Compound I Formation Is a Partially Rate-limiting Process in Chloroperoxidase-catalyzed Bromination Reactions

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R. Daniel Libby* and Nicola S. Rotberg
From the Department of Chemistry, Colby College, Waterville, Maine 04901

The kinetics of chloroperoxidase-catalyzed bromination and chlorination reactions were studied at various halide and hydrogen peroxide concentrations. At very high concentrations, both chloride ($K_f = 370 \text{ mM}$) and bromide ($K_f = 150 \text{ mM}$) are competitive substrate inhibitors versus hydrogen peroxide. Results at subinhibitory halide concentrations for bromination reactions ($k_{cat} = 4 \text{ ms}^{-1}$, $K_{cat}/K_{peroxide} = 1.6 \mu M^{-1} \text{ s}^{-1}$) and for chlorination reactions ($k_{cat} = 1.5 \text{ ms}^{-1}$, $K_{cat}/K_{peroxide} = 2.3 \mu M^{-1} \text{ s}^{-1}$) indicate that halide oxidation is rate-limiting in chlorination reactions. However, in bromination reactions, both compound I formation and bromide oxidation are partially rate-limiting. This is the first documented case where compound I formation participates in determining the overall rate of a peroxidase reaction.

Chloroperoxidase (chloride:hydrogen-peroxide oxidoreductase, EC 1.11.1.10) is a peroxidase with an unusually broad range of catalytic properties. In addition to normal peroxidase reactions, chloroperoxidase can also chlorinate and brominate nucleophilic organic substrates, and it has significant catalase activity (1). Most studies of chloroperoxidase have focused on its halogenation reactions (2). The mechanistic sequence for halogenation reactions involves an initial reaction of the native enzyme with hydrogen peroxide forming an enzymatic oxidized form of chloroperoxidase reacts with halide ion to produce an electrophilic halogenating intermediate (Equation 1, $E$). This oxidized form of chloroperoxidase reacts with halide ion to produce an electrophilic halogenating intermediate (Equation 2, $E_{OX}$). Although there is considerable controversy as to the specific mechanism of halogen transfer, $E_{OX}$ is responsible for halogenation of the organic substrate (Equation 3, $RH$).

$$E + H_2O_2 \overset{k}{\rightarrow} E \cdot H_2O_2 \rightarrow E + H_2O \quad (1)$$

$$E + X^- \rightarrow E \cdot X^- \rightarrow \text{EOX} \quad (2)$$

$$\text{EOX} + RH + H^+ \rightarrow E + RX + H_2O \quad (3)$$

The reaction sequence (Equations 1–3) suggests a ping-pong kinetic mechanism, but it is only recently that evidence for a ping-pong binding sequence has been reported (4, 5).

The data support the sequence indicated above with hydrogen peroxide binding to the native enzyme and halide binding to compound I. However, detailed kinetic studies have been limited because chloroperoxidase reactions are subject to substrate inhibition by all peroxide and halide substrates. Most rate studies have utilized optimum substrate concentrations. Relative rates at optimum halide concentrations (I$^\cdot$ > Br$^\cdot$ > Cl$^\cdot$) parallel the relative ease of oxidation of the halide ions (1, 3). Also, Lambier and Dunford (6) presented kinetic evidence that the chloride oxidation step is rate limiting in the peracetic acid-supported chloroperoxidase-catalyzed chlorinization of 2-chlorodimedone. Consequently, the oxidation of the halide ion has been assumed to be the rate-limiting step in all halogenation reactions catalyzed by chloroperoxidase.

A recent study of chloride-dependent catechol peroxidation seemed to cast doubt on the rate-limiting character of the halide oxidation step. In chloride-independent peroxidation, catechol is directly oxidized by compound I. In the chloride-dependent reaction catechol is oxidized by $E_{OX}$ (Equation 3), but both the chloride-dependent and independent pathways produce the same apparent $k_{cat}$ for catechol peroxidation (5).

