Single turnover studies of oxidative halophenol dehalogenation by horseradish peroxidase reveal a mechanism involving two consecutive one electron steps: toward a functional halophenol bioremediation catalyst.

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Abstract:

Horseradish peroxidase (HRP) catalyzes the oxidative para-dechlorination of the environmental pollutant/carcinogen 2,4,6-trichlorophenol (2,4,6-TCP). A possible mechanism for this reaction is a direct oxygen atom transfer from HRP compound I (HRP I) to trichlorophenol to generate 2,6-dichloro 1,4-benzoquinone, a two-electron transfer process. An alternative mechanism involves two consecutive one-electron transfer steps in which HRP I is reduced to compound II (HRP II) and then to the ferric enzyme as first proposed by Wiese et al. [F.W. Wiese, H.C. Chang, R.V. Lloyd, J.P. Freeman, V.M. Samokyszyn, Arch. Environ. Contam. Toxicol. 34 (1998) 217–222]. To probe the mechanism of oxidative halophenol dehalogenation, the reactions between 2,4,6-TCP and HRP compounds I or II have been investigated under single turnover conditions (i.e., without excess H2O2) using rapid scan stopped-flow spectroscopy. Addition of 2,4,6-TCP to HRP I leads rapidly to HRP II and then more slowly to the ferric resting state, consistent with a mechanism involving two consecutive one-electron oxidations of the substrate via a phenoxy radical intermediate. HRP II can also directly dechlorinate 2,4,6-TCP as judged by rapid scan stopped-flow and mass spectrometry. This observation is particularly significant since HRP II can only carry out one-electron oxidations. A more detailed understanding of the mechanism of oxidative halophenol dehalogenation will facilitate the use of HRP as a halophenol bioremediation catalyst.

Keywords: biochemistry | inorganic biochemistry | horseradish peroxidase | rapid kinetics | chlorophenol dehalogenation | phenoxy radical | bioremediation | ferryl heme intermediates

Article:

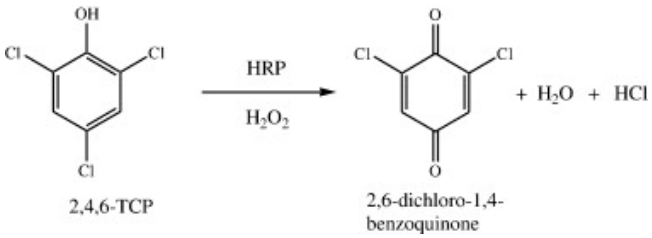
1. Introduction

Chlorinated phenols are a major class of environmental pollutants and are potential human carcinogens [1], [2], [3] and [4]. They are extensively used as disinfectants, pesticides, herbicides

and dyes; as a result they can contaminate the soil and ground water, and eventually enter the food chain. Removal of chlorophenols from industrial waste waters is achieved by a variety of treatment processes, including (a) sequential anaerobic and aerobic biodegradation [5], [6] and [7], (b) adsorption by activated carbon [8] and [9], (c) photo-degradation [10] and [11], (d) and, most recently, enzymatic degradation [12], [13], [14] and [15].

Several peroxidase enzymes are being explored for the removal of chlorophenols from industrial waste waters [15]. Detailed studies have been performed regarding the oxidation of chlorophenols by lactoperoxidase [16], lignin peroxidase (LiP) [17], horseradish peroxidase (HRP) [18], [19] and [20], dehaloperoxidase [21], [22], [23], [24], [25], [26], [27], [28], [29], [30], [31] and [32] and Caldariomyces fumago chloroperoxidase [33] and [34]. Among these peroxidases, HRP is an especially promising candidate for use as a bioremediation catalyst because of its (a) stability resulting in easier storage and handling, (b) ability to oxidize a large number of chlorophenols, (c) flexibility to function at wide ranges of temperature and pH, (d) and, most importantly, ready availability and relatively low cost [13], [14], [35], [36], [37], [38], [39], [40] and [41]. The oxidative dehalogenation of chlorophenols is catalyzed by HRP in the presence of H2O2. A better understanding of the mechanism of this process would help in the design of a more efficient HRP-based bioremediation method.

Heme peroxidases couple the oxidation of organic substrates to the reduction of hydrogen peroxide to water by a mechanism involving two ferryl heme intermediates. Peroxidase compound I (Cpd I), a ferryl (FeIV=O) porphyrin π-cation radical [42] and [43] is formed first after addition of H2O2 to the ferric enzyme. Reduction of Cpd I by a one-electron oxidizable substrate yields the second ferryl porphyrin intermediate known as compound II (Cpd II). Cpd II is then reduced by one more electron to regenerate the ferric resting state [42] and [43]. Among the chlorophenols that can be degraded by HRP, 2,4,6-trichlorophenol (2,4,6-TCP) has received considerable attention due to its higher toxicity and its environmental release by diverse industries [4], [33] and [34]. HRP catalyzes the oxidative dechlorination of 2,4,6-TCP generating 2,4-dichloro-1,4-benzoquinone (Eq. (1)) [20]. A possible mechanism of this reaction could involve a direct oxygen transfer from HRP Cpd I to 2,4,6-TCP to generate 2,4-dichloro 1,4-benzoquinone in a single two-electron oxo transfer process [44] and [45]. An alternative mechanism involves two consecutive one-electron transfer steps with sequential formation of both HRP compounds I (HRP I) and II (HRP II), as initially proposed by Samokyszyn and co-workers [20]. The first substrate intermediate in this oxidative dechlorination mechanism is a phenoxy radical, which has been shown to react directly with DNA to form covalent DNA adducts leading to the initiation of tumorigenesis [46]. The generation of these radicals in the body is explained by the peroxidase- or myoglobin Cpd II-mediated oxidation of chlorophenols which entered the body through food or water intake [46] and [47].



