Quantitating Direct Chlorine Transfer from Enzyme to Substrate in Chloroperoxidase-catalyzed Reactions*

(Received for publication, March 6, 1996, and in revised form, June 18, 1996)

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Substrate competition methods that were previously used to quantify the involvement of free Cl2 in the chloride-dependent peroxidatic reactions catalyzed by chloroperoxidase (CPO) (Libby, R. D., Shedd, A. L., Phipps, K. A., Beachy, T. M., and Gerstberger, S. M., (1992) J. Biol. Chem. 267, 1769–1775) are extended to CPO-catalyzed halogenation reactions. Relative substrate specificities of halogen acceptor substrates (RHS) antipyrine (ap), NADH, 2-chlorodimedone (2cd), and barbituric acid (ba) are compared with previously studied peroxidatic substrates catechol (cat) and 2,4,6-trimethylphenol (tmp) in their reactions with the CPO-H2O2-Cl system versus the hypochlorite-Cl system. Studies were carried out at pH 2.75 over a chloride concentration range of 1–100 mM and at pH 4.80 over a chloride concentration range of 300–400 mM. Competition studies involved successive pairwise comparisons of substrates of increasing enzyme specificity. The orders of specificities, ba > 2cd > ap > cat > tmp at pH 2.75 and ba > 2cd > NADH > ap > cat > tmp at pH 4.80, are the same for both the CPO-H2O2-Cl and hypochlorite-Cl systems. However, the magnitudes of the specificities are different between the two systems. In all comparisons except ap versus cat, the specificity of the CPO-H2O2-Cl system toward the preferred substrate is higher than that of the hypochlorite-Cl system. Quantitative comparisons between specificities of CPO-H2O2-Cl and hypochlorite-Cl systems indicate that at least 98% of the CPO-catalyzed halogenation reactions of ba, 2cd, NADH, and ap occur by mechanisms in which the substrate reacts directly with the enzyme. Thus, less than 2% of any of the CPO reactions could possibly involve a free oxidized halogen intermediate. All data are consistent with a mechanism in which RH binds to the CPO chlorinating intermediate (EOCl), and the chlorine atom is transferred directly from EOCl to RH. Further, the results indicate that any halogenation substrate with a higher CPO specificity than ap must also undergo direct chlorine transfer from the enzyme.

These results underscore the critical need for quantitative kinetic evidence in establishing the extent of involvement of any potential reaction intermediate. Finally, this work calls into question the long held assumption of the obligatory involvement of hypochlorite as an intermediate in myeloperoxidase reactions. It supports the recent kinetic evidence presented by

* This work was supported by NIGMS, National Institutes of Health, Grant 7 R15 GM42078-02. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: CPO, chloroperoxidase; tmp, 2,4,6-trimethylphenol; ba, barbituric acid; ap, antipyrine; cat, catechol; 2cd, 2-chlorodimedone; Met, methionine; ps, preferred substrate, which is preferentially consumed in a competition reaction; cs, competitive substrate, which is not preferred in a competition reaction; as, absorbing substrate, whose absorbance change was used to monitor the course of a reaction; AH2, peroxidatic substrate; RCl, halogenated product; RH, halogenation substrate; RCI, halogenated product; EOCI, enzymatic chlorinating intermediate; Prodcs, product of the competitive substrate; Prodps, product of the competitive substrate; [ps]i, initial concentration of the preferred substrate; [cs]i, initial concentration of the competitive substrate; [cs]o, concentration of the competitive substrate consumed in the nonenzymatic reaction (Scheme II, step 8); [ps]o, concentration of the preferred substrate consumed in the nonenzymatic reaction (Scheme II, step 10); [ps]n, concentration of the competitive substrate consumed in direct reaction with the enzyme (Scheme II, step 8); [ps]e, concentration of the preferred substrate consumed in direct reaction with the enzyme (Scheme II, step 7); e, molar absorption coefficient; (cat), reaction mixture containing cat as the only acceptor substrate; (ap + cat), reaction mixture containing ap and cat; (ps + cs), reaction mixture containing the preferred and competitive substrates; fcs, fraction of the preferred substrate that is consumed through direct reaction with the enzyme versus its consumption and free Cl2 production (Scheme II, step 7 versus steps 8 + 9); fcs, fraction of the preferred substrate that is consumed through direct reaction with the enzyme versus its consumption and free Cl2 production (Scheme II, step 8 versus steps 8 + 9); fcs, the fraction of the experimental determined fraction of the preferred substrate consumed in CPO-catalyzed reactions with respect to the total amount of reaction (Scheme II, steps 7 + 10 versus steps 7 + 8 + 9); fcs, the fraction of the experimental determined fraction of the preferred substrate consumed in nonenzymatic reactions with respect to the total amount of reaction (Scheme II, step 10 versus steps 10 + 11); % ENZ, the percentage of an enzymatic reaction that involves direct reactions between the enzyme and all substrates present (Scheme II, steps 7 + 8 versus steps 7 + 8 + 9); % Cl2, the percentage of an enzymatic reaction that involves a free oxidized chlorine species (Scheme II, step 9 versus steps 7 + 8 + 9); fcs, the fraction of total enzymatic reaction flux that passes through the Cl2 pathway (Scheme II, steps 7 + 8 versus steps 7 + 8 + 9).
nation of a variety of organic substrates (peroxidatic and halogenation reactions), CPO also catalyzes the dismutation of hydrogen peroxide (catalytic reaction), chlorination and bromination reactions, and some cytochrome P450 monooxygenase reactions (2-8). The mechanisms of CPO-catalyzed chlorination reactions have been studied for more than 30 years; however, there is still considerable controversy over how the chlorine becomes attached to the organic substrate. Although there is general agreement on the sequence of enzymatic intermediates outlined in Scheme I, the mechanism by which step 5 occurs is still under debate. Some reports favor direct transfer of the chlorine atom from the enzyme to the substrate (Scheme II, step 7 and 8) (9–11), while others argue for the intermediacy of a nonenzymic halogenating intermediate (Scheme II, steps 9–11) (12-14). The former conclusions were based upon kinetic and substrate specificity studies. The latter conclusions were based upon similarities between products formed in enzymatic reactions and those from corresponding reactions of hypochlorite model reactions. Substrates previously used on each side of the controversy have been different. Thus, it seems possible that the extent of involvement of a nonenzymic halogenating intermediate might be controlled by the particular acceptor substrate used. Our recent report used direct substrate competition studies to quantitate the involvement of free Cl2 in the chloride-dependent CPO-catalyzed peroxidatic reactions of cat and tmp. That work proved that the quantitative determination of the involvement of a free oxidized chlorine intermediate is possible (1). However, the reactions studied, the chloride-dependent peroxidation of cat and tmp, do not produce halogenated products (15). In this paper, we report the extension of our substrate competition studies from peroxidatic substrates to the good halogen acceptor substrates that were previously reported to involve free oxidized chlorine species in their CPO-catalyzed chlorination reactions (12-14). Our results conclusively settle the controversy over the mechanism of chlorine atom transfer from CPO to good halogen acceptor substrates.