Since chloride-dependent and independent reactions share only the compound I formation sequence (Equation 1), that process must be rate-limiting under the conditions of the study. The data were collected at 2.0 mM hydrogen peroxide, its optimum concentration at pH 2.75. However, hydrogen peroxide oxidizes the substrate, so, at its optimum hydrogen peroxide concentration, the enzyme may be considerably below saturation with hydrogen peroxide. Thus, the compound I formation step could be rate-limiting due to low substrate concentration.

To clarify the energetics of chloroperoxidase catalyzed reactions, we have carried out a detailed kinetic study of the enzymatic chlorination and bromination of 2-chlorodimedone. The results of our study are summarized in this report.

MATERIALS AND METHODS

General—Rates for both chlorination and bromination reactions were determined by following consumption of 2-chlorodimedone spectrophotometrically at 278 nm using a molar absorptivity coefficient of 12,200 M$^{-1}$ cm$^{-1}$ (7). All experiments were carried out at 25 °C in a Shimadzu UV-160 1UV-VIS spectrophotometer.

Enzyme Preparations—Chloroperoxidase was provided by Professor Lowell P. Hager of the Department of Biochemistry at the University of Illinois, Urbana, and had been purified as described previously (8). The enzyme had a specific activity of 1400 units/mg and an $R_v$ value of 1.4 (1).

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†To whom correspondence should be addressed.

The abbreviations used are: $E$, chloroperoxidase compound I; $E \cdot H_2O_2$, chloroperoxidase-hydrogen peroxide Michaelis complex; $X^-$, chloride or bromide ion; $E \cdot X^-$, compound I-halide Michaelis complex; $E_{OX}$, chloroperoxidase halogeneration intermediate, $RH$, halogenation substrate; $RX$, halogenated product.

\begin{align*}
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\end{align*}
RESULTS AND DISCUSSION

Chloride ion exhibits substrate inhibition ($K_I = 370 \text{ mM}$) in chloroperoxidase halogenation and chloride-dependent peroxidatic reactions (5, 6). To avoid these effects, all of our studies utilized chloride concentrations of 20 mM or below. Bromide also produces substrate inhibition. Fig. 1 indicates that, at high concentrations, bromide is a competitive inhibitor ($K_I = 150 \pm 10 \text{ mM}$) versus hydrogen peroxide in the chloroperoxidase-catalyzed bromination of 2-chlorodimedone. As with chloride, the $K_I$ value for bromide is essentially identical to its dissociation constant for binding with native chloroperoxidase (11). Thus, as expected, bromide and chloride seem to have parallel binding and reaction behavior except that, in general, chloroperoxidase has a higher affinity for bromide than for chloride. To avoid complicating effects of bromide inhibition, bromide concentrations of 500 $\mu\text{M}$ or lower were used in our kinetic studies.

Chloroperoxidase-catalyzed chlorination and bromination reactions exhibit the parallel lines in double-reciprocal plots characteristic of a ping-pong kinetic mechanism (Figs. 2 and 3). Table I summarizes the kinetic parameters obtained from intercept replots for the chloroperoxidase-catalyzed chlorination and bromination of 2-chlorodimedone. The $K_M$ for bromination is higher than that for chlorination. Thus, the overall free energy of activation for the reaction of substrate-saturated enzyme is lower for bromination than for chlorination. Likewise, the $k_{cat}/K_{M,\text{halide}}$ values indicate that at very low substrate concentrations, the free energy of activation for binding and reaction of the halide ion is lower for bromination than for chlorination. Since the free energies of activation for the more easily oxidized bromide are lower than those for chloride, these results are consistent with halide oxidation being rate-limiting in chloroperoxidase halogenation reactions.

The similarity of the $k_{cat}/K_M$ values for the two reactions indicates that the identity of the halide has essentially no effect on the energetics of hydrogen peroxide binding and reaction. This lack of halide ion effect is consistent with the recently proposed ping-pong mechanism in which halide binding occurs only after hydrogen peroxide has reacted and released a product (4, 5). However, a comparison of the two $k_{cat}/K_M$ values for the reaction of each halide suggests that
there are differences between the energetics of chlorination and bromination reactions.