With the use of rapid scanning stopped-flow absorption spectroscopy, the reactivities of homogeneous short-lived ferryl HRP intermediates can be investigated. Thus, the hypothesized two consecutive one-electron transfer mechanism can be directly tested with single turnover experiments using double mixing rapid-scan stopped-flow spectroscopy, in which the ferryl intermediate (HRP I or HRP II) can be generated in the first mix followed by reaction with chlorophenol in the second mix. Using rapid-scan stopped-flow spectroscopy, we have previously reported data with C. fumago chloroperoxidase that were consistent with a mechanism of oxidative dehalogenation of chlorophenols involving two consecutive one-electron oxidations [34]. However, it was not possible to carry out those studies under single turnover conditions since an excess of H2O2 was needed to form chloroperoxidase compound I. Although the turnover number (68 s− 1) for HRP-catalyzed oxidation of 2,4,6-TCP in the presence of excess (> 103 × [HRP]) amounts of H2O2 and the substrate at pH 5.4 has previously been determined by Ferrari and co-workers [21], no kinetic data on the direct reactions of HRP I and HRP II with 2,4,6-TCP have been reported.

In the present study, we demonstrate the reaction steps involved in the dehalogenation of 2,4,6-TCP and illustrate how HRP I and HRP II are reduced by 2,4,6-TCP using single turnover experiments. It has previously been shown that the number and position of chlorine substituents on the phenolic ring has an influence on the rate and the extent of removal of halogens by HRP or by biodegradation [5] and [48]. Therefore, we have also extended our studies to rapid-scan stopped-flow spectroscopy and mass spectrometry analyses of the HRP-catalyzed degradation of 2,3,6-trichlorophenol (2,3,6-TCP) and 2,6-dichlorophenol (2,6-DCP) and report the rate constants for these reactions.

2. Materials and Methods

Horseradish peroxidase (HRP) (RZ = 3.1), purchased from USB (United States Biochemical, Cleveland, OH), was dissolved in 100 mM potassium phosphate at pH 5.0. The HRP concentration was determined spectrophotometrically using the molar absorptivity of ε403 = 103 mM− 1 cm− 1[49]. Reagent grade chemicals (Sigma, ACROS, or Fisher) were used without further purification. Fresh 100 mM H2O2 stock solutions in water were made from the commercial 30% solution, and H2O2 concentrations were confirmed spectrophotometrically using ε240 = 39.4 M− 1 cm− 1[50].

Stock solutions of (100 mM) 2,6-DCP, 2,3,6-TCP and 2,4,6-TCP were made in a 50/50 ethanol/deionized-H2O mixture by first dissolving the halophenol in ethanol. The concentration of 2,4,6-TCP was confirmed spectrophotometrically using ε312 = 4.44 mM− 1 cm− 1 at pH 7.0 (20). Potassium phosphate (100 mM) at pH 5.0 was used for all experiments.

UV-visible absorption spectra were recorded at 4 °C using a Cary 400 spectrophotometer interfaced to a Dell PC for data acquisition. Rapid scan stopped-flow experiments were carried out at 4 °C on a Hi-Tech Ltd. SF-61 DX2 instrument equipped with a diode array detector. The dead time was determined to be 1.5 ms. In single and double mixing experiments, numerous scans were recorded for different run times to optimize the conditions. The kinetic data were fit to various reaction models by the KinetAsyst program (Hi-Tech Ltd.) or by the Specfit program from Spectrum Software Associates.

Either the single mixing or double mixing mode was used to optimize the formation and determine the stability of HRP ferryl intermediates, HRP I and HRP II. HRP (15 μM) in 100 mM potassium phosphate solution, pH 5.0 was mixed with H2O2 (15 μM in buffer) in the absence or presence of sodium ascorbate (7.5 μM), for the formation of HRP I and HRP II, respectively. For the reactions of ferryl intermediates with substrates, halophenol (15 μM, 7.5 μM or 3.75 μM) in buffer was mixed with the preformed intermediates. Age times of 40 ms and 8 s were utilized for reactions with HRP I and II, respectively, based on the conditions for their optimal formation. The run time was varied depending on the time taken for the completion of the reaction.

