A Monooxygenase Catalyzes Sequential Dechlorinations of 2,4,6-Trichlorophenol by Oxidative and Hydrolytic Reactions*

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**Ralstonia eutropha** JMP134 2,4,6-trichlorophenol (2,4,6-TCP) 4-monooxygenase catalyzes sequential dechlorinations of 2,4,6-TCP to 6-chlorohydroxyquinol. Although 2,6-dichlorohydroxyquinol is a logical metabolic intermediate, the enzyme hardly uses it as a substrate, implying it may not be a true intermediate. Evidence is provided to support the proposition that the monooxygenase oxidized 2,4,6-TCP to 2,6-dichloroquinone that remained with the enzyme and got hydrolyzed to 2-chlorohydroxyquinol, which was chemically reduced by ascorbate and NADH to 6-chlorohydroxyquinol. When the monooxygenase oxidized 2,6-dichloroquinol, the product was 2,6-dichlorohydroquinol, which was not further converted to 6-chlorohydroxyquinol, implying that the enzyme only converts 2,6-dichloroquinol to 6-chlorohydroxyquinol. Stoichiometric analysis indicated the consumption of one O₂ molecule per 2,4,6-TCP converted to 6-chlorohydroxyquinol, ruling out the possibility of two oxidative reactions. Experiments with ¹⁸O-labeling gave direct evidence for the incorporation of oxygen from both O₂ and H₂O into the produced 6-chlorohydroxyquinol. A monooxygenase that catalyzes hydroxylation by both oxidative and hydrolytic reactions has not been reported to date. The ability of the enzyme to perform two types of reactions is not due to the presence of a second functional domain but rather is due to catalytic promiscuity, as a homologous monooxygenase converts 2,4,6-TCP to only 2,6-dichloroquinol. Employing both conventional catalysis and catalytic promiscuity of a single enzyme in two consecutive steps of a metabolic pathway has been unknown previously.

The diversity and complexity of life-forms on Earth are the manifestation of an extensive protein repertoire performing the required activities and structural functions. It is widely accepted that the diversity is mainly evolved from pre-existing proteins via gene duplication, divergence of the duplicated sequence, recombination, and selection (for recent advancement of the theory, see Refs. 1 and 2). Likewise, the microbial metabolism of synthetic chemicals, which is mostly neoteric to this planet, is also believed to follow this same line of evolution (3). We describe here that a monooxygenase has evolved to catalyze consecutive dechlorinations of 2,4,6-trichlorophenol (2,4,6-TCP) to 6-chlorohydroxyquinol by means of oxidative and hydrolytic reactions.

Polychlorophenols are introduced into the environment in large quantities through their wide usage as wood preservatives (4–6). In addition, polychlorophenol derivatives are often used as herbicides and fungicides (7, 8); the corresponding chlorophenols are often the metabolic intermediates of their biodegradation. For examples, 2,4,5-trichlorophenol is the first metabolic intermediate in the degradation of herbicide 2,4,5-trichlorophenoxyacetate by *Burkholderia cepacia* AC1100 (9, 10), and 2,4,6-TCP is produced during fungicide prochloraz degradation by *Aureobacterium* sp. strain C964 (11). Human exposure to polychlorophenols can lead to acute hyperthermia, convulsions, and rapid death, with long-term health effects resulting from mutagenicity and carcinogenicity (4). Further health concerns are with the highly carcinogenic chlorinated dibenzo-p-dioxins and dibenzofurans (12), which are produced from polychlorophenols either during manufacturing processes (4) or by means of biotransformation in soils (13). Some polychlorophenol-degrading microorganisms have been applied successfully in the bioremediation of polychlorophenols (14, 15). The complete biodegradation pathways of pentachlorophenol (16), 2,4,5-trichlorophenol (17), and 2,4,6-TCP (18) have been elucidated to provide scientific guidance for bioremediation of these pollutants.

