the mechanism proposed for defluorination of tetrafluoro-*p*-hydroxybenzoate by *p*-hydroxybenzoate hydroxylase [25]. Pentachlorophenol monooxygenase has quite broad substrate specificity, and pentachlorophenol is in fact a poor substrate for this enzyme [26]. Thus, it is likely that pentachlorophenol monooxygenase has arisen by a rather straightforward adaptation of a previously existing flavin monooxygenase enzyme to accommodate a xenobiotic substrate.

Once again, there appears to be more than one way to accomplish the monooxygenase type of dehalogenation reaction. Although the pentachlorophenol monooxygenase from *Mycobacterium* has not yet been purified, it is membrane-associated and appears to be a P450-type enzyme [27].

Conclusions

Microbial aromatic dehalogenase enzymes provide a rich area for future mechanistic work. Detailed mechanistic studies have only been carried out for two enzymes, and there are undoubtedly many more dehalogenases that have not yet been purified and characterized. There is a particular need for studies of the aromatic dehalogenases present in anaerobic microorganisms. The analysis of the sequences, structures and mechanisms of these enzymes will provide insight into the types of protein scaffolds that can be adapted to carry out these transformations and may allow the genetic engineering of dehalogenase enzymes with novel properties.

References

1. Brown, J.F., Bedard, D.L., Brennan, M.J., Carnahan, J.C., Feng, H. & Wagner, R.E. (1987). Polychlorinated biphenyl dechlorination in aquatic sediments. *Science* **236**, 709–712.
2. Yang, G., Liang, P.-H. & Dunaway-Mariano, D. (1994). Evidence for nucleophilic catalysis in the aromatic substitution reaction catalyzed by (4-chlorobenzoyl)coenzyme A dehalogenase. *Biochemistry* **33**, 8527–8531.
3. Crooks, G.P., Xu, L., Barkley, R.M. & Copley, S.D. (1995). Exploration of possible mechanisms for 4-chlorobenzoyl CoA dehalogenase: evidence for an aryl-enzyme intermediate. *J. Am. Chem. Soc.* **117**, 10791–10798.
4. Benning, M.M., *et al.*, & Holden, H.M. (1996). Structure of 4-chlorobenzoyl coenzyme A dehalogenase determined to 1.8 Å resolution: an enzyme catalyst generated via adaptive mutation. *Biochemistry* **35**, 8103–8109.
5. Taylor, K.L., Liu, R.-Q., Liang, P.-H., Price, J. & Dunaway-Mariano, D. (1995). Evidence for electrophilic catalysis in the 4-chlorobenzoyl-CoA dehalogenase reaction: UV, Raman, and ^{13}C-NMR spectral studies of dehalogenase complexes of benzoyl-CoA adducts. *Biochemistry* **34**, 13881–13888.
6. D'Ordine, R.L., Tonge, P.J., Carey, P.R. & Anderson, V.E. (1994). Electronic rearrangement induced by substrate analog binding to the enoyl-CoA hydratase active site: evidence for substrate activation. *Biochemistry* **33**, 12635–12643.
7. Engel, C.K., Mathieu, M., Zeelen, J.P., Hiltunen, J.K. & Wierenga, R.K. (1996). Crystal structure of enoyl-coenzyme A (CoA) hydratase at 2.5 Å resolution: a spiral fold defines the CoA-binding pocket. *EMBO J.* **15**, 5135–5145.

