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Kinetics of Two-Electron Oxidations by the Compound I Derivative of Chloroperoxidase, a Model for Cytochrome P450 Oxidants

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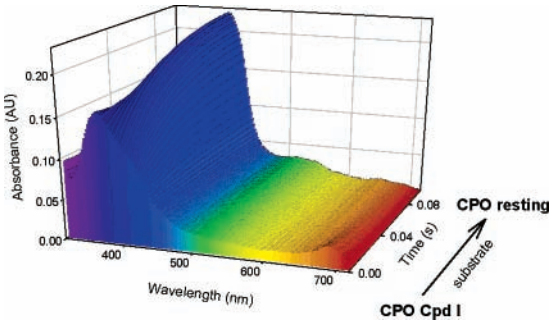
ABSTRACT



Rate constants for two-electron oxidation reactions of Compound I from chloroperoxidase (CPO) with a variety of substrates were measured by stopped-flow kinetic techniques. The thiolate ligand of CPO Compound I activates the iron–oxo species with the result that oxidation reactions are 2 to 3 orders of magnitude faster than oxidations by model iron(IV)–oxo porphyrin radical cations containing weaker binding counterions.

Enzyme-catalyzed oxidation reactions represent an area of considerable contemporary interest, and oxidations of unactivated C−H bonds to give alcohol products are of special interest. The ubiquitous cytochrome P450 enzymes (P450s) hydroxylate unactivated C−H bonds with facility, and these heme-containing enzymes serve as models for practical catalysts.[1−3] The true oxidants in P450s have not been observed under natural conditions, however, nor do they accumulate to observable amounts under cryogenic conditions when the requisite electrons are provided by γ-radiolysis[4,5] or by radioactive decay of phosphorus-32.[6]

The oxidant in a P450 enzyme is usually thought to be an iron(IV)−oxo porphyrin radical cation, termed Compound I, by analogy to the intermediates formed in peroxidase and catalase enzymes,[7,8] but differences exist between the latter

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(1) *Cytochrome P450 Structure, Mechanism, and Biochemistry*, 3rd ed.; Ortiz de Montellano, P. R., Ed.; Kluwer Academic: New York, 2005.
(2) Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H. *Chem. Rev.* **1996**, *96*, 2841−2887.
(3) Denisov, I. G.; Makris, T. M.; Sligar, S. G.; Schlichting, I. *Chem. Rev.* **2005**, *105*, 2253−2277.
(4) Davydov, R.; Macdonald, I. D. G.; Makris, T. M.; Sligar, S. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1999**, *121*, 10654−10655.
(5) Davydov, R.; Makris, T. M.; Kofman, V.; Werst, D. E.; Sligar, S. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **2001**, *123*, 1403−1415.
(6) Denisov, I. G.; Makris, T. M.; Sligar, S. G. *J. Biol. Chem.* **2001**, *276*, 11648−11652.
(7) Dawson, J. H. *Science* **1988**, *240*, 433−439.
(8) Hiner, A. N. P.; Raven, E. L.; Thorneley, R. N. F.; Garcia-Canovas, F.; Rodriguez-Lopez, J. N. *J. Inorg. Biochem.* **2002**, *91*, 27−34.

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enzymes and P450s. The oxidants in P450s are formed by a sequence of reduction, oxygen binding, and protonation steps instead of reaction with hydrogen peroxide, and P450 enzymes contain thiolate from cysteine as the fifth ligand to iron as opposed to nitrogen of histidine or oxygen of tyrosine. A more subtle difference is that the P450 enzymes are activated when substrate is bound in the active site, whereas substrates diffuse into the active sties of "activated" peroxidase and catalase enzymes. Compound I analogues in peroxidases and biomimetic porphyrin−iron models are relatively low reactivity oxidizing species, and the high reactivity of P450 oxidants typically has been ascribed to a counterion effect of the thiolate ligand that strongly activates the Compound I derivative by weakening the iron−oxygen bond.[2]

One peroxidase enzyme, chloroperoxidase (CPO) from *Caldariomyces fumago*, contains a cysteine thiolate ligand to iron.[9,10] CPO is the only known thiolate-heme enzyme that gives a well-characterized Compound I species, which has been studied by visible absorption,[11] Mössbauer,[12] resonance Raman,[13] EPR,[12] ENDOR,[14] and XAFS[15] spectroscopies. Compound I of CPO is often considered to be the best model available for the putative Compound I in P450 enzymes,[16] and it catalyzes two-electron, oxo-transfer oxidation reactions that mimic those catalyzed by P450 enzymes.[17,18] Limited kinetics of one-electron oxidations by CPO Compound I were reported,[19] and no kinetic information for two-electron oxidation reactions was available, however. We report here kinetic studies of reactions of CPO Compound I with various two-electron reductants that provide benchmark data. Most of the substrates studied are known to be oxidized to alcohols, epoxides and hypohalides by CPO under turnover conditions.[17,18,20] The general findings were that the rate constants for the CPO Compound I oxidation reactions are 2−3 orders of magnitude greater than those of models.

