'Thermodynamic' mechanism of catalysis by haloperoxidases

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Abstract A novel 'thermodynamic' mechanistic rationale of haloperoxidase catalysis is based on the following two assumptions: (i) the role of enzyme consists only in the rapid equilibration between the halogen-containing species originating from halide and hydrogen peroxide; (ii) the interaction between the enzyme and organic substrate is kinetically insignificant and halogenation occurs as a result of the electrophilic attack of the active brominating (Br_3^-, Br_2 and HBrO) or chlorinating (HClO) species at monochlorodimedon indicative of a higher chloride 'specificity' of chloroperoxidase from *C. fumago*.

Key words: Haloperoxidase; Catalysis; Mechanism

1 Introduction

A biological function of the haloperoxidase enzymes is to convert activated C-H bonds into C-Hal bonds by halide and hydrogen peroxide (Eq. 1) [1,2]. It is a primary step for functionalization

$$-\overset{|}{\underset{|}{C}}-H + Hal^- + H_2O_2 + H^+ \rightarrow -\overset{|}{\underset{|}{C}}-Hal + 2H_2O \qquad (1)$$

of organic molecules, and, therefore, the enzymes, and especially heme chloroperoxidase from *Caldariomyces fumago*, are attractive catalysts in organic synthesis [3]. The rich synthetic chemistry encouraged structural [4,5] and mechanistic studies [6–16] of various haloperoxidases and related enzymes. The mechanism of catalysis by ClP is controversial. There are two major sources of debate. The first one concerns the nature of a true halogenating species. Halide itself cannot carry out the nucleophilic cleavage of a C-H bond even if assisted by the enzyme. It is more likely that the ClP-promoted interaction between halide and H_2O_2 gives rise to a third electrophilic species that further interacts with a substrate. Hal_2 and HO-Hal are often considered as reactive agents [6–14]. However, the oxidation of halide ions by H_2O_2 may result in two series of compounds 2a or 2b and every member could, in principle, be involved in the enzymatic reaction, the relative contribution of each into the overall rate being, of course, different.

$$Br^- \leftrightarrow Br_3^- \leftrightarrow Br_2 \leftrightarrow HBrO \leftrightarrow BrO_3^- \qquad (2a)$$

$$Cl^- \leftrightarrow Cl_2 \leftrightarrow HClO \leftrightarrow HClO_2 \leftrightarrow ClO_3^- \qquad (2b)$$

The second controversy concerns the intermediates between enzyme and organic substrate [17,18], or, in other words, a meaningful, in a catalytic sense, enzyme-substrate binding. Numerous examples indicate that the ClP-catalyzed halogenation is poorly regio-and stereoselective [3]. Therefore, the role of enzyme-substrate binding might only be secondary, the dominant process being the catalytic generation of the active halogenating species shown by series 2.

In this paper, we will present evidence for the mechanistic concept outlined above which accommodates the two assumptions. (i) The enzyme is only needed for rapid equilibration between halogen-containing species (2a) or (2b). (ii) Thus formed, these are capable of enzyme-independent halogenation to afford the final products. To achieve this goal, two sets of data were obtained and compared. The first is the thermodynamic data on the equilibrium distribution in series (2a) or (2b) achieved on mixing Hal^- and H_2O_2. These data were calculated using the computer routine CPESSP developed recently [19] and accommodated to the present problem. It enables one to derive equilibrium concentrations of all species involved in a redox process. The second set is the kinetic data on oxidation of the model ClP substrate, 1,1-dimethyl-4-chloro-3,5-cyclohexanedione (MCD), at the same Hal^-/H_2O_2 concentrations.

2. Materials and methods

2.1. Kinetics of enzymatic halogenation of MCD

Enzymatic oxidation of MCD carried out at 25°C and pH 2.75 (0.4 M phosphate) was followed by measuring the decrease in absorbance at 278 nm (ε = 11 000 M^{-1} cm^{-1}) due to halogenation of MCD as described in detail elsewhere [20]. The enzymatic reactions were initiated by the addition of H_2O_2 to a thermostated solution of MCD, ClP, and halide (Cl^- or Br^-). The concentration of the enzyme in the UV-vis cell was usually 10^{-8} M, while [H_2O_2] and [Hal^-] varied in the range 0–20 and 0–300 mM, respectively. All the reagents used in this work were of the highest available quality. ClP and MCD were purchased from Sigma and used as received. Spectrophotometric measurements were made on a Shimadzu UV-160A spectrophotometer equipped with a CPS-240A cell positioner/temperature controller.

