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C. fumago Chloroperoxidase is also a Dehaloperoxidase: Oxidative Dehalogenation of Halophenols

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Chloroperoxidase from *Caldariomyces fumago* (CCPO) is the most extensively studied halogenating peroxidase, with multifunctional catalytic activities typical of peroxidase as well as cytochrome P450 enzymes.[1] CCPO, like all peroxidases, follows a reaction cycle that uses H_2O_2 to generate the high-valent ferryl oxidant, Compound I (Cpd I), which can then undergo two, one-electron reductions to regenerate the ferric resting state with concomitant substrate oxidation.[1−4] Interestingly, CCPO has none of the three conserved residues found near the heme unit in all other known peroxidases. In addition to the lack of the conserved proximal His ligand, neither the conserved distal His nor the Arg residues thought to facilitate O−O cleavage are found in CCPO.[3] Previous studies implicate Glu183 as the residue in CCPO analogous to the distal His in typical peroxidases.[5] Despite these differences in the proximal and distal regions of the heme unit, the widely accepted enzymatically active form of CCPO is Cpd I, as in all other known peroxidases.[6]

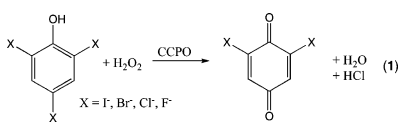
CCPO catalyzes some P450-type oxygen atom transfer reactions, such as the epoxidation of olefins and the oxygenation of sulfides.[7−10] Structurally, both CCPO and the cytochrome P450s contain protoporphyrin IX as the prosthetic group, and both are proximally ligated to the protein by a deprotonated cysteine thiolate.[1−3] Cys-ligated heme enzymes are foremost among heme proteins in their ability to catalyze the insertion of an oxygen atom, derived from either molecular oxygen or peroxide, into a variety of organic substrates.[1,2,11] This is in contrast to most His-ligated heme enzymes, which typically rely on electron-transfer-type oxidations as opposed to direct oxygen atom insertion mechanisms due to limited substrate access to the heme iron.[12]

Although CCPO demonstrates a broad range of catalytic activity, its primary biological function appears to be the halogenation of aliphatic substrates according to the reaction:

$$AH + X^- + H^+ + H_2O_2 \rightarrow AX + 2H_2O; \quad X = I^-, Br^-, Cl^-$$

where AH represents the substrate, and X^- can be Cl^-, Br^-, or I^-, but not F^-.[4,13] The pathway for peroxidase-catalyzed halogenations has been proposed to involve a ferric hypohalite intermediate known as Cpd X.[1,2] However, the mechanism of CCPO-catalyzed halogenation of organic substrates is still under investigation.[14−16]

Contrary to its primary biological function of chlorination, CCPO can also dehalogenate trihalophenols (eq 1) and *p*-halophenols, a feature that may have important environmental implications.



(1)

Large-scale production of halogenated organic compounds has resulted in contaminants that are often retained in the environment. In particular, phenols and their derivatives are commonly used in a variety of industrial processes, and chlorinated phenol toxicity has been proven both in vitro[17] and in vivo.[18] For example, exposure to high levels of chlorophenols can cause damage to the liver and the immune system. In addition, many chlorinated phenols, such as 2,4,6-trichlorophenol (TCP), persist in the environment to such an extent that they are included in the US EPA priority list of dangerous pollutants.[19] Interest in using biocatalysts in industrial and bioremediation processes continues to increase as agencies, such as the EPA, place more stringent restrictions on the use of reagents that are potentially damaging to the environment.

CCPO catalyzes the oxidative dehalogenation of trihalophenols (eq 1) and *p*-halophenols at appreciable rates and under reaction conditions often too harsh for other biocatalysts. As CCPO is significantly more robust than other peroxidases, its ability to dehalogenate halophenols could lead to its use as a bioremediation catalyst for aromatic dehalogenation reactions. Previous studies provide a well-detailed analysis of the oxidative 4-dechlorination of polychlorinated phenols by horseradish peroxidase (HRP),[20] lignin peroxidase (LiP),[21] and *Amphitrite ornata* dehaloperoxidase (DHP),[22,23] all of which are His-ligated heme proteins. This is the first example of a Cys-ligated heme-containing peroxidase that can also function as a dehaloperoxidase. It is also the first case of a peroxidase, known primarily for its halogenation function, to also dehalogenate substrates.

The H_2O_2-dependent oxidative dechlorination of TCP catalyzed by CCPO at pH 3.0 was followed by electronic absorption (EA) spectroscopy, high-performance liquid chromatography (HPLC), and gas chromatography−mass spectrometry (GC−MS).[24,25] EA spectral changes observed for the CCPO/TCP/H_2O_2 reaction mixture provided the first evidence for catalytic, oxidative dehalogenation yielding 2,6-dichloro-1,4-benzoquinone (DCQ). The product was characterized by its absorption maxima at $\lambda_{max} = 272$ and 342 nm, as reported previously for HRP[20] and LiP.[21] Analysis of the product by GC−MS (Figure 1) confirmed it to be DCQ, with a MS identical to that of a DCQ standard (Aldrich). By monitoring the absorbance at 272 nm versus time,[25] the turnover number for the CCPO-catalyzed reaction was found to be essentially identical to that reported for the same reaction carried out by HRP.[20] Formation of DCQ was shown to occur only in the presence of CCPO.

Because Cys-ligated heme enzymes are noted for their ability to catalyze O-atom transfer reactions (vide supra), efforts were initiated to establish whether the new quinone oxygen atom in DCQ was derived from H_2O_2 or H_2O. However, the exchange rate for $H_2{}^{18}O$ into the product quinone was found to be very rapid, with complete exchange occurring in a matter of seconds. Thus, simple ^{18}O incorporation studies will not be possible to probe the mechanism of oxidative dehalogenation.

