Inhibition and Inactivation of Vanadium Bromoperoxidase by the Substrate Hydrogen Peroxide and Further Mechanistic Studies†

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Received May 18, 1995⊗

ABSTRACT: Hydrogen peroxide, which is a substrate of vanadium bromoperoxidase (V-BrPO), has been shown to be a noncompetitive inhibitor of V-BrPO. Hydrogen peroxide inhibition increases with increasing pH. The inhibition is reversible under the conditions of the initial steady-state kinetic experiments. Analysis of the inhibition constants ($K_{ii}^{H_2O_2}$, $K_{is}^{H_2O_2}$) versus H^+ concentration indicates that an ionizable group with a pK_a between 6.5 and 7 is involved in the inhibition. The origin of the oxygen atoms in the dioxygen produced by the V-BrPO-catalyzed bromide-assisted disproportionation of hydrogen peroxide has been shown through $H_2{}^{18}O_2$ labeling experiments to originate from the same molecule of hydrogen peroxide. V-BrPO-catalyzed bromination is shown to be an electrophilic (Br^+) as opposed to a radical ($Br^•$) process. The stoichiometry of H_2O_2 consumed to MCD reacted or to O_2 produced is reported. The concentration of hydrogen peroxide also affects the competition of dioxygen formation during MCD bromination; competitive dioxygen formation is strongly enhanced at high pH. Turnover of V-BrPO under conditions of very high hydrogen peroxide concentration leads to irreversible inactivation at pH 4 and pH 5. Much less inactivation occurs during turnover at long reaction times at higher pH (>pH 6), and the inactivation can be fully reversed by subsequent addition of vanadate.

Interest in the biological role of vanadium has surged recently with the discovery of the first two naturally occurring vanadium enzymes, vanadium bromoperoxidase (Vilter, 1984) and vanadium nitrogenase (Eady et al., 1987; Hales, 1989), and the recognition that vanadium is involved in other important biological processes (Chasteen, 1990). Vanadium bromoperoxidase (V-BrPO)[1] has been isolated primarily from marine macroalgae (Wever & Kustin, 1979; Walker & Butler, 1993). Numerous halogenated natural products have been isolated from marine organisms. Haloperoxidases are thought to be involved in the biosynthesis of these compounds. Some of these natural products are simple, volatile halohydrocarbons (e.g., bromoform, dibromomethane, etc.) produced in very large quantities; others are more interesting halogenated indole and terpene compounds possessing a variety of biological and pharmacological activities of interest, including antiviral, antiinflammatory, antineoplastic, and antifungal activities among others. Haloperoxidases, such as V-BrPO and the marine FeHeme bromoperoxidase (Manthey & Hager, 1985), have been isolated from virtually all classes of marine algae. Thus, in the quest to elucidate the biosynthesis of these important natural products, the mechanisms of the haloperoxidase enzymes are also of interest.

V-BrPO is an acidic (i.e., pI = 4) glycoprotein (Krenn et al., 1989) that binds *ca.* 1 equiv of vanadium(V) per subunit (MW 65 000) (de Boer et al., 1986; Everett & Butler, 1989). Vanadium can be removed from V-BrPO in the presence of phosphate (Soedjak et al., 1991), producing the inactive apoenzyme derivative. The activity can be fully restored by the addition of vanadate ($H_2VO_4^-$/HVO_4^{2-}) to apo-(V)-BrPO (de Boer et al., 1988). While the structure of V-BrPO has not yet been solved, EXAFS results on V-BrPO from *Ascophyllum nodosum* suggest the vanadium(V) site may be a distorted octahedron coordinated by one short oxide ligand and five other light atom ligands, one or two of which may be histidine nitrogen ligands (Arber et al., 1989). Bond valence sum analysis is more consistent with a pentacoordinate vanadium(V) site (Butler & Clague, 1995; Carrano et al., 1994).

V-BrPO catalyzes the bromination of a variety of organic substrates, including monochlorodimedone (2-chloro-5,5-dimethyl-1,3-dimedone, MCD), a cyclic β-diketone, which is the substrate usually used to measure the specific activity of haloperoxidases. With the exception of MCD, indoles, and selected other substrates, very few organic substrates are efficiently brominated (Tschirret-Guth & Butler, 1994). For example V-BrPO can catalyze the formation of 1,3,5-tribromophenol, 5-bromocytosine, and 5-bromouracil; however, the reaction is not stoichiometric with hydrogen peroxide consumption, since much more than 1 equiv of hydrogen peroxide is consumed per equivalent of brominated product produced (Everett & Butler, 1989). The extra equivalents of hydrogen peroxide are used in the V-BrPO-catalyzed "bromide-assisted disproportionation of hydrogen peroxide" yielding dioxygen (Everett & Butler, 1989). The dioxygen is produced in the singlet excited state ($^1\Delta_g$) as

† Grant support from the National Science Foundation (MCB90-18025) is gratefully acknowledged. This paper is funded in part by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under Grant NA89AA-D-SG138, project number R/MP-53 through the California Sea Grant College, and in part from the California State Resources Agency. A.B. is a Sloan Research Fellow.