8. Lacourciere, G.M. & Armstrong, R.N. (1993). The catalytic mechanism of microsomal epoxide hydrolase involves an ester intermediate. *J. Am. Chem. Soc.* **115**, 10466–10467.
9. Verschueren, K.H.G., Seljee, F., Rozeboom, H.J., Kalk, K.H. & Dijkstra, B.W. (1993). Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase. *Nature* **363**, 693–698.
10. Pries, F., *et al.*, & Janssen, D.B. (1994). Site-directed mutagenesis and oxygen isotope incorporation studies of the nucleophilic aspartate of haloalkane dehalogenase. *Biochemistry* **33**, 1242–1247.
11. Lee, J.-Y. & Xun, L. (1997). Purification and characterization of 2,6-dichloro-*p*-hydroquinone chlorohydrolase from *Flavobacterium* sp. strain ATCC 39723. *J. Bacteriol.*, in press.
12. De Souza, M.L., Sadowsky, M.J. & Wackett, L.P. (1996). Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP: gene sequence, enzyme purification, and protein characterization. *J. Bacteriol.* **178**, 4894–4900.
13. Mulbry, W.W. (1994). Purification and characterization of an inducible *s*-triazine hydrolase from *Rhodococcus corallinus* NRRL B-15444R. *Appl. Env. Microbiol.* **60**, 613–618.
14. Ni, S., Fredrickson, J.K. & Xun, L. (1995). Purification and characterization of a novel 3-chlorobenzoate reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile* tiedjei DCB-1. *J. Bacteriol.* **177**, 5135–5139.
15. McCarthy, D.L., Navarrete, S., Willett, W.S., Babbitt, P.C. & Copley, S.D. (1996). Exploration of the relationship between tetrachlorohydroquinone dehalogenase and the glutathione *S*-transferase superfamily. *Biochemistry* **35**, 14634–14642.
16. Romanov, V. & Hausinger, R.P. (1996). NADPH-dependent reductive *ortho* dehalogenation of 2,4-dichlorobenzoic acid in *Corynebacterium sepedonicum* KZ-4 and *Coryneform* bacterium strain NTB-1 via 2,4-dichlorobenzoyl coenzyme A. *J. Bacteriol.* **178**, 2656–2661.
17. Ni, S., Fredrickson, J.K. & Xun, L. (1995). Purification and characterization of a novel 3-chlorobenzoate reductive dehalogenase from the cytoplasmic membrane of *Desulfomonile tiedjei* DCB-1. *J. Bacteriol.* **177**, 5135–5139.
18. Markus, A., Klages, U., Krauss, S. & Lingens, F. (1984). Oxidation and dehalogenation of 4-chlorophenylacetate by a two-component enzyme system from *Pseudomonas* sp. strain CBS3. *J. Bacteriol.* **160**, 618–621.
19. Fetzner, S., Müller, R. & Lingens, F. (1992). Purification and some properties of 2-halobenzoate 1,2-dioxygenase, a two-component enzyme system from *Pseudomonas cepacia* 2CBS. *J. Bacteriol.* **174**, 279–290.
20. Romanov, V. & Hausinger, R.P. (1994). *Pseudomonas aeruginosa* 142 uses a three-component *ortho*-halobenzoate 1,2-dioxygenase for metabolism of 2,4-dichloro- and 2-chlorobenzoate. *J. Bacteriol.* **176**, 3368–3374.
21. Mason, J.R. & Cammack, R. (1992). The electron-transport proteins of hydroxylating bacterial dioxygenases. *Ann. Rev. Microbiol.* **46** 277–305.
22. Pavel, E.G., Martins, L.J., Ellis, W.R., Jr & Solomon, E.I. (1994). Magnetic circular dichroism studies of exogenous ligand and substrate binding to the non-heme ferrous active site in phthalate dioxygenase. *Chem. Biol.* **1**, 173–183.
23. Xun, L. & Orser, C.S. (1991). Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. *J. Bacteriol.* **173**, 4447–4453.
24. Orser, C., Lange, C.C., Xun, L., Zahrt, T.C., Schneider, B.J. (1993). Cloning, sequence analysis, and expression of the *Flavobacterium* pentachlorophenol-4-monooxygenase gene in *Escherichia coli*. *J. Bacteriol.* **175**, 411–416.
25. Husain, M., Entsch, B., Ballou, D.P., Massey, V. & Chapman, P.J. (1980). Fluoride elimination from substrates in hydroxylation reactions catalyzed by *p*-hydroxybenzoate hydroxylase. *J. Biol. Chem.* **255**, 4189–4197.
26. Xun, L., Topp, E. & Orser, C. (1992). Diverse substrate range of a *Flavobacterium* pentachlorophenol hydroxylase and reaction stoichiometries. *J. Bacteriol.* **174**, 2898–2902.
27. Uotila, J.S., Kitunen, V.H., Saastamoinen, T., Coote, T., Häggblom, M.M. & Salkinoja-Salonen, M.S. (1992). Characterization of aromatic dehalogenases of *Mycobacterium fortuitum* CG-2. *J. Bacteriol.* **174**, 5669–5675.