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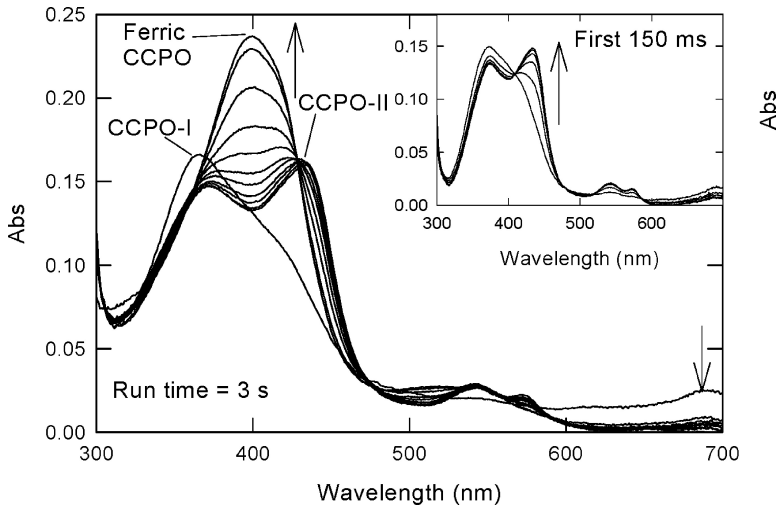

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Caldariomyces fumago Chloroperoxidase Catalyzes the Oxidative Dehalogenation of Chlorophenols by a Mechanism Involving Two One-Electron Steps

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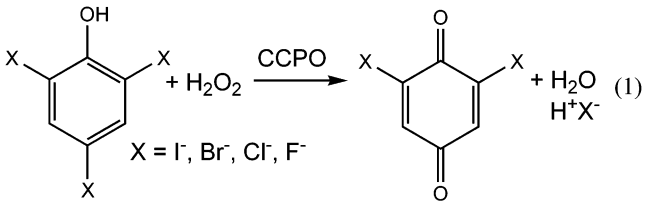
The ability of several heme-containing peroxidases, including *Amphitrite ornata* dehaloperoxidase (DHP) and *Caldariomyces fumago* chloroperoxidase (CCPO), to catalyze the oxidative dehalogenation of halophenols such as 2,4,6-trichlorophenol (TCP) (eq 1) has been reported.[1−6]



(1)

DHP is optimized to dehalogenate halophenols.[5] Structural studies have revealed that DHP has a substrate-binding pocket and a globin fold, and the modeled position of the oxygen atom bound to the heme iron led to the suggestion of a single two-electron oxidation.[7] Spectroscopic and kinetic assays have consistently demonstrated globin-like properties of DHP.[8−11] Mutagenesis and pH versus activity studies with DHP have led to a proposal that the mechanism of DHP-catalyzed oxidative dehalogenation involves a net two-electron oxidation of bound substrate, which cannot be activated starting from the compound II state.[12,13]

In contrast, peroxidases such as CCPO typically oxidize organic substrates, especially phenols, by two consecutive one-electron steps. The ferric enzyme reacts with H_2O_2 to form the high-valent ferryl/porphyrin radical cation, compound I (CCPO-I), which is reduced back to the ferric state in two one-electron steps with concomitant substrate oxidation via a second ferryl species, compound II (CCPO-II).[14] However, a two-electron oxidation mechanism involves direct oxygen atom insertion into organic substrates.[14,15] The mechanistic difference is subtle yet distinct. The relative stability of CCPO-I and -II makes CCPO an ideal catalyst with which to use rapid scan stopped-flow techniques to distinguish whether the mechanism of heme peroxidase-catalyzed oxidative dehalogenation proceeds by two consecutive one-electron transfers or by a single two-electron oxidation.