2.2. Calculation of equilibrium concentrations of the halogen species

All calculations were performed using the original computer program CPESSP [19]. The key feature in computation of any multicomponent mixture is the right choice of the so-called primary species [21,22]. These are a minimal number of the mixture components which can be used for derivation of the whole set of half-reactions. In particular, for the Br^-/H_2O_2 system the primary species are BrO_3^-, OH^-, H^+ and e (the electron being considered as an independent species). The primary species are used for the construction of all equilibria that occur in the system. In this case, these are:

1	BrO_3^-	+ 0 e	+ 0 H^+	⇄	BrO_3^-	
2	2HO	+ 0 e	+ 0 H^+	⇄	H_2O_2	
3	BrO_3^-	+ 6 e	+ 6 H^+	⇄	Br^-	+ 3 H_2O
4	$2BrO_3^-$	+ 10 e	+ 12 H^+	⇄	Br_2	+ 6 H_2O
5	$3BrO_3^-$	+ 16 e	+ 18 H^+	⇄	Br_3^-	+ 9 H_2O
6	BrO_3^-	+ 4 e	+ 5 H^+	⇄	HBrO	+ 2 H_2O
7	1/2 H_2O_2	+ 1 e	+ H^+	⇄	H_2O	

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Abbreviations: ClP, chloroperoxidase from *Caldariomyces fumago*; MCD, monochlorodimedon.

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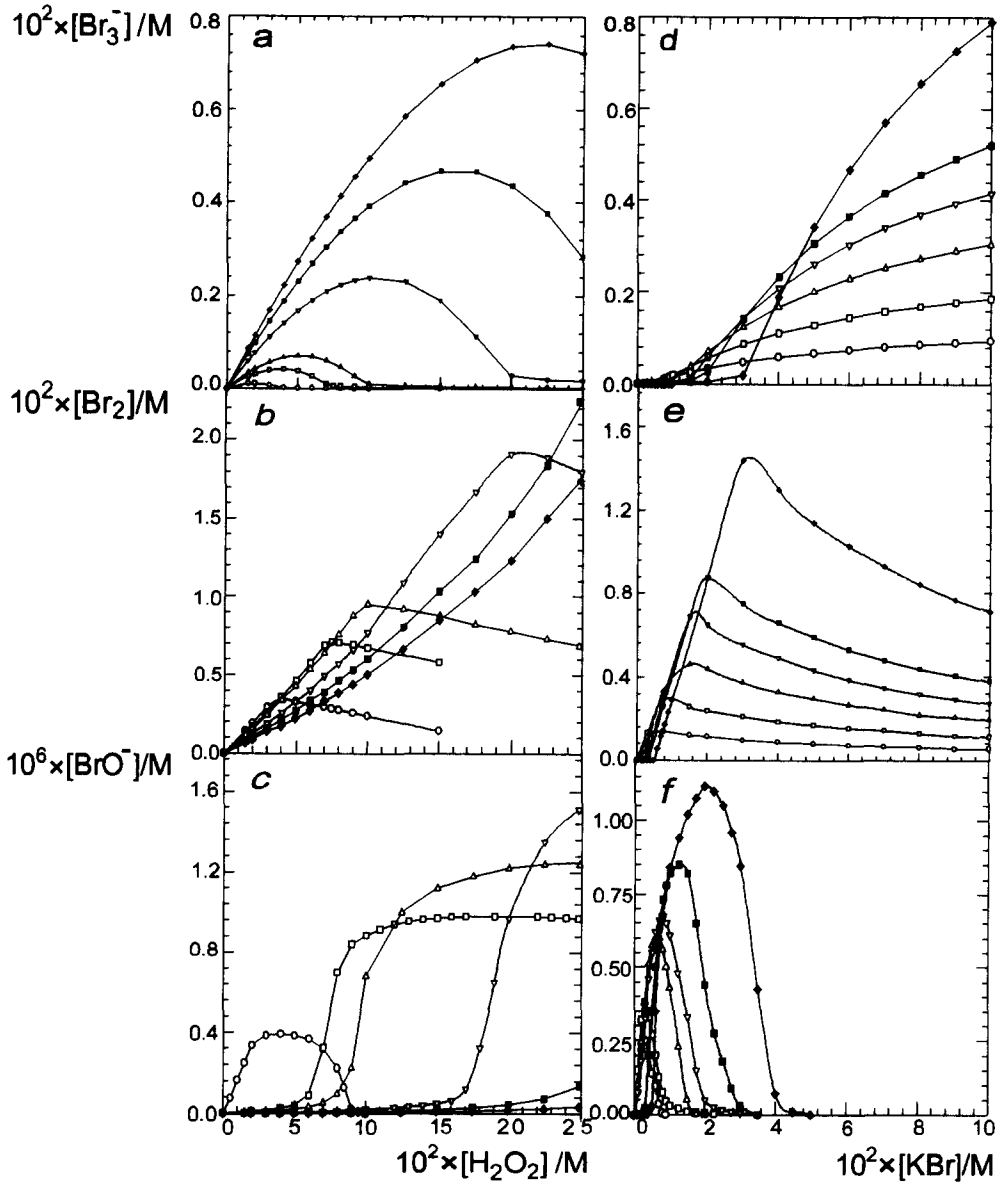