We isolated CPO from *C. fumago* and purified it by reported methods.[21,22] The enzyme purity was evaluated from the R/Z value (A_{400nm}/A_{280nm}), and CPO with $R/Z > 1.4$ was used. CPO was oxidized to the Compound I derivative in >95% yield as described[11] by using 1.5−2.0 equiv of commercial peroxyacetic acid (32%).[23] The reported rate constant for oxidation of resting CPO with peroxyacetic acid is ca. 4×10^6 M^{-1} s^{-1},[24] and comparable rate constants were observed here. Figure S1 in the Supporting Information shows typical UV−visible spectral changes upon oxidation of resting enzyme to the Compound I species.

The kinetics of CPO Compound I oxidation reactions were measured with a three-syringe, stopped-flow kinetic unit. The resting enzyme in 100 mM potassium phosphate buffer (pH 4.8) was mixed with the peroxyacetic acid solution. After a 100 ms delay, the solution containing CPO Compound I was mixed with a solution containing a large excess of substrate. Kinetics were monitored at 400 nm (growth of the Soret band of resting enzyme) or at 690 nm (decay of the Q-band of Compound I). A typical time-resolved spectrum and kinetic traces are shown in Figure 1.

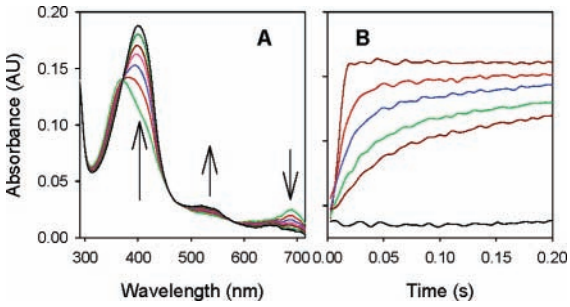


Figure 1. (A) Time-resolved UV−vis spectrum for reaction of CPO Compound I with 0.10 mM styrene over 330 ms. (B) Kinetic traces at 400 nm for reactions of CPO Compound I with styrene at (from the bottom) 0, 0.10, 0.20, 0.40, 0.8, and 1.6 mM concentrations.

In the presence of a large excess of substrate, CPO Compound I decayed with pseudo-first-order kinetics. Second-order rate constants were determined from eq 1 , where k_{obs}

$$k_{obs} = k_0 + k_{ox}[\mathrm{Sub}] \qquad (1)$$

is the observed pseudo-first-order rate constant, k_0 is the background first-order rate constant for decay in the absence of substrate, k_{ox} is the second-order rate constant, and [Sub] is the molar concentration of substrate. Plots of k_{obs} versus [Sub] typically gave straight lines with near-zero intercepts; examples are shown in Figure 2. We measured the kinetics in three or four sets of studies for each substrate with three independent kinetic runs in each set of studies, and the second-order rate constants are listed in Table 1.

Several interesting features are seen in the second-order rate constants. Many substrates were oxidized successfully

(9) Morris, D. R.; Hager, L. P. *J. Biol. Chem.* **1966**, *241*, 1763−1768.
(10) Sundaramoorthy, M.; Terner, J.; Poulos, T. L. *Structure* **1995**, *3*, 1367−1377.
(11) Palcic, M. M.; Rutter, R.; Araiso, T.; Hager, L. P.; Dunford, H. B. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1123−1127.
(12) Rutter, R.; Hager, L. P.; Dhonau, H.; Hendrich, M.; Valentine, M.; Debrunner, P. *Biochemistry* **1984**, *23*, 6809−6816.
(13) Egawa, T.; Proshlyakov, D. A.; Miki, H.; Makino, R.; Ogura, T.; Kitagawa, T.; Ishimura, Y. *J. Biol. Inorg. Chem.* **2001**, *6*, 46−54.
(14) Kim, S. H.; Perera, R.; Hager, L. P.; Dawson, J. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **2006**, *128*, 5598−5599.
(15) Stone, K. L.; Behan, R. K.; Green, M. T. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 16563−16565.
(16) Van Rantwijk, F.; Sheldon, R. A. *Curr. Opin. Biotechnol.* **2000**, *11*, 554−564.
(17) Zaks, A.; Dodds, D. R. *J. Am. Chem. Soc.* **1995**, *117*, 10419−10424.
(18) Hager, L. P.; Lakner, F. J.; Basavapathruni, A. *J. Mol. Catal. B: Enzym.* **1998**, *5*, 95−101.
(19) Lambeir, A. M.; Dunford, H. B.; Pickard, M. A. *Eur. J. Biochem.* **1987**, *163*, 123−127.
(20) Wagenknecht, H. A.; Woggon, W. D. *Chem. Biol.* **1997**, *4*, 367−372.
(21) Hollenberg, P. F.; Hager, L. P. *Methods Enzymol.* **1978**, *52*, 521−529.
(22) Hashimoto, A.; Pickard, M. A. *J. Gen. Microbiol.* **1984**, *130*, 2051−2058.
(23) The concentration of peroxyacetic acid was determined by iodometric titration.
(24) Araiso, T.; Rutter, R.; Palcic, M. M.; Hager, L. P.; Dunford, H. B. *Can. J. Biochem.* **1981**, *59*, 233−236.