The first enzyme in the 2,4,6-TCP degradation pathway of *Ralstonia eutropha* JMP134 is particularly interesting. The enzyme 2,4,6-TCP monooxygenase (TcpA) belongs to the newly discovered reduced flavin adenine dinucleotide (FADH₂)-utilizing monooxygenases that use FADH₂ as a cosubstrate rather than a cofactor (18). An NADH:FAD oxidoreductase is required to supply FADH₂ for TcpA. TcpA replaces two chlorines from 2,4,6-TCP with two hydroxyl groups to produce 6-chlorohydroxyquinol, with minor production of 2,6-dichlorohydroquinol. We question whether 2,6-dichlorohydroquinol is a metabolic intermediate because TcpA hardly consumes 2,6-dichlorohydroquinol when directly provided as a substrate (18). Because monooxygenases produce a quinone after removal of an electron-withdrawn group (such as chlorine) from a phenolic compound (19–21), the direct product of 2,4,6-TCP oxidation by TcpA should be 2,6-dichlorohydroquinone (Fig. 1). We present evidence that TcpA converts 2,4,6-TCP to 6-chlorohydroxyquinol by means of two different reactions: (i) it oxidizes 2,4,6-TCP to 2,6-dichlorohydroquinone, and then (ii) it hydrolyzes 2,6-dichlorohydroquinone to 6-chlorohydroxyquinol (Fig. 1). NADH and ascorbate can chemically reduce the latter to 6-chlorohydroxyquinol in the reaction mixture (Fig. 1), but this may occur enzymatically inside the cell (20, 21).

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1 The abbreviations used are: 2,4,6-TCP, 2,4,6-trichlorophenol; TcpA, 2,4,6-TCP 4-monooxygenase; TRD, 2,4,5-trichlorophenol 4-monooxygenase; FAD, flavin adenine dinucleotide; FADH₂, reduced FAD; GC-MS, gas chromatography-mass spectrometry; Fre, flavin reductase.

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EXPERIMENTAL PROCEDURES

Analytical Techniques—NADH concentrations were measured by a UV spectrophotometer at 340 nm. 2,4,6-TCP, 2,6-dichlorophenol, 2,6-dichloroquinol, and 6-chlorohydroxyquinol were detected and quantified by a high-performance liquid chromatography method as reported (18) and also analyzed by a reported gas chromatography-mass spectrometry (GC-MS) method (18). Oxygen consumption was determined in a closed reaction chamber (0.67 ml) fitted with a Clark-type electrode, as reported previously (22).

Reactions—Both TcpA and Fre (an Escherichia coli general flavin reductase) were purified as reported previously (18, 23). All of the reactions were done in 40 mM potassium phosphate buffer (pH 7) containing 5 μM FAD, 200 μM 2,4,6-TCP or 2,6-dichlorophenol, 2 mM ascorbate, 90 units/ml of catalase (Sigma), and various amounts of NADH, TcpA, and Fre as indicated for individual reactions. The time-course experiments were done in 250-μl volumes with 194 μg of TcpA, 2.7 μg of Fre, and 1660 μM NADH. Samples of 20 μl were taken and mixed with 20 μl of a mixture of acetonitrile and acetic acid (9:1), which stopped the reaction, at time intervals of 0.5 min. Time zero was the sample before adding NADH. The stoichiometric experiments were done in a closed chamber (0.67 ml) with 2500 μg of TcpA, 2.7 μg of Fre, and 128 μg NADH. The high TcpA concentration was used to ensure the maximal usage of the produced FADH2 for 2,4,6-TCP oxidation. NADH was added to initiate the reaction; it was completely consumed within 3 min. The H2O labeling experiments were done with 250 μl of the reaction volume containing 485 μg of TcpA, 2.7 μg of Fre, and 1680 μM NADH, and 80 μl of H2O (99% α-Dalrich). To initiate the reaction, NADH was added; after 10 min, additional 1660 μM NADH was added and incubated for an additional 20 min to ensure the maximal conversion of 2,4,6-TCP to 6-chlorohydroxyquinol. The sample was acidified by the addition of 10 μl of concentrated HCl, and the reaction end products were extracted into ethyl acetate and dried with sodium sulfate; the residues were derivatized with a 100-μl mixture of pyridine and acetic anhydride (1:3) for GC-MS analysis (18). A control was done without H2O18 under otherwise identical conditions. The 18O-labeled experiments were done with a 500-μl reaction mixture containing 970 μg of TcpA, 5.4 μg of Fre, and 1660 μM NADH in a 2-ml glass vial. In addition, the reaction mixture contained 1 mm glucose and 0.5 μg of glucose oxidase (Sigma) to remove extraneous oxygen. The samples were brought into an anaerobic chamber (95% N2 and 5% H2). Glucose was added 5 min prior to the start of the reaction to ensure complete removal of dissolved O2. NADH was then added, and the vial was sealed with a rubber stopper. 1 ml of 18O2 (23.3%) (ICON Stable Isotopes, Summit, New Jersey) was injected into the vial to start the reaction. After 20 min, the sample was acidified by HCl and the reaction end products were extracted into ethyl acetate for GC-MS analysis. All reactions were carried out at room temperature.