Fig. 1. Calculated distribution of the halogen-containing species formed on mixing bromide ions with hydrogen peroxide at 25°C, pH 2.75; for calculation details, see text. Concentrations of KBr in a–c are 0.0075 (○), 0.015 (□), 0.02 (△), 0.04 (▽), 0.06 (■) and 0.08 (◆) M; concentrations of H_2O_2 in d–f are 0.0015 (○), 0.003 (□), 0.005 (△), 0.007 (▽), 0.009 (■) and 0.015 (◆) M.

The stoichiometric coefficients at the primary species give rise to an *m*/*n* atomic matrix, where *m* is the number of independent reactions and *n* is the number of the primary species. In this case, it is a 7/4 matrix shown below together with the corresponding equilibrium constants *K*:

	Primary species				log *K*
	BrO_3^-	HO	e	H^+	
1	1	0	0	0	0.00
2	0	2	0	0	0.00
3	1	0	6	−6	141.974
4	2	0	10	−12	246.723
5	3	0	16	−18	389.949
6	1	0	4	−5	94.649
7	0	1	1	−1	29.949

The atomic matrix is then converted according to the Brincley routine [23] into the molecular matrix where the real 'primary' species, such as Cl^- or Br^-, are used instead of the electron. The calculation begins after setting the initial concentrations of the primary species together with the equilibrium constants *K* for the independent reactions and provides the equilibrium concentrations of the primary species and all other participants. The existence of only one solution for redox reactions of this type was demonstrated by Kosyak [24]. The *K* values for the half-reaction are derived from the corresponding half-wave potentials $E_{1/2}(-RT\ln K = nFE_{1/2}$, where *n* is the number of electrons and *F* is the Faraday constant). Most of the *K* and $E_{1/2}$ values used in this work were taken from the monograph of Huheey [25].

3. Results and discussion

3.1. The equilibrium concentration of the halogen species

Representative computational results demonstrating the bromine speciation at pH 2.75 are shown in Fig. 1. The equilibrium concentrations of Br_3^-, Br_2 and HBrO, as expected, vary significantly. Remarkably, there are maxima on the profiles for Br_2 and HBrO (Fig. 1e,f), viz. the potentially most reactive brominating agents. The origin of the maxima is