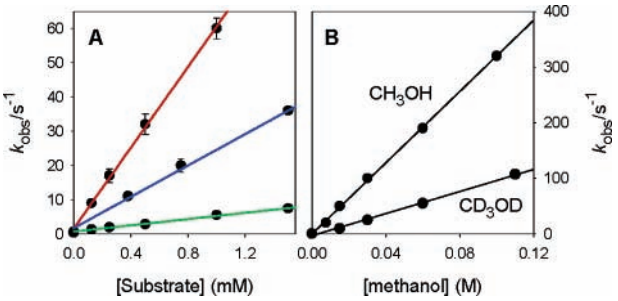


Figure 2. (A) Observed rate constants for reactions of CPO Compound I with styrene (red), cinnamyl alcohol (blue), and 2-methyl-1-heptene (green). (B) Observed rate constants for reactions of CPO Compound I with methanol.

by CPO Compound I, and it is apparent that access to the active site was possible for all but the larger substrates, *cis*-stilbene and diphenylmethane (entries 17 and 18). The seemingly large substrate 2-vinylnaphthalene displayed no retarding kinetic effect that might be ascribed to sterics, however (entry 12). The natural substrates chloride and bromide (entries 19 and 20) reacted very rapidly as expected. Chloride and bromide also are natural substrates for myeloperoxidase, and the rate constants for their reactions with myeloperoxidase Compound I at pH 7 are $k = 2.5 \times 10^4$ and 1.1×10^6 M^{-1} s^{-1}, respectively.[25]

Table 1. Second-Order Rate Constants for Reactions of CPO Compound I with Two-Electron Reductants[a]

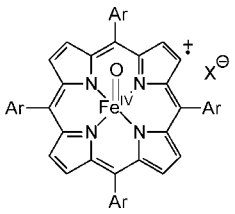
entry	substrate	k_{ox} (M^{-1} s^{-1})
1	toluene	$(5.5 \pm 0.2) \times 10^2$
2	ethylbenzene	$(9.6 \pm 0.2) \times 10^2$
3	ethylbenzene-d_{10}	$(3.7 \pm 0.5) \times 10^2$
4	methanol	$(3.2 \pm 0.1) \times 10^3$
5	methanol-d_4	$(9.9 \pm 0.1) \times 10^2$
6	cinnamyl alcohol	$(2.3 \pm 0.1) \times 10^4$
7	styrene	$(6.1 \pm 0.5) \times 10^4$
8	4-chlorostyrene	$(4.0 \pm 0.3) \times 10^4$
9	4-fluorostyrene	$(6.1 \pm 0.4) \times 10^4$
10	4-methylstyrene	$(6.7 \pm 0.6) \times 10^4$
11	4-methoxystyrene	$(8.3 \pm 0.5) \times 10^4$
12	2-vinylnaphthalene	$(1.1 \pm 0.1) \times 10^5$
13	2-methyl-1-heptene	$(4.3 \pm 0.4) \times 10^3$
14	2-methyl-2-heptene	$(3.6 \pm 0.2) \times 10^3$
15	10-undecenoic acid	$(6.4 \pm 0.5) \times 10^3$
16	lauric acid	ND[b]
17	*cis*-stilbene	ND[b]
18	diphenylmethane	ND[b]
19	chloride	$(2.5 \pm 0.1) \times 10^4$
20	bromide	$(3.8 \pm 0.1) \times 10^5$

[a] Reactions at 22 ± 1 °C in 100 mM phosphate buffer (pH 4.8). In the absence of substrate, the Compound I derivative decayed with a first-order rate constant of ca. 0.5 s^{-1}. [b] Not detected; no acceleration in the rate of decay of Compound I was observed with these substrates; see the Supporting Information.