EXPERIMENTAL PROCEDURES

Enzyme Preparations

The CPO used in these studies was produced and purified in our laboratories as described previously (16). The enzyme had a specific activity of 3400 units/mg and an RZ value of 1.4 (8). Concentrations of CPO solutions were determined from their absorbance at 398 nm using a molar absorption coefficient of 85,000 M−1 cm−1.

Spectral Measurements

All uv-visible absorption measurements were determined in a Shimadzu UV-160 spectrophotometer using 1-cm path length quartz cuvettes.

Error Analysis

The standard deviations in all functions, F, reported in Tables I and II were estimated using the following equation (17).

$$S.D. = \left( \frac{\sum (F - \bar{F})^2}{n-1} \right)^{1/2}$$  (Eq. 1)

The partial derivatives of each function with respect to each experimentally determined absorbance value, δF/δAi, were determined from the function using the Mathematica system run on a Macintosh IIsi computer system (18). The variance values, S2 Ai, were determined directly from experimentally observed absorbance values, Ai.

Reagents

All hydrogen peroxide solutions were prepared from 40% hydrogen peroxide, Alfa Products Reagent. Concentrations of solutions were determined by the method of Cotton and Dunford (19). Hypochlorite solutions were prepared from Clorox bleach and assayed by their ability to oxidize iodide ion under the conditions described by Cotton and Dunford (19). 2dd was synthesized as described previously (8). NADH, disodium salt (approximately 98%) was from Sigma; ap (98%), ba (99%), cat (>99%), and tmp (97%) were from Aldrich. All other reagents were of the highest quality available from commercial sources. Water used in these studies was purified by reverse osmosis and then further purified by passage through two activated charcoal columns, two deionizer columns, and a 0.2-μm filter using a Barnstead Nanopure II system.

Substrate Competition Studies

General Considerations—The relative specificities of enzymatic and nonenzymatic reactions toward the substrates were determined by direct pairwise competition studies under conditions in which the oxidizing reagent was limiting (1). For each combination of substrates, the chloride-dependent CPO-catalyzed reaction was compared with the nonenzymatic reaction of the substrates with hypochlorite in the presence of chloride. Both sets of reactions were carried out in 100 mM phosphate buffer, at either pH 2.75 and 1–100 mM chloride or pH 4.80 and 100–400 mM chloride. The concentrations of the organic substrates were varied depending upon their relative reactivities toward the enzyme system, but in all cases, the ratio of concentrations of a particular pair of substrates in any given competition reaction was the same for both the enzymatic and nonenzymatic systems. Also, the initial concentration of oxidant was always one-half of that of the lower concentration substrate. Thus, in all competition reactions no more than one-half of any substrate was consumed. The enzymatic system utilized 50 nM CPO and H2O2 as the oxidizing reagent. The H2O2 was completely consumed within 10 s. The nonenzymatic system utilized hypochlorite as the oxidizing reagent. The hypochlorite was completely consumed within 10 s. Reactions were monitored spectrophotometrically. The absorbance characteristics of cat and tmp allowed direct independent determinations of each substrate in the same reaction mixture (see below). Other substrates did not provide appropriate absorbance changes to allow direct independent determination of both substrates in the same reaction mixture. However, it was possible to monitor reactions of pairs of substrates other than cat and tmp by determining the change in absorbance due to the reaction of one substrate, the absorbing substrate. Our experimental data consist of determinations of the total reaction flux and the part of the total reaction flux attributable to the absorbing substrate. The relative specificities of substrates are expressed as fractions of total reaction flux attributable to the preferred substrate rather than as ratios of amounts of substrates consumed in each reaction as they were previously (1). It was generally assumed that the rate of reaction of each substrate is proportional to the initial concentration of the substrate (see "Results"). Thus, Equation 2 is the general equation for calculating the fraction of reaction flux attributable to the preferred substrate in a competition between any two substrates.

$$f_{io} = \frac{\Delta[ps]}{\Delta[ps] + \Delta[cs]}$$  (Eq. 2)

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The specific methods for determining the total reaction flux and the part of the total reaction flux attributable to the preferred substrate varied somewhat with each pair of competing substrates; thus, the particular equations used varied somewhat from reaction to reaction (see Appendix).