RESULTS

TcpA used both 2,6-dichlorophenol and 2,4,6-TCP as its substrates. When 2,6-dichlorophenol was used as its substrate, TcpA converted it to 2,6-dichloroquinol without detectable 6-chlorohydroxyquinol (Fig. 2A). When 2,4,6-TCP was the substrate, TcpA converted it mainly to 6-chlorohydroxyquinol with minor accumulation of 2,6-dichloroquinol (Fig. 2B). Hydroxylation of the 4-position of 2,6-dichlorophenol by TcpA occurs through a simple monoxygenase reaction without the formation of 2,6-dichloroquinol as an intermediate. On the other hand, removing an electron withdrawing group, such as a chlorine or nitro group, from a substituted phenol produces a quinone as the immediate product (19–21). The formation of 2,6-dichloroquinone from 2,4,6-TCP oxidation (Fig. 1) must be necessary for the subsequent conversion to 6-chlorohydroxyquinol. This conversion is enzymatic because 2,6-dichloroquinone added directly into a buffer solution remains as 2,6-dichloroquinone, which can be reduced to 2,6-dichloroquinol upon the addition of borohydride or ascorbate (23). Further, 2,4,5-trichlorophenol monoxygenase (TftD), which is homologous to TcpA with 85% identity and with no gap in their amino acid sequence alignment, does not have the ability to convert 2,4,6-TCP all the way to 6-chlorohydroxyquinol. Instead, TftD oxidizes 2,4,6-TCP to 2,6-dichloroquinol, which is chemically reduced to 2,6-dichloroquinol in the reaction mixture (23, 24), indicating that only TcpA has the catalytic ability for the second step of dechlorination of 2,4,6-TCP. The production of a small amount of 2,6-dichloroquinol during 2,4,6-TCP metabolism by TcpA is probably due to the reduction of the enzyme-bound 2,6-dichloroquinone (Fig. 1) by small reducing agents, such as NADH or ascorbate in the reaction system, to 2,6-dichloroquinol, which can then be released from the enzyme. Alternatively, a small amount of 2,6-dichloroquinone can be released from TcpA and be reduced by reducing agent in the solution.