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: BCA, bicinchoninic acid; BrPO, bromoperoxidase; MCD, monochlorodimedone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; $^1O_2$, singlet oxygen.

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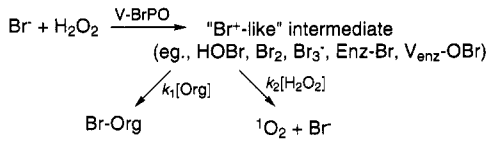
Scheme 1



identified by its characteristic emission at 1268 nm and the effect of specific singlet oxygen quenchers on the emission intensity and lifetime (Everett et al., 1990a; Kanofsky, 1989a,b).

The mechanisms of dioxygen formation and MCD bromination occur via the formation of a common intermediate, produced in a rate-limiting step (Scheme 1; Everett & Butler, 1989; Everett et al., 1990b). The identity of the oxidized bromine intermediate as enzyme-bound or released appears to depend on the nature of the organic substrate (Tschirret-Guth & Butler, 1994) and other reaction conditions (e.g., pH, [Br^-], etc.; de Boer & Wever, 1988). When MCD is the organic substrate, the k_1[MCD] and $k_2[H_2O_2]$ pathways are competitive processes since the rate of dioxygen formation in the absence of MCD is equal to the sum of the rates of MCD bromination and dioxygen formation in the presence of MCD (Everett et al., 1990b). The steady-state kinetic studies of dioxygen formation (Everett et al., 1990b) and of MCD bromination (Everett et al., 1990b; de Boer & Wever, 1988) are consistent with a bromide-inhibited bi−bi ping−pong mechanism. The kinetic parameters (K_m^{Br}, $K_m^{H_2O_2}$, K_{ii}^{Br}, K_{is}^{Br}) for both reactions are equal, consistent with the generation of a common intermediate, which is produced in a rate-limiting step (Everett et al., 1990b). Contrary to bromide, which is a substrate and an inhibitor, hydrogen peroxide reportedly does not inhibit V-BrPO (Wever & Kustin, 1990; de Boer & Wever, 1988; Wever et al., 1985). However, in a previous steady-state kinetic study (Everett et al., 1990b), it appeared that hydrogen peroxide could inhibit V-BrPO under certain conditions. Hydrogen peroxide can inhibit myeloperoxidase, a chloroperoxidase, although the mechanism is not known (Bakkenist et al., 1980), and horse radish peroxidase (Baynton et al., 1994; Arnao et al., 1990). In this study, we have investigated the mechanism of inhibition by the substrate hydrogen peroxide, the stoichiometry of the bromination and dioxygen formation reactions, the electrophilic versus radical nature of the bromination reaction, the competition between the bromide-assisted disproportionation of hydrogen peroxide and MCD bromination, and the origin of each oxygen atom in the dioxygen that is produced.

MATERIALS AND METHODS

Bromoperoxidase Preparation. Vanadium bromoperoxidase was isolated from *A. nodosum* collected at Kornwerderzand, Holland, in April 1989. The isolation procedure has been described previously (Everett et al., 1990b). The enzyme stock solution was stored in 50 mM HEPES, pH 7.0 or water at 0 or 4 °C.

Bromoperoxidase Activity Measurements. The standard assay for determining the specific bromoperoxidase activity of V-BrPO from *A. nodosum* is the bromination of 50 μM monochlorodimedone (MCD), which is monitored spectrophotometrically at 290 nm under conditions of 0.1 M Br^-, 2 mM H_2O_2, and pH 6.5. The change in extinction coefficient between MCD and Br-MCD is 19 900 cm^{-1} M^{-1} for pH > 5, 19 600 cm^{-1} M^{-1} at pH 5, and 18 200 cm^{-1} M^{-1} at pH 4.

Dioxygen Measurements. Rates of dioxygen formation were measured with a Yellow Springs Instrument (YSI) oxygen probe (YSI 5331) and monitor (YSI 5300). The reaction medium was sparged with nitrogen gas to minimize the dioxygen concentration so that the reaction solution and oxygen probe would not become saturated during the reaction. The reaction was initiated by the addition of V-BrPO. The rate of dioxygen formation was calculated directly from a plot of the percent dioxygen in solution versus time using an oxygen concentration of 0.247 mM in air-saturated water as the standard (Thomas et al., 1970). The reported rates were corrected for an apparent background rate of dioxygen formation or drift in the electrode of 0.2−0.6% per minute, which was always measured before the addition of V-BrPO.

Conditions for Steady-State Kinetic Analysis of Dioxygen Formation. The reaction medium for the steady-state kinetic studies consisted of 0.1 M sodium citrate at pH 4.0−5.5 or 0.1 M sodium phosphate at pH 6.0−8.0 with 0.1 M potassium bromide and sufficient sodium sulfate to produce an ionic strength of 0.97 M, unless otherwise specified. The hydrogen peroxide concentration was varied from 0.5 to 400 mM. The concentration of V-BrPO was 3−12 nM depending on the pH, unless otherwise specified.