A Restek RTX-5 GC capillary column (30 m × 0.32 mm) was used for product analysis. The initial temperature of the column was set at 50 °C for 2 min and the temperature was subsequently increased at a rate of 10 °C/min up to a final temperature of 300 °C and held for 7.5 min. MS detection was in the electron impact ionization mode, and a VG70S mass spectrometer scanning from 50 to 450 (m/z) was used for product identification. Authentic samples of 2,6-DCP, 2,3,6-TCP, and 2,4,6-TCP gave identical corresponding retention times for GC/MS analyses.

All kinetic rate constants were analyzed by using the non-linear regression program (Sigma Plot) for absorbance vs. time plots to a single exponential curve with three parameters mode. The values reported here were based on three separate measurements and are expressed as mean values ± standard deviations.

3. Results and discussion

3.1. Oxidative halophenol dehalogenation

We have performed single turnover experiments employing rapid scan stopped-flow spectroscopy to examine whether the mechanism of oxidative dehalogenation catalyzed by HRP involves two consecutive one-electron steps or a single two-electron oxidation. The present study represents an important step forward in our understanding of the mechanism of this reaction in that we are able to carry out the stopped-flow investigation under single turnover conditions, meaning that we can generate one equiv. of HRP I in the first mix and then react it vs. the chlorophenol substrate in the second mix. First, we optimized the formation of HRP I (FeIV = O/porphyrin π-cation radical) and HRP II (FeIV = O) for use in double mixing rapid-scan stopped-flow spectroscopy experiments. To do this, HRP was reacted with an equimolar amount of H2O2 to generate HRP I (plus one-half molar equiv. of ascorbate, a two-electron donor substrate, to form HRP II) at pH 5.0 and 4 °C to generate the ferryl intermediate without any excess H2O2. This ensures that only a single turnover can be achieved. We chose pH 5.0 because the products are stable at acidic pH and can be isolated for GC/MS analysis. Even though potassium phosphate is not a good buffer at pH 5, it still helps maintain a constant pH value at 100 mM (ionic strength: I = ~ 0.1 M). Similar results were observed at pH 7.0.

Furthermore, despite the fact that an excess (> 10) amount of 2,4,6-TCP over HRP would provide kinetic data that can be readily analyzed as pseudo-first order reactions, the highest ratio used for the reaction of HRP I with 2,4,6-TCP in this study was only 1:2. Unexpectedly, kinetic traces for conversion of HRP I to HRP II were well fit to single exponential reactions to the completion of the reaction even when the ratios of [2,4,6-TCP] /[HRP] were near (2.0 and 1.0) or sub-stoichiometric (0.5). Similarly, single exponential fits were also obtained for conversion of

HRP II to ferric HRP in the presence of 0.5, 1.0, 2.0, 2.5, 5.0 and 10 equiv. of 2,4,6-TCP. These apparently unusual results will be discussed later in terms of likely involvement of a 2,4,6-TCP radical in the reactions. All the kinetic data (rate constants) that were obtained for three or more separate runs in this study are summarized in Table 1 as mean ± standard deviation values.

Table 1. Kinetics constants for the reactions of halophenols with HRP ferryl intermediates (HRP I and HRP II).

Substrate	[Substrate]/[HRP] at $t = 0$	Rate constant (s^{-1})	
		HRP I to HRP II [a]	HRP II to Ferric HRP [b]
No substrate		0.027 ± 0.003	0.0043 ± 0.0006
2,4,6-TCP	0.5 (HRP I)	56.6 ± 5.5	
	0.5 (HRP II)		0.68 ± 0.09
	1.0 (HRP I)	114 ± 12	0.96 ± 0.03[c]
	1.0 (HRP II)		1.15 ± 0.17
	2.0 (HRP I)	238 ± 8	2.50 ± 0.20[c]
	2.0 (HRP II)		1.56 ± 0.13
2,3,6-TCP	2.0 (HRP I)	30.1 ± 0.9	1.24 ± 0.09[c]
	2.0 (HRP II)		1.37 ± 0.04[d]
2,6-DCP	2.0 (HRP I)	19.7 ± 0.7	0.37 ± 0.02[c]
	2.0 (HRP II)		0.26 ± 0.03

a HRP I formed by mixing HRP (15 μM) with one equiv. of H_2O_2 (15 μM). The resulting HRP I (7.5 μM) was then mixed with buffer/halophenol (15 μM, 7.5 μM or 3.75 μM) in the second mix (age time 40 ms) (concentrations after mixing, [HRP] = [H_2O_2] = 3.75 μM, [halophenol] = 7.5 μM, 3.75 μM or 1.875 μM). The reaction was monitored at 419 nm to follow the formation of HRP II.

b HRP II formed by mixing HRP (15 μM) with one equiv. of H_2O_2 (15 μM) and one-half equiv. of sodium ascorbate (7.5 μM). The resulting HRP II (7.5 μM) was then mixed with buffer/halophenol (15 μM, 7.5 μM or 3.75 μM) in the second mix (age time 8 s) (final concentrations, [HRP] = [H_2O_2] = 3.75 μM, [ascorbate] = 1.875 μM, [halophenol] = 7.5 μM, 3.75 μM or 1.875 μM). The reaction was monitored at 403 nm to follow the regeneration of ferric HRP.

c Second phase reaction following HRP I to HRP II conversion.

d First (~ 60%) of two phases. When 2 molar equiv. of 2,3,6-TCP was reacted with HRP II, the reaction trace was better fit to double-exponential decays (r^2 = ~ 0.999) with fast (1.37 s^{-1}, ~ 60%) and slow (0.37 s^{-1}, ~ 40%) phases rather than to a single-exponential decay (0.73 s^{-1}, r^2 = 0.990).