2,6-Dichloroquinol was unstable when added directly to the reaction mixture, and it was rapidly reduced to 2,6-dichloroquinol by ascorbate, as previously reported (23, 24); after a short incubation, 2,6-dichloroquinol was detectable, but neither 2,6-dichloroquinol nor 6-chlorohydroxyquinol was detectable. 2,6-Dichloroquinone was also added directly into a reaction mix containing TcpA without ascorbate. Upon the addition of ascorbate to reduce quinones to quinols, two additional major peaks were detectable besides the 2,6-dichloroquinone peak, but the 6-chlorohydroxyquinol was not detectable by high-performance liquid chromatography analysis. The two major additional peaks were tentatively assigned as monothiol-
Upon the addition of catalase, half of the consumed O2 was indicated that 222-ductase, which used NADH to reduce FAD to FADH2. In the oxidative process. The reaction mixture contained a flavin reaction, and the removal of the second chlorine is by a non-

2,4,6-TCP to 6-chlorohydroxyquinol by only a single oxidative reaction, and the removal of the second chlorine is by a non-

2,4,6-TCP oxidation. Of the 200 µM 2,4,6-TCP present in the reaction mixture, 93 µM was used by TcpA for 2,4,6-TCP oxidation. Without TcpA, NADH was completely consumed with concomitant and quantitative consumption of O2 (Table I).

Stoichiometric analysis further suggests that TcpA converts 2,4,6-TCP to 6-chlorohydroxyquinol by a single oxidative reaction, and the removal of the second chlorine is by a non-oxidative process. The reaction mixture contained a flavin reductase, which used NADH to reduce FAD to FADH2. In the absence of TcpA, FADH2 was oxidized back to FAD and H2O2. When TcpA was present, TcpA competed with free O2 for FADH2. Without TcpA, NADH was completely consumed with concomitant and quantitative consumption of O2. Upon the addition of catalase, half of the consumed O2 was released as expected, because two H2O2 molecules were converted to one O2 molecule and two H2O molecules. With TcpA, 112 µM O2 was consumed after the addition of 128 µM NADH (Table I). Upon addition of catalase, 11 µM O2 was released, indicating that 22 µM O2 was used for direct FADH2 oxidation. Thus, of the 112 µM O2 consumed, 90 µM was used by TcpA for 2,4,6-TCP oxidation. Of the 200 µM 2,4,6-TCP present in the reaction mixture, 93 µM was consumed and 81 µM 6-chlorohydroxyquinol was produced. The produced 2,6-dichloroquinol was less than 2 µM. Of the 128 µM NADH added, only 16 µM of NADH was not used for oxygen consumption, presumably being used to reduce the quinone to quinol. According to Fig. 1, the reducing equivalent for quinone reduction should be around 90 µM. We contend the additional reducing power is supplied by the 2 mM ascorbate residing in the reaction mixture because ascorbate has been previously shown to reduce quinone to quinol (23). In summary, the reaction stoichiometry shows the consumption of only one O2 per 2,4,6-TCP converted to 6-chlorohydroxyquinol.

Direct evidence that the conversion of 2,4,6-TCP to 6-chlorohydroxyquinol occurs by means of both oxidative and hydrolytic reactions came from labeling experiments with H18O or 15O2. The reaction end products were extracted, acetylated, and analyzed by GC-MS. The acetylated 6-chlorohydroxyquinol was eluted off the column at 10.62 min (Fig. 3), and its mass spectra came from labeling experiments with H18O (A), with 23.3% of 18O2 (B), or with 30% of H18O (C). Our data show that TcpA converts 2,4,6-TCP to 6-chlorohydroxyquinol, according to the scheme presented in Fig. 1. First, TcpA oxidizes 2,4,6-TCP at about 100 times faster than it consumes 2,6-dichloroquinol, as reported previously (18), yet, 2,6-dichloroquinol is only marginally accumulated during 2,4,6-TCP degradation by TcpA (Fig. 2). The lack of transitory accumulation of 2,6-dichloroquinol during 2,4,6-TCP oxidation by TcpA only indicates that the intermediate is not released, but the evidence cannot rule out that 2,6-dichloroquinol is a metabolic intermediate. Second, TcpA oxidizes 2,6-dichlorophenol to 2,6-dichloroquinol (Fig. 6A) but not further to 6-chlorohydroxyquinol (Fig. 2), which argues against the idea that 2,6-dichloroquinol is a metabolic intermediate during 2,4,6-TCP oxidation. If it were, TcpA would have converted 2,6-

| Substrate consumed | NADH | 2,4,6-TCP | O2 | Product produced |
|---------------------|-------|-----------|----|-----------------|
| Controla | 128 | 0 | 128 ± 2 | 6CHQ |
| Reactionb | 128 | 93 ± 4 | 112 ± 1 | 2,6 DiCH H2O2 |