Reactions Involving cat—When cat was used as a substrate in CPO reactions, its change in concentration was corrected for the amount of cat passing through the chloride-independent reaction (Scheme I, step 2) using control reactions containing Met as described previously (1) (see Appendix).

cat versus tmp Reactions—Amounts of cat consumed were determined directly from absorbance increases of reaction mixtures at 388 nm using a molar absorption coefficient of 1300 M−1 cm−1. Amounts of tmp consumed were determined from absorbance decreases of reaction mixtures at 260.5 nm using a molar absorption coefficient of 1400 M−1 cm−1. Reactions of cat exhibit an isosbestic point at 260.5 nm, and reactions of tmp produce no change in absorbance at 388 nm, so in reaction solutions containing both substrates, the reaction of each can be observed separately (1). cat reaction flux through the CPO halide-independent path (Scheme I, step 2) was determined from absorbance increases at 388 nm of parallel reaction mixtures containing the same initial concentration of oxidant but with both cat and Met as substrates (1).

ap versus cat—Consumption of the competitive substrate, cat, was determined from absorbance increases at 388 nm of reaction mixtures containing cat and ap. Total reaction flux was determined from absorb-
ance increases at 388 nm of parallel reaction mixtures containing the same initial concentration of oxidant but only cat as substrate. cat reaction flux through the CPO halide-independent path was determined as described above for cat versus tmp reactions. Consumption of ap was taken as the difference between the corrected consumption of cat in the absence and presence of ap (see Appendix, Equations 7–10).

2cd versus ap—Consumption of the preferred substrate, 2cd, was determined from absorbance decreases at 278 nm of reaction mixtures containing 2cd and ap (8). The total reaction flux was determined from absorbance decreases at 278 nm of parallel reaction mixtures containing the same initial concentration of oxidant but only 2cd as substrate (see Appendix, Equations 11–13).

ba versus 2cd—Consumption of the competitive substrate, 2cd, was determined from absorbance decreases at 278 nm of reaction mixtures containing ba and 2cd. Total reaction flux was determined from absorbance decreases at 278 nm of parallel reaction mixtures containing the same initial concentration of oxidant but only 2cd as substrate. Consumption of ba was taken as the difference between the consumption of 2cd in the absence and presence of ba (see Appendix, Equations 14–16).

NADH versus ap—Consumption of the preferred substrate, NADH, was determined from absorbance decreases at 340 nm of reaction mixtures containing NADH and ap. Total reaction flux was determined from absorbance decreases at 340 nm of parallel reaction mixtures containing the same initial concentration of oxidant but only NADH as substrate (see Appendix, Equations 11–13).

2cd versus NADH—Consumption of the competitive substrate, NADH, was determined from absorbance decreases at 340 nm of parallel reaction mixtures containing the same initial concentration of oxidant but only NADH as substrate. Consumption of 2cd was taken as the difference between the consumption of NADH in the absence and presence of 2cd (see Appendix, Equations 14–16).

ba versus NADH—Consumption of the competitive substrate, NADH, was determined from absorbance decreases at 340 nm of reaction mixtures containing ba and NADH. Total reaction flux was determined from absorbance decreases at 340 nm of parallel reaction mixtures containing the same initial concentration of oxidant but only NADH as substrate. Consumption of ba was taken as the difference between the consumption of NADH in the absence and presence of ba (see Appendix, Equations 14–16).

RESULTS

Approach to Calculations—Previously, we used competition studies between cat and tmp to show that CPO can produce kinetically significant amounts of free Cl₂ (1). Those studies compared the relative specificities of the CPO-H₂O₂-Cl and hypochlorite-Cl systems toward 1:1 mixtures of cat and tmp. Since tmp was shown to react only with free Cl₂ and not directly with the enzyme (1, 20), we were able to use the relative specificity data to determine the percentage of the total reaction flux of the cat reaction that passes through the Cl₂ pathway. The extent of Cl₂ pathway involvement in the CPO-catalyzed reaction of cat increases with chloride concentration, reaching essentially 100% above 80 mM chloride. We had hoped to use tmp as a competing substrate to quantitate any Cl₂ pathway involvement in CPO-catalyzed reactions of other substrates previously proposed to react entirely by the Cl₂ pathway. However, the difference in specificity between tmp and all other substrates, which have very low Kₘ values in their CPO-catalyzed reactions, made direct comparisons of these substrates experimentally impossible. In competition toward each of these substrates, tmp was not measurably consumed in either the CPO-H₂O₂-Cl or the hypochlorite-Cl system.

As illustrated in the Appendix, Equations 26 and 27, the extent of involvement of the direct chlorine transfer and the free Cl₂ pathways (Scheme II, step 9 versus 7 + 8, respectively) cannot be directly determined from two-substrate competition studies unless one substrate, such as tmp as in the Appendix, Equation 32, is known or assumed to have no direct reaction with the enzyme (i.e. Scheme II, step 8, is not available). However, the required information can be gleaned by linking each substrate to tmp through successive comparisons of substrates of ever increasing specificities in their CPO-catalyzed reactions (see Tables I and II).

Assumptions—Our previous work with the halide-dependent CPO-catalyzed peroxidation of cat and tmp demonstrated that, at very high chloride concentrations, the relative specificities of the CPO-H₂O₂-Cl system are identical to those of the hypochlorite-H₂O₂ system. Thus, we can be confident that we have an appropriate model for the nonenzymatic halogenating intermediate that CPO is capable of producing. However, determination of the values in Tables I and II, required two assumptions. 1) The rates of all reactions, both enzymatic and nonenzymatic, of a substrate are directly proportional to the initial concentration of the substrate. 2) fₑₑₑ and fₑₑ are independent of the presence or identity of any other substrate.