3.2. Reaction of ferric HRP with H_2O_2 in the absence of organic substrate

To study the steps involved in the HRP-catalyzed oxidation of halophenols and to probe the roles of high-valent intermediates in the reaction mechanism, the rate constants for the spontaneous reaction must first be measured. Rapid scan, stopped-flow spectroscopy was thus used to initially optimize the formation and then establish the stability of the HRP ferryl intermediates (HRP I and HRP II) at 4 °C, pH 5.0. The absorption spectra of ferric HRP, HRP I and HRP II recorded at pH 5.0 (Fig. 1) [51] for this work were essentially identical to those previously reported at neutral pH value [52] and [53]. Reaction of HRP with an equimolar amount of H_2O_2 (final concentrations = 3.75 μM) resulted in the homogenous formation of HRP I in 40 ms as demonstrated by the characteristic absorption spectrum of HRP I (Soret peak at 400 nm). After 3 s, HRP I gradually converted to HRP II (k_{obs} = 0.027 ± 0.003 s^{-1}), which has a Soret band at 419 nm and distinct α (558 nm) and β (527 nm) transitions in the visible region. The spectrum of HRP II remained unchanged for about 55 s and then decayed very slowly (k_{obs} = 0.0043 ± 0.0006 s^{-1}) to regenerate native ferric HRP (Soret maximum at 403 nm). If the reaction of HRP I with a reducing substrate proceeds via HRP II, then the reaction mechanism involves two consecutive one-electron steps; if HRP I directly converts back to the ferric state without involvement of HRP II, then the reaction mechanism is a direct two-electron oxidation with the O-atom transfer from HRP I. The spontaneous (i.e., no added organic substrate) reduction of HRP I to ferric HRP, under the given experimental conditions (HRP: H_2O_2 = 1:1, pH 5.0, 4 °C), clearly proceeded through the homogeneous formation of HRP II.

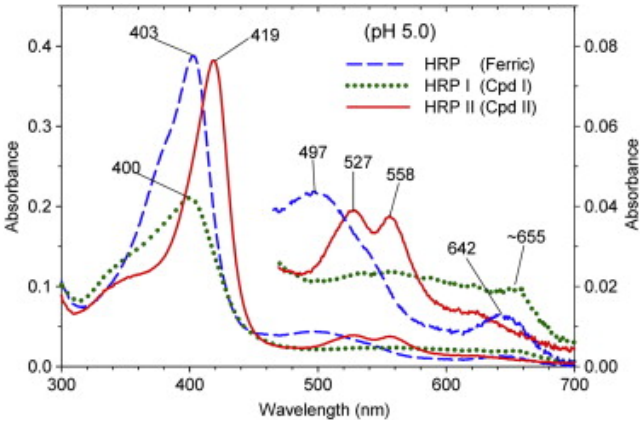


Fig. 1. Rapid scan, stopped-flow spectra illustrating formation of the ferryl intermediates and the regeneration of the ferric state after a single mixing of ferric HRP (15 μM) with H_2O_2 (15 μM) in 100 mM potassium phosphate solution at 4 °C and pH 5.0. The traces represent (green dotted line) HRP I formed at 40 ms; (red solid line) HRP II formed at 200 s; (blue dashed line) ferric HRP regenerated at > 45 min.

3.3. Reaction of HRP I with 2,4,6-TCP

As just discussed, spontaneous conversion of preformed HRP I to HRP II (monitored at 419 nm) occurred slowly with a rate constant of 0.027 ± 0.003 s^{-1}. The reaction of HRP I with 2,4,6-TCP is a much faster process. When HRP I was mixed with 2 equiv. of 2,4,6-TCP, HRP I was rapidly converted to HRP II in a first phase (k_{obs} = 238 ± 8 s^{-1}) (representative results shown in Fig. 2A). Note that, under these conditions, ~ 50% of HRP I had been converted to HRP II within the dead time (~ 1.5 ms) of the instrument. Then HRP II was slowly reduced to the ferric state in the second phase (k_{obs} = 2.50 ± 0.20 s^{-1}) (Fig. 2B). The HRP I / 2,4,6-TCP reaction, followed at

419 nm, shows single exponential behavior (Fig. 2A, *inset*); the rate of formation of HRP II (k_{obs} = 238 ± 8 s^{-1}) was ~ 8800 -fold faster with 2,4,6-TCP than without (k_{obs} = 0.027 ± 0.003 s^{-1}). The rate of subsequent conversion of HRP II to the ferric state (k_{obs} = 2.50 ± 0.20 s^{-1}) was also faster (by ~ 580-fold) than in the absence of the substrate (k_{obs} = 0.0043 ± 0.0006 s^{-1}) under these conditions where 1 equiv. of 2,4,6-TCP is expected to remain unoxidized upon formation of HRP II.