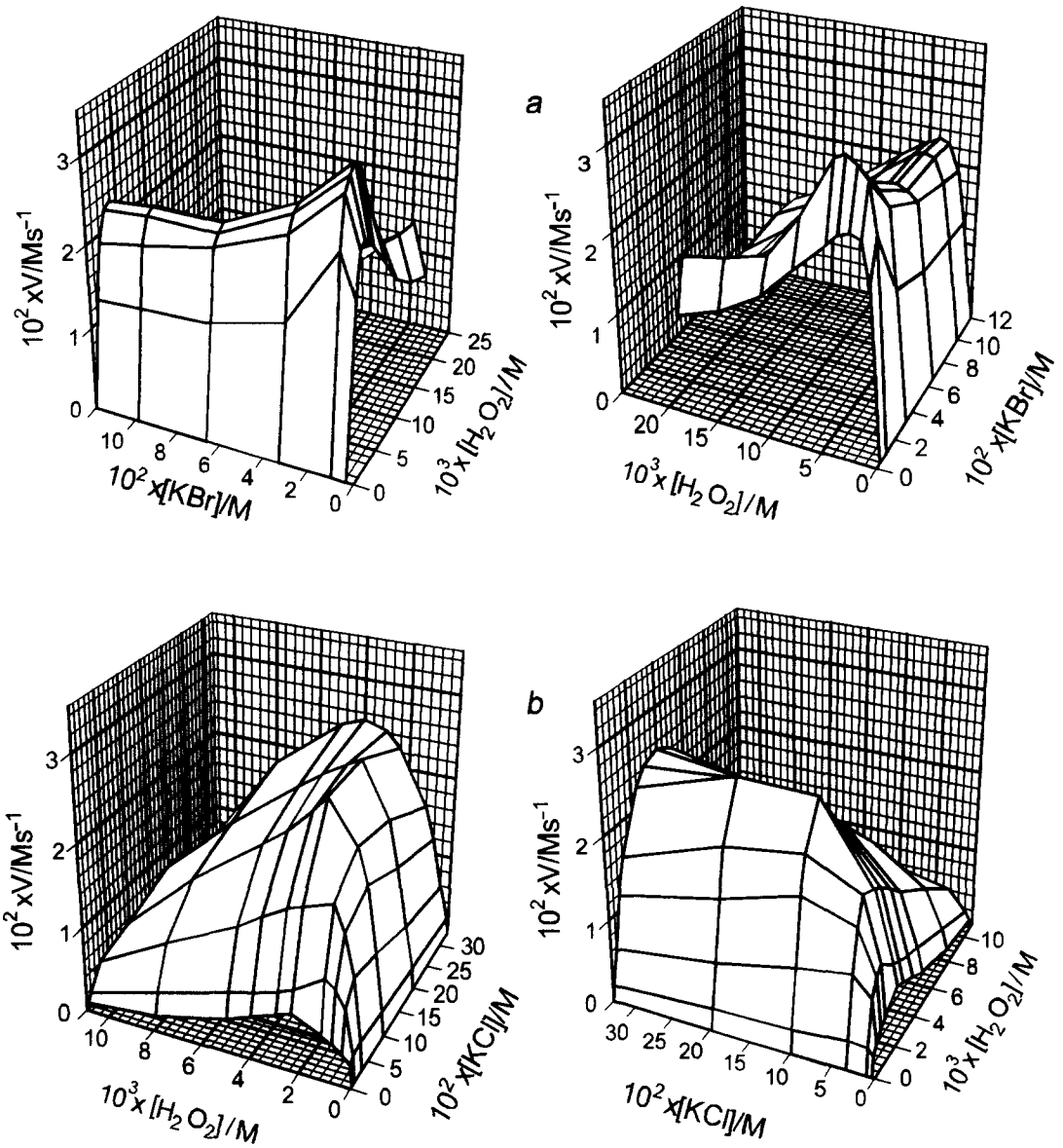

Fig. 2. Initial rate of ClP-catalyzed monochlorodimedon halogenation in the bromide (a) and chloride (b) systems at 25°C, pH 2.75. [ClP] = 10^{-8} M, [MCD] = 10^{-4} M.

clear. The bromide ion acts as a reducing agent for Br_2 and HBrO and, thus, increases the 'population' of more reduced species in series 2a. In accord with this is the sharpness of the profiles for more oxidized species in Fig. 1e,f. The drop in [HBrO] is more pronounced compared to that of [Br_2], since the former has a higher capacity for reduction, viz. into both Br^- and Br_2. It should also be pointed out that the [BrO_3^-] vs. the total bromide concentration [Br^-]$_t$ profile matches that in Fig. 1f. Unfortunately, we could not take into account the bromous acid, $HBrO_2$, because of the lack of corresponding thermodynamic data.

The data for chlorine speciation is noticeably different. The plots of [Cl_2], [HClO], and [$HClO_2$] (Cl_3^- was not considered here, since the stability of Cl_3^- is a factor of 100 lower as compared to Br_3^- [26]) against [Cl^-]$_t$ are predominantly monotonically increasing functions without sharp maxima in the concentration ranges chosen. A broad maximum is observed for ClO_3^- only at low [H_2O_2]$_t$=0.003 M. Maxima are more frequent on the corresponding profiles against [H_2O_2]$_t$, but only at low [Cl^-]$_t$.