Assumption 1 was necessary, because, even with successive
comparisons, substrate specificities vary so much that higher concentrations of the competing substrate than of the preferred substrate were required to determine the relative specificities precisely. Certainly, assumption 1 is reasonable for all nonenzymatic reactions. However, in the CPO-catalyzed reactions, concentrations of substrates are generally above their Km values. Thus, one might be concerned that the relative rates of direct enzymatic reactions could be largely independent of the relative substrate concentrations. We believe that assumption 1 is warranted in this study for two reasons. 1) The purpose of this study is to quantitate the possible involvement of the Cl2 pathway in CPO reactions. Assumption 1 clearly applies to the competition of substrates in nonenzymatic reactions with Cl2, whether its origin is an enzymatic or nonenzymatic system. Consequently, if a significant proportion of the reaction flux of a CPO reaction involves the Cl2 pathway, there would be no problem. 2) The Vmax values of chloride-dependent reactions of all substrates used in this study are independent of the identity of the substrate (9, 10, 22, 23). Thus, if relative reactivities were disproportionately controlled by Vmax, the fps-cpo values would tend toward 0.5; i.e. the specificity of the enzymatic reaction toward the preferred substrate would be reduced compared with the nonenzymatic system. With all substrates the fps-cpo values are much higher than 0.5, and for all except ap, the enzymatic specificity for the preferred substrate is higher than that of the hypochlorite-Cl system. Thus, some of the reaction flux must involve a direct reaction between the enzyme and organic substrate. Further, the reaction on the enzyme surface must have a higher specificity toward the preferred substrate than does the hypochlorite-Cl reaction. Since assumption 1 seems to apply to substrates with higher enzymatic specificities than ap, it seems unlikely that ap reactions would be more significantly controlled by Vmax than those of the higher specificity substrates. Thus, we believe that assumption 1 does not introduce any significant error into our determinations.

Assumption 2 was required to provide the mathematical link between each substrate and the one substrate, tmp, that does not react directly with the enzyme (1, 20). Since fpsE compares the reaction of Scheme II, step 7, with the sum of steps 7 and 9, it depends only upon the concentrations of the organic substrate and chloride. The presence of a competing substrate, which could react through Scheme II, steps 8 and 11, can only decrease the effective concentration of EOCl but not the ratio between the flux passing through Scheme II, step 7 versus that utilizing step 9. Also, if the same substrate is the competing substrate, its fcat, ratio of Scheme II, step 8 to step 9, represents its competition with chloride and therefore is identical to its fpsE. Thus, the fpsE determined for cat in competition with tmp can be used as fcat for cat in its competition with ap. The fpsE for ap can in turn be used as fcat, in the ap competition with 2cd and so on (see Tables I and II and Appendix, Equation 31).

Percentage of CPO Reaction Flux Involving Direct Reaction between Enzyme and Substrate—Since the extent of involvement of free Cl2 is vanishingly small, we have reported our data in terms of the percentage of the total reaction flux of a pair of substrates involving direct reaction between enzyme and substrates, % ENZ (see Tables I and II). The reactions were studied at pH 2.75 and 4.8, the pH values at which most previous studies were done (9–14). Chloride concentrations were varied to determine the possible effects of chloride concentration on the proportion of the reaction involving direct chlorine transfer between the enzyme and the organic substrate. Higher chloride concentrations were used at pH 4.8 because of the lower affinity of CPO for chloride ion at this pH versus pH 2.75 (8). CPO-H2O2-Cl specificities, fps-cpo values, and % ENZ values for the cat competition with tmp are strongly chloride-dependent. However, none of the values for any other reaction was significantly affected by chloride concentration, and all other % ENZ values are essentially 100.

The orders of relative reactivity, ba > 2cd > ap > cat > tmp at pH 2.75 and ba > 2cd > NADH > ap > cat > tmp at pH 4.8, are the same for both CPO-H2O2-Cl and hypochlorite-Cl reactions; however, with the exception of the ap versus cat competition, the specificity of CPO-H2O2-Cl for the preferred substrate is higher than that of the corresponding hypochlorite-Cl reactions. All reactions containing ba, 2cd, NADH, or ap occur essentially 100% by direct reaction between the enzyme and substrate as indicated by their fpsE values of essentially 1.0.

The ap reaction specificity at pH 4.8 was affected by chloride concentration. However, at 100 mM chloride, an optimum chloride concentration at pH 4.8, essentially 100% of the ap reaction flux involves direct reaction between enzyme and substrate. And even at very high chloride concentrations, the extent of direct reaction is still 90% for the ap-cat pair (see Table II).

DISCUSSION

Earlier work has clearly established that chloride becomes productively involved in CPO reactions only after the native enzyme has been oxidized to the compound I intermediate (15, 21, 22). However, the exact course of chloride involvement has been a topic of considerable controversy (9–14). All of our data are consistent with the mechanism for chloride involvement indicated in Scheme II in which chloride competes with organic substrates for reaction with EOCI. Thus, very low Km substrates effectively exclude chloride from reaction with EOCI and therefore eliminate involvement of any free oxidized halogen species in their CPO-catalyzed reactions.