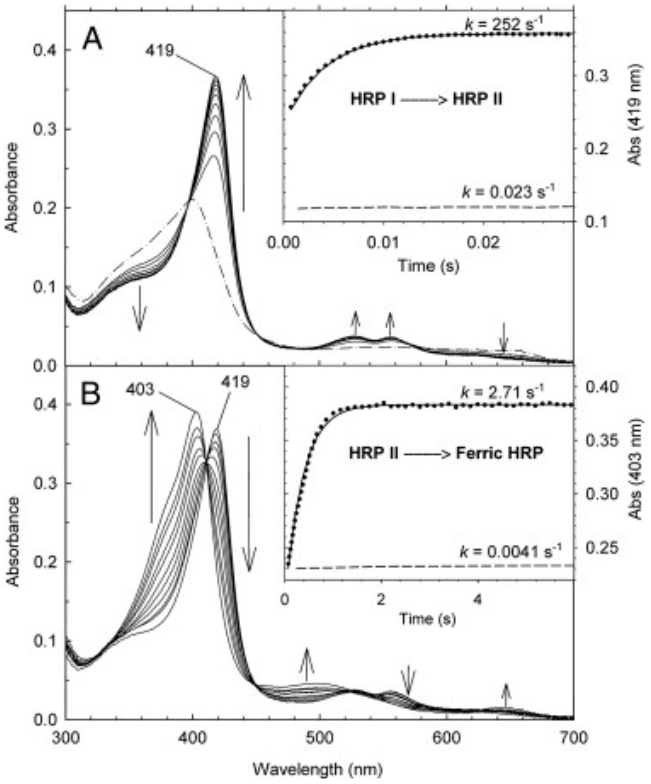


Fig. 2. Conversion of HRP I to HRP II in 100 mM potassium phosphate solution at 4 °C, pH = 5.0. Ferric HRP was first mixed with one equiv. of H_2O_2 for the formation of HRP I (age time 40 ms), which was then mixed with TCP to observe the reaction course (final concentrations, [HRP] = [H_2O_2] = 3.75 μM, [TCP] = 7.5 μM). Representative results (spectral changes and kinetic traces and their analysis) are displayed here; statistical data based on three or more runs are summarized in Table 1. (A) First phase of the reaction showing the conversion of HRP I to HRP II. Since the absorption spectrum of HRP I can not be recorded under the conditions (see text, Section 2.3), its spectrum (dot-dashed line) taken from Fig. 1 is overlaid as reference. *Inset*, The same reaction monitored at 419 nm shows, upon mixing with 2 mol equiv. of TCP, HRP I fully converting to HRP II with k_{obs} = 252 s^{-1} (dotted line), a single exponential kinetic fit (solid line), spontaneous conversion without TCP (dashed line). (B) Second phase of the reaction showing the conversion of HRP II to ferric HRP. *Inset*, The same reaction monitored at 403 nm shows HRP II that was formed by mixing 2 mol equiv. of TCP with HRP I, fully converting to ferric HRP in the subsequent reaction with k_{obs} = 2.71 s^{-1} (dotted line), a single exponential kinetic fit (solid line), spontaneous conversion without TCP (dashed line). In both panels A and B, the vertical arrows indicate the directions of absorbance changes.

The mass spectrum of the dehalogenated product matches that of the authentic sample of 2,6-dichloro-1,4-benzoquinone confirming the product formation (m/z 176, data not shown). The data indicate that in the 2,4,6-TCP dehalogenation mechanism, the first electron is transferred from the substrate to HRP I to generate the phenoxy radical and HRP II. Thus the oxidative dehalogenation of TCP to benzoquinone product is mediated by both HRP I and HRP II as initially hypothesized by Samokyszyn and co-workers [20] and subsequently by Ferrari and co-workers [21].

3.4. Reaction of HRP I with other halophenols

The spectral changes observed for the oxidation of 2,3,6-TCP and 2,6-DCP by HRP I (data not shown) were the same as those seen for 2,4,6-TCP (Fig. 2). The HRP I to HRP II conversion rates are also much higher (> 103-fold) in the presence of these substrates than the spontaneous reduction. HRP II is again seen as an intermediate between HRP I and the ferric enzyme, consistent with a mechanism involving two consecutive one-electron oxidation steps. However, the rates for these substrates are not as high as in the 2,4,6-TCP reaction (2,4,6-TCP ≫ 2,3,6-TCP > 2,6-DCP ⋙ No substrate) (Table 1). Although the result in this study using the very limited number of substrates may not be sufficient enough to draw a general conclusion, the number of chlorine substituent for the substrates and whether a chlorine atom is absent at 4 (para) position appear to have a significant effect on the rate constants for reduction of HRP I and HRP II. This is likely due to the fact that the slower substrates are harder to oxidize due to the differences in chloro substitution pattern. Laurenti and co-workers reported two products for the HRP/H2O2/2,6-DCP reaction, 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl and 3,3′,5,5′-tetrachlorodiphenoquinone [18]. In the reaction of HRP I with 2 molar equiv. of 2,6-DCP in this study, the mass spectrum of the reaction product (m/z 324, data not shown) is consistent with the generation of 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl; however, further oxidation of this product to 3,3′,5,5′-tetrachlorodiphenoquinone was not seen. This is because only one equiv. of HRP I was generated (single turnover with HRP:H2O2 = 1:1) consumption of which would leave no remaining oxidant to carry out the further oxidation of 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl to the corresponding quinone. In the case of 2,3,6-TCP oxidation, a different dimeric product [mass spectrum (m/z 311, 313, 315, data not shown)] was formed, the structure of which is under investigation.