\( ^{18}O \) from one H218O into one 6-chlorohydroxyquinol. Although 2,6-dichloroquinol was produced in trace amounts (Fig. 2), it was detected by the same GC-MS analysis. It gave a GC peak at 9.97 min (Fig. 3), and the mass peak at 178 represented the fragment with loss of all two acetyl groups from acetylated 2,6-dichloroquinol (Fig. 5). The peak cluster consisted of mass peaks at 178, 180, and 182, consistent with the presence of two chlorines in the molecule. In the control experiment (Fig. 5A) and the labeling experiment with H218O (Fig. 5C), the mass peaks were identical, but the mass shifted higher in the labeling experiment with 18O2 (Fig. 5B), indicating that O2 is involved in the first hydroxylation of 2,4,6-TCP.

**DISCUSSION**

Our data show that TcpA converts 2,4,6-TCP to 6-chlorohydroxyquinol, according to the scheme presented in Fig. 1. First, TcpA oxidizes 2,4,6-TCP at about 100 times faster than it consumes 2,6-dichloroquinol, as reported previously (18), yet, 2,6-dichloroquinol is only marginally accumulated during 2,4,6-TCP degradation by TcpA (Fig. 2). The lack of transitory accumulation of 2,6-dichloroquinol during 2,4,6-TCP oxidation by TcpA only indicates that the intermediate is not released, but the evidence cannot rule out that 2,6-dichloroquinol is a metabolic intermediate. Second, TcpA oxidizes 2,6-dichlorophenol to 2,6-dichloroquinol (Fig. 6A) but not further to 6-chlorohydroxyquinol (Fig. 2), which argues against the idea that 2,6-dichloroquinol is a metabolic intermediate during 2,4,6-TCP oxidation. If it were, TcpA would have converted 2,6-

**Fig. 3.** The GC/MS chromatogram of acetylated chloroaromatic compounds from a TcpA reaction mixture. 2,4,6-TCP was converted to 6-chlorohydroxyquinol by TcpA. The aromatic compounds were extracted into ethyl acetate, dried, and derivatized by acetylation. The acetylated products were analyzed by GC/MS. Peak 1, glycerol; peak 2, unknown; peak 3, trichlorophenol (9.86 min); peak 4, possible acetic anhydride derivative; peak 5, dichloroquinol (9.97 min); peak 6, 6-chlorohydroxyquinol (10.62 min); peak 7, unknown.

**Fig. 4.** The GC/MS chromatogram of acetylated chloroaromatic compounds from a TcpA reaction mixture. 2,4,6-TCP was converted to 6-chlorohydroxyquinol by TcpA. The aromatic compounds were extracted into ethyl acetate, dried, and derivatized by acetylation. The acetylated products were analyzed by GC/MS. Peak 1, glycerol; peak 2, unknown; peak 3, trichlorophenol (9.86 min); peak 4, possible acetic anhydride derivative; peak 5, dichloroquinol (9.97 min); peak 6, 6-chlorohydroxyquinol (10.62 min); peak 7, unknown.