This study definitively establishes that essentially all chlorine transfer in CPO-catalyzed reactions of ap, NADH, 2cd, and ba occur directly from enzyme to substrate without any involve-
ment of a free oxidized chlorine intermediate. It is probable that some species such as hypochlorite does form on the enzyme, but the specificity data clearly indicate that the enzyme must be directly involved in the actual reaction of the organic substrate. The parallel specificities between the CPO-H$_2$O$_2$-Cl and hypochlorite-Cl systems support previous suggestions that EOCI may be an iron hypochlorite species such as that illustrated in Scheme II, EOCI (10). Thus, it is not surprising that products of CPO-catalyzed chlorination reactions are the same as those produced by corresponding hypochlorite-Cl reactions (12–14).

The lack of significant effects of chloride concentration on either the $f_{\text{ps-cpo}}$ or % ENZ values for ap, NADH, 2cd, and ba is consistent with their $f_{\text{ps-cl}}$ values of essentially 1.0. An $f_{\text{ps-cl}}$ value of 1.0 indicates that the reaction flux through step 9 of Scheme II is essentially 0. Thus, the substrate completely excludes chloride from reacting with EOCI. These results further predict that any substrate that is more preferred by the CPO-H$_2$O$_2$-Cl system than is ap should also completely exclude chloride from EOCI and thus have an $f_{\text{ps-cl}}$ value of 1.0. Consequently, this work establishes a very simple method for determining the substrate than that for ap, 2cd, NADH, or ba would exclude the substrate (1.0). Consequently, this work establishes a very simple method for determining the substrate than that for ap, 2cd, NADH, or ba would exclude the

### Table I

Relative specificities and percentage of enzymatic chlorine transfer of CPO substrates at pH 2.75

| CPO substrates (ps/cps) | Cl | $f_{\text{ps-cpo}}$ | $f_{\text{ps-ne}}$ | % ENZ | $f_{\text{ps-cl}}$ |
|------------------------|----|--------------------|--------------------|-------|------------------|
| cat/tmp                | 1  | 0.94 ± 0.015       | 0.40 ± 0.025       | 90.5 ± 2.5 | 0.905 ± 0.061    |
| cat/tmp                | 20 | 0.51 ± 0.007       | 0.420 ± 0.005      | 15.7 ± 1.4  | 0.156 ± 0.015    |
| cat/tmp                | 100| 0.463 ± 0.043      | 0.398 ± 0.013      | 10.8 ± 7.5  | 0.108 ± 0.076    |
| ap/cat                 | 1  | 0.977 ± 0.022      | 1.0000 ± 0.0001    | 99.8 ± 0.3  | 0.997 ± 0.003    |
| ap/cat                 | 20 | 0.996 ± 0.003      | 0.9999 ± 0.00001   | 98.0 ± 1.5  | 0.980 ± 0.016    |
| ap/cat                 | 100| 0.9999 ± 0.0005    | 0.9999 ± 0.0001    | 99.9 ± 0.5  | 0.999 ± 0.004    |
| 2cd/ap                 | 1  | 0.995 ± 0.002      | 0.953 ± 0.003      | 100.0 ± 0.01| 1.000 ± 0.00002  |
| 2cd/ap                 | 20 | 0.995 ± 0.002      | 0.953 ± 0.003      | 99.99 ± 0.01| 1.000 ± 0.0001   |
| 2cd/ap                 | 100| 0.9999 ± 0.0002    | 0.996 ± 0.016      | 100.0 ± 0.01| 1.000 ± 0.0001   |
| ba/2cd                 | 1  | 0.831 ± 0.008      | 0.759 ± 0.010      | 100.0 ± 0.0002| 1.000 ± 0.0001  |
| ba/2cd                 | 20 | 0.831 ± 0.017      | 0.757 ± 0.020      | 100.0 ± 0.002| 0.99999 ± 0.0003 |
| ba/2cd                 | 100| 0.818 ± 0.012      | 0.747 ± 0.015      | 100.0 ± 0.001| 1.000 ± 0.0001   |

### Table II

Relative specificities and percentage of enzymatic chlorine transfer of CPO substrates at pH 4.80

Fractions and percentages were determined as described in the Appendix. See “Experimental Procedures” for specific reaction protocols.

| Substrates (ps/cps) | Cl | $f_{\text{ps-cpo}}$ | $f_{\text{ps-ne}}$ | % ENZ | $f_{\text{ps-cl}}$ |
|--------------------|----|--------------------|--------------------|-------|------------------|
| cat/tmp            | 100| 0.960 ± 0.067      | 0.370 ± 0.090      | 94 ± 11| 0.936 ± 0.223    |
| cat/tmp            | 400| 0.541 ± 0.050      | 0.395 ± 0.072      | 24 ± 12| 0.241 ± 0.148    |
| ap/cat             | 100| 0.989 ± 0.006      | 0.9999 ± 0.0001    | 99.9 ± 0.1 | 0.999 ± 0.003 |
| ap/cat             | 400| 0.964 ± 0.004      | 0.9996 ± 0.0003    | 89 ± 8 | 0.885 ± 0.093    |
| NADH/ap            | 100| 0.976 ± 0.027      | 0.924 ± 0.054      | 100.0 ± 0.004| 1.000 ± 0.0001 |
| NADH/ap            | 400| 0.862 ± 0.013      | 0.739 ± 0.023      | 98.3 ± 1.3 | 0.980 ± 0.018   |
| 2cd/NADH           | 100| 0.888 ± 0.012      | 0.539 ± 0.029      | 100.0 ± 0.0005| 1.000 ± 0.005  |
| 2cd/NADH           | 400| 0.885 ± 0.007      | 0.526 ± 0.026      | 99.8 ± 0.17| 0.997 ± 0.003   |
| ba/2cd             | 100| 0.912 ± 0.009      | 0.778 ± 0.010      | 100.0 ± 0.0001| 1.000 ± 0.005  |
| ba/2cd             | 400| 0.893 ± 0.023      | 0.807 ± 0.046      | 100.0 ± 0.02 | 1.000 ± 0.0004 |
| ba/NADH            | 100| 0.979 ± 0.007      | 0.741 ± 0.017      | 100.0 ± 0.0001| 1.000 ± 0.009  |
| ba/NADH            | 400| 0.979 ± 0.003      | 0.742 ± 0.012      | 100.0 ± 0.03  | 1.000 ± 0.001   |