3.5. Reaction of HRP II with 2,4,6-TCP, 2,3,6-TCP and 2,6-DCP

In the peroxidase reaction, the high-valent oxidant Cpd II can also carry out one-electron oxidations. Therefore, the ability of HRP II to dehalogenate halophenols was studied using double-mixing stopped-flow experiments. In reactions of HRP I with the three chlorophenol substrates described above, the HRP II formed in the first phase of the reaction was reduced back to ferric HRP in the second phase. The rate constants for the second phase reaction were much smaller than those for the first phase by the factors of 94 (2,4,6-TCP), 24 (2,3,6-TCP) and 54 (2,6-DCP) (Table 1). This reflects intrinsic differences between HRP I and HRP II for their substrate oxidation rates. The somewhat lower concentrations of oxidizable substrate available for HRP II relative to HRP I may also contribute a minor factor to the observed differences.

Alternatively, HRP II can be generated by mixing ferric HRP (7.5 μM before mixing) with one equiv. of H2O2 containing one-half equiv. of the two-electron reductant sodium ascorbate (to reduce the initially formed HRP I to HRP II). Complete HRP II formation occurs in 2 s (kobs = ~

2.1 s− 1) resulting in its characteristic absorption spectrum (Soret, β and α bands at 419 nm, 527 nm and 558 nm, respectively), which is essentially unchanged over a period of ~ 10 s. The spontaneous reduction of HRP II to the ferric resting state is ~ 6.2-fold slower (0.0043 ± 0.0006 s− 1) (Fig. 3 and Table 1) than that of HRP I to HRP II (0.027 ± 0.003 s− 1) (Fig. 1A and Table 1). When HRP II was preformed in the first mix and reacted with 0.5 equiv. of 2,4,6-TCP in the second mix (Fig. 3), the rate of regeneration of the ferric state (0.68 ± 0.09 s− 1) was > 150-fold faster than the spontaneous rate. Formation of the ferric state from HRP II in the presence of 2 equiv. of 2,4,6-TCP (k = 1.56 ± 0.13 s− 1) and the other chlorophenol substrates (2,3,6-TCP, 2,6-DCP) has the same order (2,4,6-TCP > 2,3,6-TCP ≫ 2,6-DCP ⋙ No substrate) with respect to the chloro substituent patterns of the substrate as seen with HRP I (Table 1).

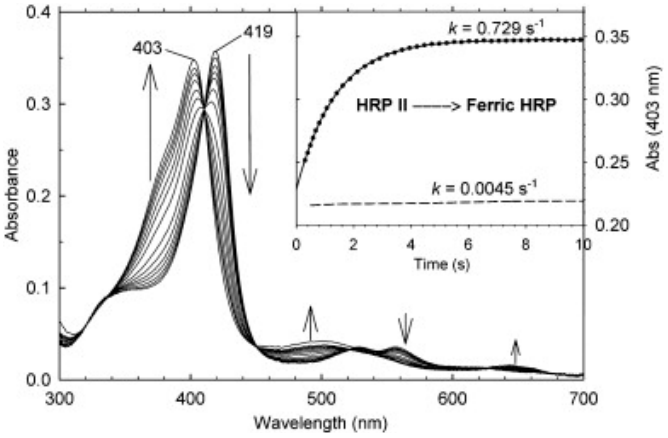


Fig. 3. Conversion of HRP II to ferric HRP in 100 mM potassium phosphate solution at 4 °C, pH = 5.0. Ferric HRP was first mixed with one equiv. of H_2O_2 and one-half equiv. of sodium ascorbate for the formation of HRP II (age time 8 s), which was then mixed with TCP to observe the reaction course (final concentrations, [HRP] = [H_2O_2] = 3.75 μM, [ascorbate] = 1.875 μM, [TCP] = 1.875 μM). The vertical arrows indicate the directions of absorbance changes. Representative results (spectral changes and kinetic traces and their analysis) are shown here; statistical data based on three or more runs are summarized in Table 1. *Inset*, The same reaction monitored at 403 nm shows, upon mixing with 0.5 mol equiv. of TCP, HRP II fully converting to ferric HRP with k_{obs} = 0.729 s^{-1} (dotted line), a single exponential kinetic fit (solid line), spontaneous conversion without TCP (dashed line).