3.2. Kinetics of ClP-catalyzed halogenation of MCD

Kinetic measurements were undertaken over broad concentration ranges to compare the rates with the computed thermodynamic data. In order to minimize the ClP inactivation, the reaction was initiated by addition of H_2O_2 and only the initial rates v were analyzed. The rates as a function of [Hal^-]$_t$ and [H_2O_2]$_t$ are demonstrated in Fig. 2. The bromide and chloride cases are different. There are maxima on all the rate versus [Br^-]$_t$ cuts in the concentration range $(1–4)\times10^{-2}$ M, while the maxima on the corresponding chloride cuts are either much less pronounced, or even unobserved, and these look like the customary Michaelis graphs.

There is an obvious similarity between the thermodynamic (Fig. 1) and kinetic data (Fig. 2). Maxima are present on the both figures for bromide while the leveling-off is more typical

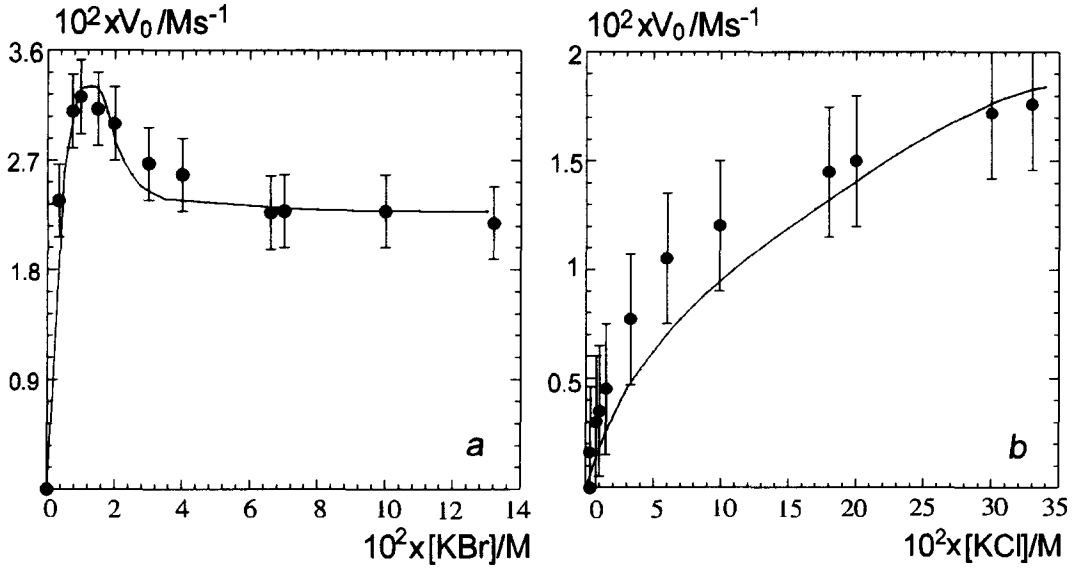

Fig. 3. Comparison of the experimental and calculated rates of ClP-catalyzed monochlorodimedon halogenation in the bromide (a) and chloride (b) systems at 25°C, pH 2.75. Solid lines are the calculated data based on the rate constants from Table 1.

of the chloride graphs. These observations point to a relationship between the enzymatic activity and the equilibrium concentrations of the halogen species in solution. In other words, the enzyme may carry out the rapid thermodynamically driven equilibration of the halogen species, and these then interact with organic substrate with a minor involvement of ClP.

3.3. Thermodynamic data in kinetic analysis

Let us assume that the enzyme-catalyzed halogen equilibration occurs much faster than the following halogenation of MCD taking place without ClP. The most general rate law may be written as

$$\frac{v}{[MCD]_t} = k_1[Hal_3^-] + k_2[Hal_2] + k_3[HHalO] + k_4[HHalO_2] \quad (3)$$

where v is the measured initial rate, $[MCD]_t$ is the total concentration of MCD, while other symbols in square brackets are the computed equilibrium concentrations of the corresponding species, the latter being computed as described above. The data presented in Fig. 2 were fitted to Eq. 3 using the computed 'thermodynamic' concentrations of the reactive species formed at given $[Hal^-]_t$ and $[H_2O_2]_t$. The best-fit rate constants are summarized in Table 1. Interestingly, the meaningful values are obtained for three rate constant k_1, k_2 and k_3 in the Br^- case, i.e. the species Br_3^-, Br_2 and HBrO do contribute to the overall rate. However, only k_3 is valid for the Cl^- case, indicating that only HClO might be the true halogenating species.