### Notes

1. Reaction conditions: 100 mM phosphate, pH 2.75, 400 μM cat, 400 μM tmp, chloride concentrations as indicated, and either 200 μM H$_2$O$_2$, 50 nm CPO, and, when present, 2 mM Met or 200 μM hypochlorous acid. Calculated from results originally reported in Ref. 1.
2. Reaction conditions: 100 mM phosphate, pH 2.75, 600 μM cat, 18 mM cat, chloride concentrations as indicated, and either 300 μM H$_2$O$_2$, 50 mM CPO (cPo), and, when present, 2 mM Met or 300 μM hypochlorous acid.
3. Reaction conditions: 100 mM phosphate, pH 2.75, 16 mM ap, 80 μM 2cd, chloride concentrations as indicated, and either 40 μM H$_2$O$_2$ and 50 nm CPO or 40 μM hypochlorous acid.
4. Reaction conditions: 100 mM phosphate, pH 2.75, 80 μM ba, 160 μM 2cd, chloride concentrations as indicated, and either 40 μM H$_2$O$_2$ and 50 nm CPO or 40 μM hypochlorous acid.
substrate's reaction. Thus, substrates such as Met, thiourea, and thiuracil, which have been shown to be preferred by CPO over 2Cd, clearly react directly with the enzyme and do not allow any significant involvement of free Cl₂ in their reactions (10).

These results demonstrate that the production of identical products between enzymatic and nonenzymatic model reactions is not sufficient evidence to establish a nonenzymic species as an intermediate in the enzymatic reaction. Nor does the detection of the formation of the intermediate in the absence of an acceptor substrate or in the presence of a poor acceptor substrate such as cat or tmp directly implicate the intermediate in reactions of all substrates. The intermediate must be shown to be kinetic competitor to support the major pathway for product formation. In short, kinetic evidence is essential for establishing the involvement of an intermediate in any reaction pathway.

These results also call into question the obligatory involvement of hypochlorite proposed for myeloperoxidase reactions (23). Production of a freely dissociable halogenating species has been demonstrated with immobilized myeloperoxidase in the absence of an acceptor substrate. That freely dissociable species has been assumed to be hypochlorous acid. However, it is possible that myeloperoxidase operates by a mechanism similar to that proposed here for CPO. A recent report by Marquet and Dunford (24) presents kinetic evidence that direct chlorine transfer occurs between myeloperoxidase and taurine when both are present in the same reaction mixture. Competition studies such as those described here should be able to determine the extent of involvement of a freely dissociable species in any myeloperoxidase-catalyzed chlorination reaction and to determine whether the species released is hypochlorous acid or Cl₂. As with CPO, competition studies and detailed kinetic studies would reinforce each other (1, 11).

With this report we have settled a controversy, which has existed for at least 20 years, over the mechanism of halogen transfer in CPO-catalyzed reactions, and we have established a set of criteria for testing reactions of other CPO substrates. In addition, we have described and tested a simple approach for quantitating the involvement of any freely dissociable reactive intermediate in an enzyme-catalyzed reaction. This method of successive pairwise competition reactions should be generally applicable for exploring the involvement of any potential enzyme-generated reactive intermediate that can also be generated nonenzymatically.

APPENDIX

Calculations of the Fraction of Reaction Flux Attributable to the Preferred Substrate in Enzymatic and Nonenzymatic Substrate Competition Reactions

General Approach

The fraction of reaction flux attributable to the preferred substrate in a competition reaction (see Tables I and II) was determined from absorbance changes as described below. The particular equations used varied somewhat depending upon the particular protocol for the reaction. The current data consist of determinations of total reaction flux and the part of the reaction flux attributable to the absorbing substrate (see "Experimental Procedures"). Thus, the relative specificities for substrates are expressed as fractions of reaction flux attributable to the preferred substrate rather than as ratios of amounts of substrates consumed as they were previously (1). All changes in concentrations were determined from changes in absorbance of the reaction mixtures. When cat was used as a substrate for CPO reactions, its change in concentration was corrected for the amount of cat passing through the chloride-independent reaction using reactions containing Met as described previously (1). Met closes the CPO chloride-dependent pathway for cat (Scheme 1, step 4) by completely consuming any EOCI that forms. Thus, reactions containing Met allow measurement of the amount of cat consumed in the chloride-independent CPO reaction pathway. No corrections are required for the nonenzymatic reactions of cat.