We report herein the ability to differentiate between one- and two-electron oxidations catalyzed by CCPO.[16] Reaction of ferric CCPO with H_2O_2 immediately forms CCPO-I, which remains spectrally unchanged for ∼1 s.[16b] Upon reaction with TCP (Figure 1), CCPO-I is quickly (<150 ms) reduced to the steady state CCPO-II intermediate (inset) and then, once all H_2O_2 has been consumed, to the ferric resting state within 3 s; quinone formation (eq 1) has been previously established.[3] A clean isosbestic point at 408 nm

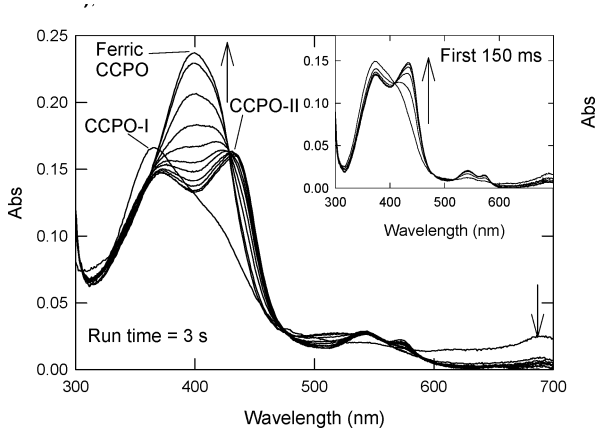


Figure 1. Reaction of CCPO-I with 2,4,6-trichlorophenol (3 s run time) in 100 mM potassium phosphate solution, pH 3.6 and 4 °C. CCPO-I was formed in the first mixture by reacting the ferric enzyme with H_2O_2 for 100 ms. Inset: Reaction of CCPO-I with 2,4,6-trichlorophenol (150 ms run time). The concentrations upon final mixing are 3 μM CCPO, 250 μM H_2O_2, and 125 μM 2,4,6-trichlorophenol.

(Figure 1 inset) indicates that CCPO-I is directly reduced to CCPO-II upon reaction with TCP, likely with formation of the phenoxy radical as previously suggested.[3] Without organic substrate, CCPO-I is slowly reduced to CCPO-II and then the ferric state, but only after ∼75 s (data not shown).

The same experimental approach demonstrates that TCP can reduce CCPO-II back to the ferric resting state. CCPO-II, formed as previously reported,[18] remains spectrally unaltered for ∼10 s.[16b] Upon reaction with TCP, CCPO-II is quickly reduced to the ferric enzyme within 1.5 s (Figure 2). As a one-electron oxidant, CCPO-II can only oxidize TCP to the quinone (eq 1) by two one-electron steps, via the phenoxy radical. The lack of a clean isosbestic point in Figure 2 is likely due to a small spectral change following interaction with substrate.

A first-order dependence on substrate concentration is seen in plots of k_{obs} versus [TCP] for reactions of both CCPO-I and CCPO-II with TCP.[16b] This confirms that the reductions of CCPO-I to CCPO-II and of CCPO-II to ferric CCPO depend directly on TCP. The reaction of CCPO-I with TCP is significantly faster ($k = 2.8 \times 10^5$ M^{-1} s^{-1}) than the reaction of CCPO-II with TCP ($k = 5.0 \times 10^3$ M^{-1} s^{-1}). Therefore, CCPO-II accumulates under steady-state turnover conditions (data not shown). The ability of CCPO-I and -II to oxidize TCP is consistent with an electron transfer oxidation process involving two consecutive one-electron steps.[3]

To validate the ability to discriminate between one- and two-electron oxidations using rapid scan stopped-flow techniques, we examined the reaction of CCPO with thioanisole. Labeling studies

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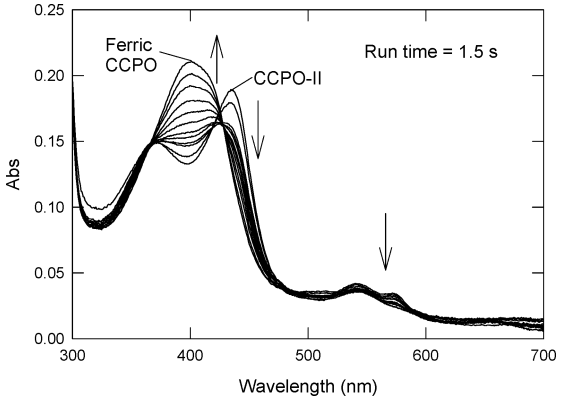

Figure 2. Reaction of CCPO-II with 2,4,6-trichlorophenol (1.5 s run time) in 100 mM potassium phosphate solution, pH 3.6 and 4 °C. CCPO-II was formed in the first mixture by reacting ferric CCPO with a solution containing H_2O_2 and ascorbate for 1 s. The concentrations upon final mixing are 3 μM CCPO, 250 μM H_2O_2, 750 μM ascorbate, and 125 μM 2,4,6-trichlorophenol.