**Fig. 4.** The mass spectra of acetylated 6-chlorohydroxyquinol. The mass spectra of derivatized 6-chlorohydroxyquinol (Fig. 3, peak 6) were from reactions without added 18O(A), with 23.3% of 18O2 (B), or with 30% of H18O (C).
dichlorophenol to 2,6-dichloroquinol and then to 6-chlorohydroxyquinol. Binding of 2,6-dichloroquinol to TcpA should not be a problem, for it is bound to TcpA when it is produced from 2,6-dichlorophenol. The difference between 2,6-dichlorophenol oxidation and 2,4,6-TCP oxidation by a monoxygenase is the direct product: 2,6-dichloroquinol is converted directly to 2,6-dichloroquinone (Fig. 6A), and 2,4,6-TCP is converted directly to 6-chlorohydroxyquinone (Fig. 6B). TcpA apparently has the ability to convert the latter to 6-chlorohydroxyquinone, which is chemically reduced to 6-chlorohydroquinol (Fig. 1). On the other hand, a TcpA homologue (TftD) of B. cepacia cannot further convert 2,6-dichloroquinone, which is chemically reduced to 2,6-dichloroquinol (Fig. 6B; Ref. 24). Third, our stoichiometric analysis indicates the consumption of one O₂ molecule per 2,4,6-TCP converted to 6-chlorohydroquinol, suggesting that only one oxidative reaction is involved for the overall conversion. Fourth, ¹⁸O-labeling experiments show the incorporation of oxygen into 6-chlorohydroquinol from both O₂ and H₂O. The detected amount of ¹⁸O from ¹⁸O₂ or H₂¹⁸O in 6-chlorohydroquinol is consistent with the incorporation of one oxygen atom from O₂ and one oxygen atom from H₂O, as predicted. Thus, the first reaction is oxidative, producing 2,6-dichloroquinone. The enzyme then uses the first product as its second substrate and removes the second chlorine by hydrolysis to generate 6-chlorohydroxyquinol. The latter is reduced by ascorbate and NADH in the reaction mixture to 6-chlorohydroxyquinol. Given the reactive nature of chloroquinones and the small mass of chemical reducing agents, it is likely that the enzyme-bound chlorohydroxyquinone is reduced before releasing. However, it is yet to be determined whether the chemical reduction step is on the enzyme or not.

Most monoxygenases catalyze a single hydroxylation of their substrates, but there are some monoxygenases that catalyze the sequential hydroxylation of certain substrates. Toluene 2-monooxygenase of B. cepacia G4 oxidizes toluene to o- cresol and then to 3-methylcatechol (25). Human cytochrome P450 2B6 monoxygenase oxidizes efavirenz, an HIV-1 reverse transcriptase inhibitor, to 8-hydroxyefavirenz and then to 8,14-dihydroxyefavirenz with similar kinetic parameters (26). A bacterial FMNH₂-utilizing monoxygenase oxidizes EDTA to ethylenediaminodiacetate and then to ethylenediaminetetraacetate (27, 28). A bacterial two-component monoxygenase of Bacillus sphaericus JS905 catalyzes the sequential conversion of p-nitrophenol to 4-nitrocatechol and then to hydroxyquinol (29). However, all the sequential hydroxylations reported so far are implied to be by means of two similar oxidative reactions. A monoxygenase catalyzing sequential oxidative and hydrolytic reactions for a single substrate has not been reported previously.

4-Hydrophenylacetate 3-monoxygenase of E. coli (23, 30), TcpA of Ralstonia eutropha JMP134 (18), and TftD of B. cepacia AC1100 (24) are the only characterized FADH₂-utilizing monoxygenases. Sequence analysis reveals that these three enzymes are part of a group of monoxygenases that are found in the GenBank database by 41 representatives in Bacteria and Archaea. Many homologues have no assigned function. These proteins have recently been grouped as a single protein family (pfam03241) (31). Because most of the proteins are composed of about 515 amino acid residues, it does not seem that TcpA contains an additional domain for the hydrolytic reaction. Further, TcpA is highly homologous to TftD with no gaps in amino acid sequence alignment. Both TcpA and TftD oxidize 2,4,6- TCP, but TftD only converts it to 2,6-dichloroquinone, which is chemically reduced to 2,6-dichloroquinol (Fig. 6B; Ref. 24). Therefore, TcpA differs from bifunctional enzymes that have two functional domains.