**Reactions Containing cat**

\[
\Delta[cat] = \varepsilon_{388}(A_{388}(cat + tmp) - A_{388}(cat + met))
\]  
(Eq. 3)

\[
\Delta[tmp] = \varepsilon_{260.5}(A_{260.5}(cat + tmp))
\]  
(Eq. 4)

For these reactions cat is the preferred substrate, so combining Equations 2, 3, and 4 yields Equations 5 and 6.

\[
f_{ps-cpo} = \frac{\varepsilon_{388}(A_{388}(cat + tmp) - A_{388}(cat + met))}{\varepsilon_{388}(A_{388}(cat + tmp) - A_{388}(cat + met)) + \varepsilon_{260.5}(A_{260.5}(cat + tmp))}
\]  
(Eq. 5)

\[
f_{ps-ne} = \frac{\varepsilon_{388}(A_{388}(cat + tmp))}{\varepsilon_{388}(A_{388}(cat + tmp) + \varepsilon_{260.5}(A_{260.5}(cat + tmp)))}
\]  
(Eq. 6)

In these reactions, since \( f_{ps-cpo} \) and \( f_{ps-ne} \) are ratios of concentrations, all of which were determined from absorbances of the same species, the molar absorption coefficients divide out, and changes in absorbance can be used directly to represent changes in concentrations. For these reactions ap is the preferred substrate; thus, combining Equations 2, 7, and 8 yields Equations 9 and 10.

\[
f_{ps-cpo} = \frac{(\Delta A_{388}(cat) - \Delta A_{388}(ap + cat))}{(\Delta A_{388}(cat) - \Delta A_{388}(ap + cat)) + (\Delta A_{388}(ap + cat)) - \Delta A_{388}(cat + met)} \left( \frac{[ap]}{[cat]} \right)
\]  
(Eq. 9)

\[
f_{ps-ne} = \frac{(\Delta A_{388}(cat))}{(\Delta A_{388}(cat) - \Delta A_{388}(ap + cat)) + (\Delta A_{388}(ap + cat)) \left( \frac{[ap]}{[cat]} \right)}
\]  
(Eq. 10)

**Reactions Containing Only Substrates Other Than Cat**

Since none of the substrates in this group reacts significantly with CPO compound I under the reaction conditions, no corrections are needed in measurements of concentration changes for their CPO reactions. Consequently, the equations for calculating \( f_{ps-cpo} \) and \( f_{ps-ne} \) are identical and are given as \( f_{ps} \). Each reaction was monitored by the absorbance change due to one of the substrates, the absorbing substrate. Equations for calculating the \( f_{ps} \) depend upon whether the absorbing substrate is the preferred substrate or the competing substrate.

Reactions in Which the Absorbing Substrate Is the Preferred
Substrate—These reactions include 2cd versus ap and NADH versus ap. In these reactions, [ps]cPO = [cs]. The Δ[ps] and the sum of Δ[ps] + Δ(cs) were determined from changes in absorbance at the wavelength indicative of the preferred substrate (Equations 11 and 12).

$$\Delta[ps] = \epsilon_\text{opt}(\Delta A_{\text{opt}}[ps + cs]) \tag{Eq. 11}$$

$$\Delta[ps] + \Delta(cs) = \epsilon_\text{opt}(\Delta A_{\text{opt}}[ps]) \tag{Eq. 12}$$

The combination of Equations 2, 11, and 12 yields Equation 13 for both the CPO and nonenzymatic reactions.

$$f_{\text{ps}} = \frac{\Delta A_{\text{opt}}[ps + cs]}{\Delta A_{\text{opt}}[ps] + \Delta(cs) + \Delta A_{\text{opt}}[ps + cs]} \tag{Eq. 13}$$

Reactions in Which the Absorbing Substrate is the Competing Substrate—These reactions include ba versus 2cd, 2cd versus NADH, and ba versus NADH. In all cases, [ps]cPO ≠ [cs]. The Δ(cs) and the sum of Δ[ps] + Δ(cs) were determined from changes in absorbance at the wavelength indicative of the competing substrate (Equations 14 and 15).

$$\Delta(cs) = \epsilon_\text{opt}(\Delta A_{\text{opt}}[ps + cs]) \tag{Eq. 14}$$

$$\Delta[ps] + \Delta(cs) = \epsilon_\text{opt}(\Delta A_{\text{opt}}[cs]) \tag{Eq. 15}$$

The combination of Equations 2, 14, and 15 yields Equation 16 for both the CPO and nonenzymatic reactions.

$$f_{\text{ps}} = \frac{\Delta A_{\text{opt}}(cs) - \Delta A_{\text{opt}}(ps + cs)}{\Delta A_{\text{opt}}(cs) - \Delta A_{\text{opt}}(ps + cs) + \Delta A_{\text{opt}}(ps + cs)} \tag{Eq. 16}$$

Determination of the Relative CPO Chloride-dependent Reaction Flux through the Direct Chlorine Transfer Pathway

The percentage of the CPO chloride-dependent reaction flux passing through the direct chlorine transfer pathway (see Tables I and II, and Scheme II, steps 7 and 8 versus steps 9–11) was determined from a comparison of the fractions of preferred substrate consumed in enzymatic versus nonenzymatic reactions as described below. To simplify the calculations we will define four calculated values. The fraction of the total reaction flux of a pair of substrates that passes through the free Cl2 pathway, fCl2, (Scheme II steps 9–11) is as follows.

$$f_{\text{Cl2}} = \frac{[ps_{\text{ne}}] + [cs_{\text{ne}}]}{[ps_{\text{ne}}] + [cs_{\text{ne}}] + [ps_{\text{ne}}] + [cs_{\text{ne}}]} \tag{Eq. 17}$$

The fraction of the total reaction flux of a pair of substrates that involves direct transfer of chlorine to substrates, fenz, (Scheme II, steps 7 and 8) is as follows.

$$f_{\text{enz}} = \frac{[ps] + [cs]}{[ps] + [cs] + [ps_{\text{ne}}] + [cs_{\text{ne}}]} \tag{Eq. 18}$$