Recent studies suggest that Compound II of CPO is relatively basic.[26] We evaluated pH effects on the reactivity of CPO Compound I. At six pH values in the range pH 3 to 7, we observed no effect on the rates of reaction of CPO Compound I with either styrene or methanol (see the Supporting Information).

In general, the CPO Compound I kinetic results indicate an expected high reactivity of the iron−oxo species due to the thiolate ligand. As shown by Raman spectroscopy,[27] the Fe−O bond strengths of Compound I species weaken as the counterion binding strength increases, which results in increased reactivity of iron−oxo species with stronger binding anions. The kinetic effect of the thiolate counterion in CPO Compound I is large. For example, the second-order rate constants for oxidations of ethylbenzene by the Compound I models **1**, **2**, and **3** are 1.6, 4.5, and 6.2 M^{-1} s^{-1}, respectively,[28,29] whereas CPO Compound I reacts with PhEt with a rate constant that is 2−3 orders of magnitude greater (entry 2). Similarly, the rate constant for epoxidation of styrene by CPO Compound I (entry 7) is 600 and 3200 times greater than the rate constants for oxidation of styrene by **2** and **1**, respectively.[28] A thiolate-substituted model porphyrin−iron complex was reported to oxidize substrates about 2 orders of magnitude faster than tetraphenylporphyrin−iron(III) chloride under turnover conditions,[30] but those rates of oxidation might reflect a combination of rates for oxidations of the catalysts by the sacrificial oxidants and substrate oxidation reactions.

The kinetic isotope effects observed for oxidations of nondeuterated and perdeuterated ethylbenzene and methanol (entries 2−5) reflect the high reactivity and concomitant low selectivity of the CPO Compound I species. For ethylbenzene, $k_H/k_D = 2.6$, which can be compared to a value of $k_H/k_D = 4.4$ for oxidations of ethylbenzene and ethylbenzene-d_{10} by the model Compound I species **3**.[29] For methanol, a similar small kinetic isotope effect was observed, i.e., $k_H/k_D = 3.2$.



1: Ar = $Me_3C_6H_2$, X = ClO_4
2: Ar = $Me_3C_6H_2$, X = Cl
3: Ar = F_5C_6, X = ClO_4

A further reflection of the high reactivity of CPO Compound I is seen in the linear Hammett plot for oxidations

(25) Furtmüller, P. G.; Burner, U.; Obinger, C. *Biochemistry* **1998**, *37*, 17923−17930.
(26) Green, M. T.; Dawson, J. H.; Gray, H. B. *Science* **2004**, *304*, 1653−1656.
(27) Czarnecki, K.; Nimri, S.; Gross, Z.; Proniewicz, L. M.; Kincaid, J. R. *J. Am. Chem. Soc.* **1996**, *118*, 2929−2935.
(28) Pan, Z.; Zhang, R.; Newcomb, M. *J. Inorg. Biochem.* **2006**, *100*, 524−532.
(29) Zhang, R.; Chandrasena, R. E. P.; Martinez, E., II; Horner, J. H.; Newcomb, M. *Org. Lett.* **2005**, *7*, 1193−1195.
(30) For studies with thiolate-ligated iron−porphyrin models, see: (a) Higuchi, T.; Uzu, S.; Hirobe, M. *J. Am. Chem. Soc.* **1990**, *112*, 7051−7053. (b) Higuchi, T.; Shimada, K.; Maruyama, N.; Hirobe, M. *J. Am. Chem. Soc.* **1993**, *115*, 7551−7552. (c) Urano, Y.; Higuchi, T.; Hirobe, M.; Nagano, T. *J. Am. Chem. Soc.* **1997**, *119*, 12008−12009. (d) Suzuki, N.; Higuchi, T.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 9622−9628.

of the series of substituted styrenes in entries 7−11 (Figure 3). For the model oxidant species **1** with a weakly binding

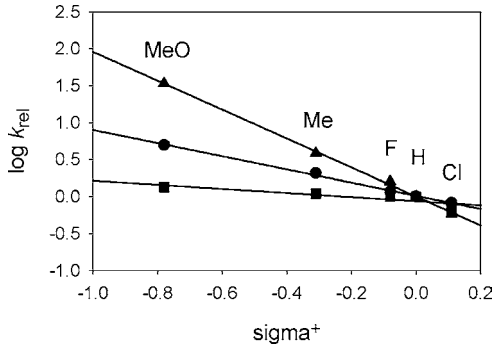


Figure 3. Hammett plots for reactions of para-substituted styrenes with the model Compound I species **1** (triangles), the model Compound I species **2** (circles), and CPO Compound I (squares). The data for species **1** and **2** are from ref 28.