Quinones are formed from monoxygenase reactions after the removal of an electron-withdrawing group, such as a chlorine or a nitro group. The phenomenon is first observed with tetrafluoro-p-hydroxybenzoate oxidation by p-hydroxybenzoate 3-monoxygenase (19). After a fluoride elimination, the direct

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**Fig. 5.** The mass spectra of acetylated 2,6-dichloroquinol. The mass spectra of derivatized 2,6-dichloroquinol (Fig. 3, peak 5) were from reactions without added ¹⁸O (A), with 23.3% of ¹⁸O₂ (B), or with 30% of H₂¹⁸O (C).
product is an unstable quinone, which is chemically reduced by NADH to produce trifluoro-3,4-dihydroxybenzoate, and the overall reaction consumes two NADH. The formation of quinones from substituted phenols has subsequently been reported for nitrite elimination and dechlorination. Methyl-5-nitroacetophenol 5-monooxygenase of Burkholderia strain DNT4 converts 4-methyl-5-nitroacetophenol to relatively stable 2-hydroxy-5-methylquinone with nitrite elimination, and the bacterium has a quinone reductase to reduce the quinone to 2-hydroxy-5-methylquinol (22). Spingomonas chlorophenolicum pentachlorophenol 4-monooxygenase converts pentachlorophenol to tetrachloroquinol with the consumption of 1 O₂ and 2 NADH (32); however, the enzyme really produces tetrachloroquinone, which is not stable and is immediately reduced to tetrachloroquinol by chemical reaction or enzymatic reactions at the expense of NADH (21). A direct comparison of normal oxidation and dechlorinating oxidation by TRD of B. cepacia AC1100 is shown in Fig. 6. Oxidation of 2,6-dichlorophenol directly produces 2,6-dichloroquinol, whereas oxidation of 2,4,6-TCP generates 2,6-dichloroquinone, which can be chemically reduced to 2,6-dichloroquinol (24).

The chlorines in chloroquinones (non-aromatic) are more reactive than those of chloroquinolines (aromatic) toward nucleophilic attacks, leading to rapid reactions with biological thiols of cysteine, glutathione, and proteins to form stable conjugates (33, 34). Hence, 2,6-dichloroquinone is relatively reactive, which can be reduced to less reactive 2,6-dichloroquinol by either small reducing chemicals (i.e. NADH, ascorbate; Refs. 20, 21) or by quinone reductases (20, 21). It is likely the produced 2,6-dichloroquinol has two fates: the minor one is to be reduced to 2,6-dichloroquinol by reducing agents in the reaction mixture, and the major one is to be hydrolyzed by TcpA to 6-chlorohydroxyquinone, which is chemically reduced to 6-chlorohydroxyquinol. Hence, TcpA takes advantage of its reactive product, 2,6-dichloroquinol, to remove another chlorine from the parent compound. Many hydrolytic dechlorinases have been reported, and all of the characterized reactions are nucleophilic substitutions with the initial nucleophilic attack by a carboxylate or a metal-coordinated hydroxide ion (3). Because TcpA is not a metal-containing protein, it may use a glutamate or aspartate residue for the catalysis. The phenomenon that TcpA catalyzes two different reactions is known as “catalytic promiscuity,” which refers to the ability of some enzymes to catalyze more than one type of reactions (35); however, TcpA is the first example that this promiscuity is employed to catalyze the next reaction in the same metabolic pathway. The observed sequential reactions of TcpA give a specific example of catalysis evolution because of the presence of a relatively reactive intermediate and the necessity for a new metabolic pathway, reflecting the ability of nature to explore the possibilities of chemical reactions, to deal with synthetic chemicals.

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