For ping-pong reaction mechanisms, the $k_{cat}/K_M$ for each substrate allows the independent analysis of each segment of the mechanism (13). The $k_{cat}$ values for chloroperoxidase reactions are sensitive to the rate constants for the first irreversible step in each half-reaction of the proposed ping-pong reaction mechanism.

$$k_{cat} = \frac{k_{21}k_3}{k_2 + k_3},$$

while $k_{cat}/K_{Halide}$ is only affected by events between halide binding and 2-chlorodimedone binding. (See “Appendix” for kinetic equations.

Consequently, the $k_{cat}/K_{Halide}$ will reflect the energetics of just the halide binding and oxidation (Equation 2) while $k_{cat}/K_{Peroxide}$ reflect the energetics of binding and reaction of hydrogen peroxide.

For the chlorination reaction, $k_{cat}/K_C$ is nearly a factor of 10 smaller than $k_{cat}/K_{Peroxide}$. Since rates of chloroperoxidase halogenation reactions are generally independent of the structure of the organic halogen acceptor substrate (14), the binding and reaction of 2-chlorodimedone (Equation 3) is most likely fast compared with the first two segments of the pathway. Thus, binding and reaction of chloride ion is the most difficult process in chlorination reactions. This relationship between $k_{cat}/K_{Peroxide}$ and $k_{cat}/K_C$ is consistent with the chloride oxidation step being rate-limiting (15, 16).

Analysis of the bromide reaction is less straightforward. In this case, $k_{cat}/K_B$ is a factor of 2 larger than $k_{cat}/K_{Peroxide}$. Thus, binding and reaction of hydrogen peroxide is more difficult than binding and reaction of bromide. However, the difference is small, so both peroxide and bromide reactions are partially rate-limiting in chloroperoxidase-catalyzed bromination processes. There is considerable controversy concerning the definition of the rate-limiting step of an enzymatic reaction (15, 16). Our data do not allow us to determine a specific step that can be called rate-limiting, but it certainly shows that the halide half-reaction is the rate-controlling process in chloroperoxidase chlorination of 2-chlorodimedone and that both half-reactions are partially rate-controlling in the bromination processes.

Data for peroxidases from a variety of sources are consistent with the rate-limiting steps for halogenation reactions (1, 3, 4, 6), halide oxidation reactions (17, 18), oxygen transfer reactions (19), and peroxidatic reactions (20-23) occurring in the half-reaction involving reduction of compound I. Thus, it has been generally assumed that for all peroxidase reactions compound I formation is fast relative to other steps. This is the first documented example of a peroxidase reaction in which compound I formation has been shown to participate in determining the overall rate of the reaction.

If the trend from chlorination to bromination continues, compound I formation could be totally rate-limiting in iodide-dependent chloroperoxidase-catalyzed reactions. However, direct kinetic studies of iodide reactions catalyzed by chloroperoxidase are difficult because iodide competes very favorably with the organic substrate thus producing molecular iodine as the initial product (24, 25). Molecular iodine reacts with free iodide to form triiodide ion which is essentially unreactive toward most halogenation substrates. Under proper conditions, the kinetics of the iodide reaction could be studied by spectrophotometrically determining the rate of triiodide formation. However, earlier kinetic studies indicate that the $K_M$ for iodide in chloroperoxidase reactions is probably lower than the $K_M$ for bromide in bromination reactions (1.0 mM, see Table I) (24, 25). Thus, at the low iodide concentrations necessary to obtain accurate saturation kinetics of the chloroperoxidase-catalyzed reactions, significant concentrations of both molecular iodine and triiodide would be present and the proportion of the two species would vary over the course of the reaction even under stopped flow conditions. So spectrophotometric studies would not be practical.

### APPENDIX

The mechanism illustrated in Equations 1–3 is a uni-unibidi-ping-pong process. It is a minimal kinetic mechanism in which steps 3, 5, and 9 most likely represent multi-step processes. If the product release steps (3 and 9) are assumed to be unidirectional under initial velocity conditions, the following initial velocity expression describes the steady state initial velocity kinetic behavior predicted by the mechanism:

$$v_0 = \frac{E}{[H_2O_2][X][I]} \left( [K_{cat}[H_2O_2][X][I] + K_{halide}[H_3O_3][I] + K_{Peroxide}[I][R][H] + [H_2O_2][X][I][R]] \right)$$