Mechanistic information suggesting that the CCPO-catalyzed oxidative dehalogenation involves an electron-transfer process rather

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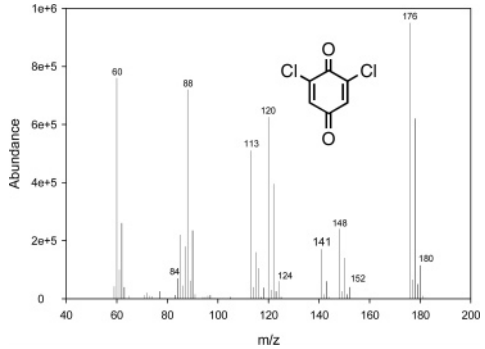
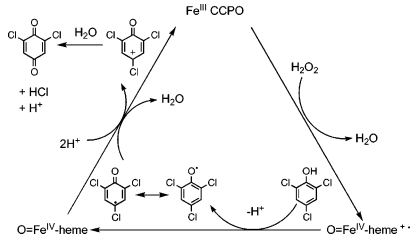


Figure 1. Mass spectrum of 2,6-dichloro-1,4-benzoquinone extracted from the CCPO (0.1 μM), TCP (0.5 mM), and H_2O_2 (1 mM) reaction in 100 mM potassium phosphate solution, pH 3.0.[24]

Scheme 1. Proposed Reaction Scheme of the CCPO/TCP/H_2O_2 Oxidative Dehalogenation Reaction



than direct O-atom insertion has been derived from examination of the reaction with monohalogenated phenols. Here, the major product is a dimer,[25,26] with *p*-benzoquinone as the minor product. The formation of a dimer from *p*-chlorophenol strongly implicates a free radical intermediate. A mechanism involving such an intermediate, based on that proposed for HRP- and LiP-catalyzed halophenol dehalogenation, is shown in Scheme 1.[20,21] After formation of CCPO Cpd I, the halophenol substrate is oxidized by one electron to form a phenoxy radical and CCPO Cpd II. Dimerization can occur from the resonance form of the phenoxy radical having an unpaired electron on the carbon ortho to the phenol oxygen, but only when that carbon is not sterically blocked by a halogen substituent (i.e., with monohalophenols but not with trihalophenols). For halophenol dehalogenation (Scheme 1), further one-electron oxidation of the phenoxy radical yields ferric CCPO and a cation intermediate. Attack of water at the 4-position followed by deprotonation and elimination of HCl results in formation of the 2,6-dichloro-1,4-benzoquinone.

We have also found that CCPO catalyzes H_2O_2-dependent defluorination, debromination, and deiodination reactions (data not shown). As with *p*-chlorophenol, two main products, *p*-benzoquinone (minor) and the halophenol dimer (major), are observed for all *p*-halophenol-CCPO-catalyzed dehalogenation reactions. Likewise, only one product, the dihaloquinone, is obtained from 2,4,6-tribromo- or 2,4,6-triiodophenol. These results show clearly that CCPO not only possesses the ability to incorporate halogens into aromatic substrates[27] but can also remove those same groups in an H_2O_2-dependent manner from the reaction products.

CCPO is an easily obtainable protein (relatively low cost), and we are interested in identifying the various factors that control this novel activity, which could be applicable toward the biodegradation of polychlorinated phenols and other noxious haloaromatic compounds. The activity illustrated herein extends the already wide range of reactions that can be carried out by CCPO. The types of reactions catalyzed by CCPO now include H_2O_2-dependent dehalogenation of mono- and trihalophenols, the exact opposite reaction type to its primary biological function of chlorination.[4]

In conclusion, we have presented the first evidence for catalytic, oxidative dehalogenation by *C. fumago* chloroperoxidase. Not only is the activity novel, but the rate of dechlorination is comparable to that of the same reaction catalyzed by HRP.[20] CCPO is significantly more robust than other peroxidases and functions under harsher reaction conditions compared to other biocatalysts. Expanding the scope of reactivity achieved by CCPO may be beneficial for industrial and biotechnological functions in the future. This considerable extension of already known activities could lead to the use of CCPO as a biocatalyst in the field of bioremediation and a broader understanding of both how peroxidases and cytochrome P450s react with halogenated organic substrates.

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Supporting Information Available: EA, HPLC, and GC/MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) EA spectral changes observed for the CCPO (0.1 μM), TCP (100 μM), and H_2O_2 (400 μM) reaction mixture in 100 mM potassium phosphate solution, pH 3.0. Authentic 2,6-dichloro-1,4-benzoquinone gave nearly identical spectral characteristics. After a 1 h incubation, 25 μL aliquots of the reaction mixtures were injected onto a C_{18} HPLC column (150 mm × 4.6 mm) under isocratic conditions (10% acetonitrile, 90% H_2O, and 0.1% trifluoroacetic acid) with a flow rate of 1 mL/min. The reaction products were extracted in ethyl acetate for GC analysis. A Restek RTX-50 GC capillary column (30 m × 0.32 mm) i.d. × 0.25 μm d.f. was used. Initial temperature of the column was set at 70 °C for 3.5 min, and from there the temperature was increased at a rate of 15 °C/min up to a final temperature of 280 °C and held for 7.5 min. MS detection was in the positive ion mode. Authentic samples of 2,4,6-trichlorophenol and 2,6-dichloro-1,4-benzoquinone gave identical retention times for both HPLC and GC analyses.
(25) See Supporting Information containing supplemental data.
(26) The mass spectral molecular ion pattern is consistent with an isomer of a chlorophenol dimer having a $C_{12}H_8O_2Cl_2$ composition.
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