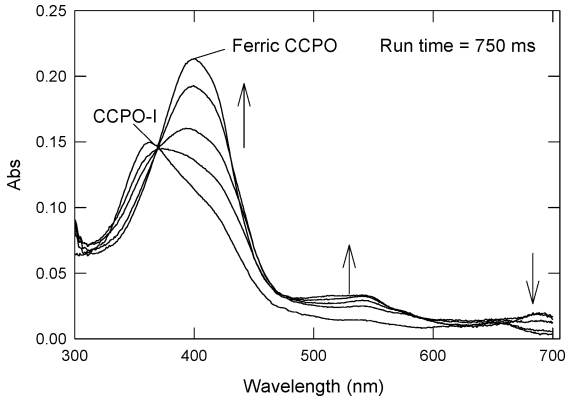

Figure 3. Reaction of CCPO-I with thioanisole (750 ms run time) in 100 mM potassium phosphate solution, pH 3.6 and 4 °C. CCPO-I was formed in the first mixture by reacting ferric CCPO with H_2O_2 for 100 ms. The concentrations upon final mixing are 3 μM CCPO, 250 μM H_2O_2, and 62.5 μM thioanisole.

show that the oxygen atom of $H_2{}^{18}O_2$ is incorporated into the product of CCPO-catalyzed sulfoxidations as expected for an oxygen atom transfer two-electron oxidation process.[19] Reaction of CCPO-I with thioanisole results in complete conversion of CCPO-I to ferric CCPO with no spectroscopic evidence of CCPO-II (Figure 3). Similar results have been reported for the reaction of CCPO-I with olefins and other two-electron substrates.[15] The absorption spectra collected for reaction of thioanisole with CCPO-I are consistent with a reaction proceeding primarily via a single two-electron oxidation.

Best known for catalyzing halogenation reactions, CCPO is a versatile heme enzyme that exhibits peroxidase, catalase, and cytochrome P450-like activities.[14,15] Like P450, the CCPO heme iron is proximally ligated by a cysteine thiolate.[14,20,21] CCPO and P450s are foremost among heme enzymes in catalyzing two-electron oxidations via oxygen atom insertion.[14] However, similar to the more common histidine-ligated peroxidases, CCPO typically oxidizes organic substrates by two consecutive one-electron transfers. We previously reported that CCPO catalyzes the oxidative dehalogenation of halophenols (eq 1). Reaction of CCPO with mono-*p*-halophenols resulted in dimeric products, suggesting a mechanism involving two consecutive one-electron oxidations involving CCPO-I and -II[3] rather than a single two-electron oxidation as proposed for DHP-catalyzed reactions.[7]

In conclusion, we present strong evidence that the mechanism of oxidative dehalogenation of halophenols catalyzed by CCPO, and presumably by other heme-containing peroxidases, involves two consecutive one-electron steps. A single two-electron oxidation mechanism has been previously suggested for this reaction based on structural studies.[7] Additionally, CCPO-I and CCPO-II are both active oxidants during catalysis, and CCPO-II itself, a one-electron oxidant, can catalyze the dehalogenation of TCP. Reaction of CCPO-I with thioanisole results in a direct conversion to ferric CCPO with no evidence of CCPO-II, consistent with a single two-electron oxidation by insertion of an oxygen atom. The relative stability of CCPO-I and -II has allowed us to differentiate between one- and two-electron substrate oxidations using rapid scan stopped-flow techniques.

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Supporting Information Available: Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) (a) CCPO was isolated and purified as described.[17] The enzyme purity was evaluated from the R_Z value (A_{400nm}/A_{280nm}), and CCPO with R_Z > 1.4 was used. Fresh H_2O_2 stocks were made daily in DI-H_2O. Fresh 10 mM halophenol stocks were made in 50/50 mixture of DI-H_2O/ethanol. Preparation of CCPO-I and CCPO-II was achieved by published procedures.[18] A four-syringe, rapid scanning stopped-flow instrument (Hi-Tech SF-61DX2) was used. When 4-chlorophenol and 2,4,6-tribromophenol were reacted with CCPO-I and -II, the results were similar to what is reported in Figures 1 and 2. (b) See Supporting Information.
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