GC/MS analysis confirmed that the products formed from the reaction of HRP II with halophenol substrates are the same as seen by Laurenti et al. in multiple turnover studies of the HRP/H2O2 system [18]: 2,6-dichloro 1,4-benzoquinone quinone with TCP and 3,3′,5,5′-tetrachloro-4,4′-dihydroxybiphenyl with 2,6-DCP. Further, these chlorophenol substrates reacted faster with HRP I than HRP II, which is usually the case with most peroxidase substrates [54]. These results clearly indicate that HRP II is directly capable of oxidatively dechlorinating 2,4,6-TCP and coupling 2,3,6-TCP and 2,6-DCP to biphenyl products. Given the fact that HRP II can only catalyze one-electron oxidations, the ability of this intermediate to catalyze the two electron oxidation of 2,4,6-TCP can only be the result of a mechanism involving two consecutive one-electron steps via a phenoxy radical intermediate.

3.6. Kinetics of the reactions of HRP I and HRP II with 2,4,6-TCP - Determination of the 2nd order rate constants

As mentioned above, kinetic traces for conversion of HRP I to HRP II as well as HRP II to ferric HRP were best fit to single exponential curves from the beginning [t = ~ 0.75 and ~ 50 ms (i.e., the earliest times of recording) after stopped-flow single mixing for HRP I and double mixing for HRP II, respectively] to the completion of the reaction even when the ratios of [2,4,6-TCP] /[HRP] were near or sub-stoichiometric (0.5–2.0). The only exception was for the reaction of 2,3,6-TCP with HRP II where two phases were observed (see Table 1, footnote d).

If a substrate is a one-electron donor, the reduction of HRP I to HRP II in the presence of 1 molar equiv. of substrate would be expected to completely consume the substrate at the end of the reaction. A single exponential mode under such conditions would not be expected. Yet the reaction proceeded as if the effective substrate concentration did not change during the entire reaction. Furthermore, the reduction of HRP II to ferric HRP (either with initial 1 molar equiv. of 2,4,6-TCP added to HRP I or 0.5 molar equiv. added to HRP II) proceeded also in a single exponential fashion at rates that are respectively 158 and 223-fold faster than the spontaneous conversion (Table 1).

A similar observation was reported in the reaction of lignin peroxidase compound I (LiP I) with thioanisole (and p-methoxylthioanisole) by Harvey and co-workers [55]. Using the rapid scan stopped-flow spectroscopic technique, the authors found that even under single turnover conditions (where stoichiometric amounts (0.75 μM) of pre-formed LiP I (ferric LiP + 1 equiv. H2O2 in the first mix) and the substrate were mixed), LiP II that was quickly formed upon reduction of LiP I by the substrate in the first phase, was converted to ferric LiP in a single exponential fashion in the second phase. A thioanisole radical(s) is presumably formed and used as substrate (reductant) in the second-phase reaction [55]. The LiP II to ferric LiP conversion rates under these conditions were ~ 20 times faster than the spontaneous decay of LiP II to ferric enzyme (in the absence of substrate).

The above results with HRP can be explained in part by assuming that (a) the initially formed phenoxy radical molecules are involved as an electron donor for HRP I and HRP II or (b) the radicals rapidly disproportionate to generate the two-electron oxidized quinone product and more phenol, effectively preventing a rapid decrease of the concentration of phenol. Fox and co-workers have reported that the phenoxy radical of 2,4,5-trichlorophenol decays very rapidly with a rate constant of $(7.7 \pm 1.7) \times 108\ M^{-1}\ s^{-1}$, presumably by disproportionation [56].

It should be pointed out that although a single exponential decay of HRP I to HRP II was still observed even in the presence of only 0.5 molar equiv. of 2,4,6-TCP ($k_{obs} = 56.6 \pm 5.5\ s^{-1}$), the subsequent second phase reaction for conversion of HRP II to ferric HRP occurred at the same rate as for the spontaneous reaction. This is to be expected since all of the substrate would have been consumed in the first phase and shows that 2,4,6-TCP cannot provide more than two electrons.

We have used the kinetic results for the conversion of HRP I to HRP II and of HRP II to ferric HRP in the initial presence of various molar equivalents (0.5–2.0) of 2,4,6-TCP (Table 1) to determine the second order rate constants for the reactions of 2,4,6-TCP with HRP I and HRP II.

When the observed rate constants (kobs) for all of these reactions are plotted against [2,4,6-TCP] /[HRP I] and [2,4,6-TCP]/[HRP II] values at t = 0 (Fig. 4A and B, respectively, where subscript 0 is added to their concentrations at t = 0), a good linear correlation is seen for the HRP I reaction in which the rate constants are proportional to [TCP]0/[HRP I]0 (bottom X-axis scale) or [TCP]0 (top X-axis scale) (Fig. 4A).1 For the HRP II case, a similar but slightly poorer linear correlation is observed when the origin (X = Y = 0) is included (Fig. 4B). Accordingly, the second order rate constants for the reaction of HRP I with 2,4,6-TCP (from the slope in Fig. 4A), 2,3,6-TCP and 2,6-DCP (based on the data for [chlorophenol]0/ [HRP I]0 = 2.0 for the latter two) were determined to be 3.2 × 107, 4.0 × 106 and 2.6 × 106 M− 1 s− 1, respectively. In a similar way, the corresponding second order rate constants for HRP II were calculated as ~ 1.4 × 105, ~ 1.8 × 105 and ~ 3.5 × 104 M− 1 s− 1, respectively.