where

$$k_{cat} = \frac{k_{21}k_3}{k_2 + k_3}, \quad K_{RH} = \frac{k_{21}k_3}{k_2[H^+](k_3 + k_3)}, \quad K_{Halide} = \frac{k_3(k_3 + k_3)}{k_3(k_3 + k_3)}$$

and

$$K_{Peroxide} = \frac{k_2(k_2 + k_2)}{k_2(k_3 + k_3)}.$$

The above expressions then yield

$$\frac{k_{cat}}{K_{Halide}} = \frac{k_{cat}}{K_{Halide}} = \frac{k_{cat}}{K_{Halide}} = \frac{k_3}{k_3 + k_3}$$

and

$$\frac{k_{cat}}{K_{Peroxide}} = \frac{k_{cat}}{K_{Peroxide}} = \frac{k_{cat}}{K_{Peroxide}} = \frac{k_2}{k_2 + k_2}.$$
REFERENCES

1. Hager, L. P., Morris, D. R., Brown, F. S., and Eberwein, H. (1966) J. Biol. Chem. 241, 1769-1777
2. Hewson, W. D., and Hager, L. P. (1979) in The Porphyrins (Dolphin, D., ed) Vol. 7, pp. 295-332, Academic Press, New York
3. Libby, R. D., Thomas, J. A., Kaiser, L. W., and Hager, L. P. (1982) J. Biol. Chem. 257, 5080-5087
4. Dunford, H. B., Lambier, A. M., Kashan, M. A., and Pickard, M. (1987) Arch. Biochem. Biophys. 252, 292-302
5. Libby, R. D., Rotberg, N. S., Emerson, J. T., White, T. C., Yen, G. M., Friedman, S. H., Sun, N. S., and Goldweski, R. (1989) J. Biol. Chem. 264, 15984-15992
6. Lambier, A. M., and Dunford, H. B. (1983) J. Biol. Chem. 258, 13558-13563
7. Morris, D. R., and Hager, L. P. (1966) J. Biol. Chem. 241, 1763-1768
8. Palcic, M. M., Rutter, R., Ariaso, T., Hager, L. P., and Dunford, H. B. (1980) Biochem. Biophys. Res. Commun. 94, 1123-1127
9. Cotton, M. L., and Dunford, H. B. (1973) Can. J. Chem. 51, 582-587
10. Cleland, W. W. (1967) Adv. Enzymol. 29, 1-92
11. Sono, M., Dawson, J. H., Hall, K., and Hager, L. P. (1986) Biochemistry 25, 347-356
12. Cleland, W. W. (1982) Methods Enzymol. 87, 390-406
13. Gates, C. A., and Northrop, D. B. (1984) Biochemistry 23, 3834-3842
14. Thomas, J. A., Morris, D. R., and Hager, L. P. (1970) J. Biol. Chem. 245, 3129-3134
15. Northrop, D. B. (1981) Annu. Rev. Biochem. 50, 103-131
16. Ray, W. J. (1983) Biochemistry 22, 4625-4631
17. Ronsen, R., and Dunford, H. B. (1972) Biochemistry 11, 2076-2082
18. Bjorksten, F. (1970) Biochim. Biophys. Acta 212, 396-406
19. Kobayashi, S., Nakano, M., Kimura, T., and Schaap, A. P. (1987) Biochemistry 26, 5019-5022
20. Corbett, M. D., Baden, D. G., and Chipko, B. R. (1979) Bioorg. Chem. 8, 91-95
21. Rönberg, M., Ariaso, T., Ellfolk, N., and Dunford, H. B. (1981) Arch. Biochem. Biophys. 207, 197-204
22. Kalyanaranman, B., Felix, C. C., and Sealy, R. C. (1984) J. Biol. Chem. 259, 7584-7589
23. Nakamura, M., Yamasaki, I., Kotani, T., and Ohtaki, S. (1985) J. Biol. Chem. 260, 13546-13552
24. Thomas, J. A., and Hager, L. P. (1968) Biochem. Biophys. Res. Commun. 32, 770-775
25. Thomas, J. A., and Hager, L. P. (1969) Biochem. Biophys. Res. Commun. 35, 444-450