The rate constants from Table 1 were used for the simulation of the rate versus $[Hal^-]_t$ and $[H_2O_2]_t$ profiles. The typical plots shown in Fig. 3 reveal an agreement between the calculated and experimental data. The successful simulation of the maximum in Fig. 3a (and similar maxima on the rate versus $[H_2O_2]_t$ plots) in terms of the 'thermodynamic' mechanism described here suggests that the latter might be a sound alternative to the traditional treatment of rate versus $[Hal^-]$ or rate versus $[H_2O_2]$ profiles in the ClP-catalyzed reactions, where the rate retardation was usually associated with the enzyme inhibition by one of the two substrates [7–14]. It should be indicated that we do not assume that all the previous mechanistic views on haloperoxidases should be revised. Rather, we wanted to emphasize an alternative, basically different mechanistic rationalization of catalysis by haloperoxidases regardless of their heme or vanadium nature.

The values of rate constants in Table 1 deserve a comment. There is nothing unusual in the fact that hypohalous acids are the most reactive, but the absolute values of k_3 still seem very large. However, this might be explained on taking into account that MCD must be strongly enolized at pH 2.75 [27] and hence highly reactive toward HHalO. Also, it is well known that the rate of halogenation of simpler ketones is independent of $[Hal_2]$ implying that the electrophilic attack occurs much faster than the rate-limiting enolization [27]. Thus, very high values for k_3 are to be expected. Further, the experimental estimate for the second-order rate constant for the reaction between crotonic acid and HClO is approx. 1.5×10^4 M^{-1} s^{-1} at pH 3, $[Cl^-]$ = 1 M and 25°C [28].

The trends in varying the rate constants (Table 1) are mechanistically pertinent. The finding that the involvement of hypochlorous acid is sufficient to account for the catalysis in the Cl^--H_2O_2 system provides strong evidence for the fact that HClO is a true halogenating species in these reactions. In the Br^- case, hypobromous acid is much more reactive than Br_2 and Br_3^-. The formation of Br_3^- could be viewed as a complexation of Br_2 with the nucleophilic anion Br^-. Hence, the electrophilicity of the Br_3^- is lower, but, nevertheless, the rate constants k_1 and k_2 are similar. The meaningful reactivity of

Table 1
The best-fit rate constants (M^{-1} s^{-1}) for halogenation of monochlorodimedon at pH 2.75, 25°C.

	Br^-	Cl^-
k_1	$(2.2\pm0.8)\times10^4$	–
k_2	$(2.4\pm0.6)\times10^4$	–
k_3	$(1.7\pm0.7)\times10^8$	$(1.3\pm0.3)\times10^8$
k_4	–	–

Br_3^- and Br_2, in our opinion, does not seem very surprising in terms of the mechanism proposed, since the corresponding rate constants k_1 and k_2 designate the electrophilicity of the species toward enolized MCD. Therefore, it seems reasonable to expect an enhanced reactivity for Br_3^- and Br_2 compared to the corresponding chlorine species. This is probably why the rate constant k_3, which characterizes the reactivity of HClO, dominates in the chloride system. Alternatively, a more enzymologically oriented mechanism will account for the narrow reactivity spectrum in the chloride system by the higher 'specificity' of chloroperoxidase toward chloride compared to bromide. Being less 'specific' in the bromide system, the enzyme displays the experimentally observed broader spectrum of reactivity.

In conclusion, we have described the 'thermodynamic' mechanism of action of haloperoxidases with a new vision of the haloperoxidase catalysis. In our opinion, it deserves at least to be known by the scientific community. The 'thermodynamic' mechanism gives a clue for understanding a number of features typical of the catalysis. Among these are generally poor regio-and stereoselectivity[1], but enormous potency of the enzymes in various processes associated with the generation of highly oxidizing media.

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[1]The stereoselectivity is observed in the halide-free systems (see [29]), when peroxide is used as the only co-substrate and ClP is involved in the oxygen, rather than halogen transfer. Naturally, the peroxidase-type mechanism is to be expected under such conditions.