The fraction of the reaction flux of the preferred substrate that involves direct transfer of chlorine to the substrate, fpsE, (Scheme II, step 7 versus steps 7 + 9) is as follows.

$$f_{\text{ps}} = \frac{[ps]}{[ps] + [ps_{\text{ne}}] + [cs_{\text{ne}}]} \tag{Eq. 19}$$

The fraction of the reaction flux of the competing substrate that involves direct transfer of chlorine to the substrate, fceE, (Scheme II, step 8 versus steps 8 + 9) is as follows.

$$f_{\text{ce}} = \frac{[cs]}{[ps_{\text{ne}}] + [cs_{\text{ne}}] + [cs_{\text{ne}}]} \tag{Eq. 20}$$

Thus, the percentage of the reaction flux of a pair of substrates that involves direct transfer of chlorine to substrates, % ENZ, is as follows.

$$% \text{ENZ} = 100(f_{\text{ps}}) \tag{Eq. 21}$$

Combining Equations 17 and 18 shows the following.

$$f_{\text{ps}} + f_{\text{ce}} = 1 \tag{Eq. 22}$$

Note that fpsE and fceE depend only upon the competition between the organic substrate and chloride for reaction with EOCI (Scheme II); thus, they are independent of the presence of additional substrates or the identities of those substrates. For the case where no competing substrate is present, the Cl2 intermediate will be completely consumed by the single substrate so that the fraction of total reaction flux involving direct chlorine transfer will be the same as when additional substrates are present.

From Scheme II, the fraction of preferred substrate consumed in a chloride-dependent CPO-catalyzed reaction can be defined as in Equation 23.

$$f_{\text{ps-cpo}} = \frac{\Delta[ps_{\text{ne}}] + \Delta[ps_{\text{ne}}]}{\Delta[ps] + \Delta(cs) + \Delta[ps_{\text{ne}}] + \Delta(cs_{\text{ne}})} \tag{Eq. 23}$$

Likewise, the fraction of preferred substrate consumed in a nonenzymatic halogenation reaction can be defined as in Equation 24.

$$f_{\text{ps-ne}} = \frac{\Delta[ps_{\text{ne}}]}{\Delta[ps_{\text{ne}}] + \Delta(cs_{\text{ne}})} \tag{Eq. 24}$$

Combining Equations 17, 19, 23, and 24 yields Equation 25.

$$f_{\text{ps-cpo}} = \frac{(f_{\text{ps}})(f_{\text{ps}}) + (f_{\text{ps}})(f_{\text{ps-ne}})}{(1 - f_{\text{ps}})(f_{\text{ps-ne}})} \tag{Eq. 25}$$

Also, combining Equations 17, 18, 19, and 20 and solving for fenz gives Equation 26.

$$f_{\text{enz}} = \frac{(f_{\text{ps}})(f_{\text{ps}})}{(1 - f_{\text{ps}})(1 - f_{\text{ps}})} \tag{Eq. 26}$$

Combining Equations 22 and 26 and solving for fCl2 gives Equation 27.

$$f_{\text{Cl2}} = \frac{(1 - f_{\text{ps}})(1 - f_{\text{ps}})}{(1 - f_{\text{ps}})(1 - f_{\text{ps}})} \tag{Eq. 27}$$

Combining Equations 22 and 26 and solving for fenz gives Equation 28.

$$f_{\text{enz}} = \frac{(f_{\text{ps}})(1 - f_{\text{ps}}) + (f_{\text{ps}})(1 - f_{\text{ps}})}{(1 - f_{\text{ps}})(1 - f_{\text{ps}})} \tag{Eq. 28}$$

Combining Equations 21 and 28 gives Equation 29.

$$% \text{ENZ} = 100(f_{\text{ps}})(1 - f_{\text{ps}}) + f_{\text{ps}}(1 - f_{\text{ps}}) \tag{Eq. 29}$$

Combining Equations 22 and 25 and collecting terms yields Equation 30.

$$f_{\text{ps-cpo}} = \frac{(1 - f_{\text{ps}})(f_{\text{ps}}) + (f_{\text{ps}})(1 - f_{\text{ps}})}{(1 - f_{\text{ps}})(1 - f_{\text{ps}})} \tag{Eq. 30}$$

Combining Equations 28 and 30 and solving for fpsE yields the following.

$$f_{\text{ps}} = \frac{(f_{\text{ps-cpo}} - f_{\text{ps-ne}})(1 - f_{\text{ps}})}{(1 - f_{\text{ps-cpo}})(1 - f_{\text{ps-ne}})} \tag{Eq. 31}$$
As described in the first section of this Appendix, $f_{ps-cpo}$ and $f_{ps-ne}$ can be experimentally determined for competitive reactions of any pair of substrates studied. However, there are two remaining unknowns in Equation 31, $f_{psE}$ and $f_{csE}$. Since, as described above, these two values are independent of each other, one must be determined by an independent method.

In this study we have used the competitive reaction between cat and tmp to obtain the $f_{catE}$ and then used the $f_{catE}$ to determine $f_{apE}$ and so on (see "Results"). Since tmp has been shown to react only through the $Cl_2$ pathway, $f_{tmpE} = 0$ (1). Consequently, for the competition between cat and tmp, where cat is the preferred substrate, Equation 31 simplifies to Equation 32.

\[
   f_{catE} = \frac{[f_{cat-cpo} - f_{cat-ne}]}{[1 - f_{cat-ne}]} \tag{Eq. 32}
\]

Thus, $f_{catE}$ can readily be calculated and used as the $f_{csE}$ in competition with more preferred substrates to determine their $f_{psE}$ values and the % ENZ for each set of reactions (see Tables I and II).

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