counterion perchlorate, the ρ^+ value was −1.96,[28] which is similar to the values found with other Compound I species with weak binding counterions.[31,32] For model oxidant species **2** with the stronger binding counterion chloride, $\rho^+ = -0.89$,[28] again a value similar to that for other chloride complexed Compound I species.[32,33] For CPO Compound I, however, $\rho^+ = -0.28$, which is the smallest ρ^+ value yet found for oxo transfer reactions from porphyrin−metal−oxo species.

One of the more noteworthy aspects of the CPO Compound I kinetics is the fact that no reaction was apparent with lauric acid (entry 16). The successful oxidation of 10-undecenoic acid with a rate constant similar to that for oxidations of other alkenes (entries 13−15) suggests that the long hydrocarbon chain in this substrate, and by analogy the hydrocarbon chain of lauric acid, readily accessed the CPO Compound I active site. We observed no increase in the rate of decay of CPO Compound I species in the presence of lauric acid, however. A pseudo-first-order rate constant of $k_{obs} = 1\ s^{-1}$, which would result from a second-order rate constant for reaction of 1 mM laurate of $k_{ox} = 500\ M^{-1}\ s^{-1}$ as found for toluene, would have been measured readily in these studies. Thus, the limit for the free energy of activation for the oxidation of lauric acid is $\Delta G^{\ddagger} > 13.5$ kcal/mol. In contrast, low-temperature studies of camphor oxidations by $P450_{cam}$ suggest that functionalization of the unactivated C−H bond in the substrate has a small activation energy.[4−6]

An apparent attenuated reactivity of CPO Compound I in comparison to the P450 oxidants is indicated in our kinetic results in general. The second-order rate constants we obtained for reactions of CPO Compound I clearly are not diffusional rate constants, which would be found if the oxidation reactions were so fast that substrate could not leave the active site. If that were the case, then the measured second-order rate constants would be much greater than found here, substrates of similar size would react with the same rate constants, the slope of the Hammett plot in Figure 3 would be zero, and kinetic isotope effects of $k_H/k_D = 1.0$ would be found. In contrast, the catalytic sequence for P450 enzymes is triggered only after the substrate is bound in the active site, and the substrate does not escape from the active site after the oxidant is formed.

The kinetic results for CPO Compound I two-electron oxidations of organic substrates provide kinetic and energetic information that is relevant to the P450 oxidants. The thiolate ligand in CPO Compound I results in acceleration of oxidation reactions in comparison to Compound I models with weaker binding counterions,[28] but the reactivity of this transient apparently is not as great as that required for the true oxidants in P450s. The CPO results are similar to the finding in a recent study where a cytochrome P450 Compound I species produced via a photooxidation reaction from the Compound II species was found not to react with lauric acid even though laurate is a substrate for the P450 under catalytic turnover conditions.[34] One possible explanation for these results is that the active oxidant in P450 enzymes is not a Compound I species but a high-energy isomer of Compound I, i.e., a highly reactive iron(V)−oxo species.[35−37]

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Supporting Information Available: Experimental details, time-resolved UV−visible spectrum of the formation of CPO Compound I from reaction of the resting enzyme with peroxyacetic acid, and results of background control experiments and pH effect studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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(31) Groves, J. T.; Watanabe, Y. *J. Am. Chem. Soc.* **1986**, *108*, 507−508.
(32) Song, W. J.; Ryu, Y. O.; Song, R.; Nam, W. *J. Biol. Inorg. Chem.* **2005**, *10*, 294−304.
(33) Lindsay Smith, J. R.; Sleath, P. R. *J. Chem. Soc., Perkin Trans. 2* **1982**, 1009−1015.
(34) Newcomb, M.; Zhang, R.; Chandrasena, R. E. P.; Halgrimson, J. A.; Horner, J. H.; Makris, T. M.; Sligar, S. G. *J. Am. Chem. Soc.* **2006**, *128*, 4580−4581.
(35) Newcomb, M.; Chandrasena, R. E. P. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 394−403.
(36) Harischandra, D. N.; Zhang, R.; Newcomb, M. *J. Am. Chem. Soc.* **2005**, *127*, 13776−13777.
(37) Pan, Z.; Zhang, R.; Fung, L. W.-M.; Newcomb, M., Submitted for publication.