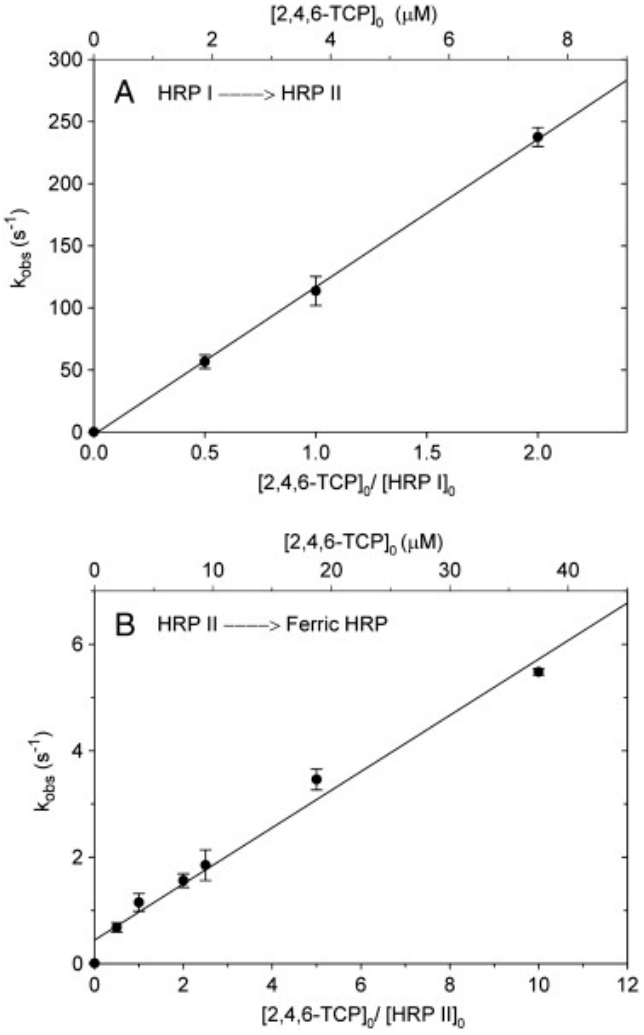


Fig. 4. Plots for observed pseudo-first rate constants vs. (A) [2,4,6-TCP]0/[HRP I]0 or (B) [2,4,6-TCP]0/[HRP II]0 (bottom abscissa scale) or [2,4,6-TCP]0 (top abscissa scale) for reduction of (A) HRP I and (B) HRP II with 2,4,6-TCP, where the subscripts “0” indicate the initial concentrations. [HRP I]0 = [HRP II]0 = 3.75 μM. Spontaneous reduction rates are plotted for [2,4,6-TCP]0 = 0 μM for the both panels. Data derived from Table 1, see Table footnotes for

additional reaction conditions. Straight lines shown are linear regression fits ($r^2 = 0.9994$ for A and 0.979 for B).

4. Conclusions

In summary, single turnover experiments show that both HRP I and HRP II are active catalysts for the oxidative dehalogenation of halophenol substrates. It has been previously proposed that HRP catalyzes this reaction by a mechanism involving two consecutive one-electron transfer steps via a phenoxy radical intermediate [20] and [21]. The data presented herein show that the reaction of HRP I with 2,4,6-TCP in the absence of excess H_2O_2 leads rapidly to HRP II and then more slowly to the ferric resting state. This constitutes direct and compelling evidence for a mechanism involving two consecutive one-electron oxidations of the substrate via a phenoxy radical intermediate. HRP II, which can only carry out one-electron oxidations, also directly dechlorinates 2,4,6-TCP. In addition, the reactions with 2,3,6-TCP and 2,6-DCP, in which the substrates are not dehalogenated, also appear to involve one-electron processes. All evidence points to a mechanism involving two consecutive one-electron steps for the oxidative dehalogenation of halophenols by HRP. Further development of HRP as a halophenol bioremediation catalyst will benefit from the more detailed understanding of the mechanism of oxidative halophenol dehalogenation described in this paper.

Abbreviations

HRP
Horseradish peroxidase
HRP I
HRP compound I
HRP II
HRP compound II
LiP
Lignin peroxidase
LiP I
LiP compound I
LiP II
LiP compound II
Cpd I
Compound I
Cpd II
Compound II
2,4,6-TCP
2,4,6-Trichlorophenol
2,3,6-TCP
2,3,6-Trichlorophenol
2,6-DCP
2,6-Dichlorophenol

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1 Under non-pseudo-first-order reaction conditions ([2,4,6-TCP]/[HRP II] = 0.5–2.0), the initial rates (vo = k[S]o[HRP I]o) need to be used to obtain the true second order rate constants (k). However, the slopes in these plots, where the “apparent” pseudo-first order rate constants were plotted vs. [S]o for the conversion of HRP I to HRP II (or HRP II to ferric HRP), would still give the same second order rate constants. Note that [S]o and [HRP I]o are substrate and enzyme concentrations at t = 0.