Steady-State Kinetic Analyses. The initial rates, v, as a function of hydrogen peroxide or bromide concentration were fit to the Michaelis−Menton expression (i.e., $v = V[H_2O_2]/\{K + [H_2O_2]\}$) by an iterative process (Cleland, 1979), where V and K are functions of K_m^{Br}, $K_m^{H_2O_2}$, V_{max}, and $K_i^{H_2O_2}$ (see Kinetic Derivations below). The kinetic parameters were obtained from appropriate fits of initial rate data as a function of hydrogen peroxide and bromide concentrations using Cleland's programs COMP and NONCOMP (Cleland, 1979). All measurements were performed in at least triplicate. The figures show the least square fits to the data and not the best fit by the NONCOMP or COMP programs.

Conditions To Investigate Competition between Dioxygen Formation and MCD Bromination. MCD bromination experiments were performed using the same stock solutions of buffer, enzyme, and hydrogen peroxide as for the dioxygen formation measurements, except that the volume of the reaction mixture for the dioxygen formation measurements was three times the volume of the MCD bromination experiments (i.e., 3 and 1 mL, respectively). Unless otherwise specified, the concentration of MCD was 50 μM.

Preparation of ^{18}O-Labeled Hydrogen Peroxide and Mass Spectral Analysis of Dioxygen Produced. ^{18}O-Labeled hydrogen peroxide ($H_2{}^{18}O_2$) was prepared by a published procedure (Sitter & Terner, 1985) from ^{18}O-labeled dioxygen (Isotech, Inc.). The dioxygen produced in the bromide-assisted disproportionation of hydrogen peroxide was analyzed by mass spectral analysis. The reaction was run on a 15-mL reaction volume in a 25-mL round-bottom flask attached to a 50-mL gas collection container, which could be sealed off at the end of the reaction and attached to the mass spectrometer. The reaction solution and the apparatus were degassed under vacuum (i.e., three cycles of freeze, evacuation, and thaw). The reaction conditions were 0.1 M or 1 mM KBr in 0.1 M phosphate buffer at pH 6.0, containing approximately 60 mM $H_2{}^{18}O_2$ or a mixture of $H_2{}^{18}O_2$ and $H_2{}^{16}O_2$. The reaction was initiated by the addition of V-BrPO to a final concentration of approximately

60 nM. The concentration of hydrogen peroxide was sufficient to produce enough dioxygen to be detected by the VG mass spectrometer.

General Reagents and Procedures. The concentration of H_2O_2 was determined spectrophotometrically by the formation of triiodide (I_3^-) (Cotton & Dunford, 1973). Protein concentrations were determined by the bicinchoninic acid assay (BCA) (Smith et al., 1985), with reagents purchased from Pierce Chemical Co. MCD was purchased from Sigma. All other chemicals were reagent grade. All glassware was soaked with NoChromix (Godax), and the buffers were passed through a Bio-Rex Chelex ion exchange membrane before use. If these precautions were not followed, high rates of dioxygen formation could occur in the absence of enzyme.

Kinetic Derivations. The rate of the equation for an enzyme-catalyzed ping−pong reaction with two substrates (H_2O_2 and Br^-) is

$$v = \frac{V[H_2O_2][Br^-]}{K_m^{H_2O_2}[Br^-] + K_m^{Br}[H_2O_2] + [H_2O_2][Br^-]} \quad (1)$$

which can be cast in the form of a rectangular hyperbola for fixed values of $[H_2O_2]$:

$$v = \frac{V[Br^-]}{K + [Br^-]} \quad (2)$$

where

$$V = \frac{V_{max}}{1 + \frac{K_m^{H_2O_2}}{[H_2O_2]}} \quad (3)$$

$$K = \frac{K_m^{Br}}{1 + \frac{K_m^{H_2O_2}}{[H_2O_2]}} \quad (4)$$

Plots of $1/v$ versus $1/[Br^-]$ are linear and parallel at varying $[H_2O_2]$ since

$$\frac{1}{v} = \frac{1}{V} + \frac{K}{V[Br^-]} \quad (5)$$

Noncompetitive inhibition by hydrogen peroxide was fit to

$$v = \frac{V_{max}[Br^-]}{K_m^{Br}\left(1 + \frac{[H_2O_2]}{K_{is}^{H_2O_2}}\right) + [Br^-]\left(1 + \frac{[H_2O_2]}{K_{ii}^{H_2O_2}}\right)} \quad (6)$$

$K_m^{H_2O_2}$ is not present in eq 6 because the high H_2O_2 concentration required for H_2O_2 inhibition saturates the $K_m^{H_2O_2}$ equilibrium. The full rate equation including $K_m^{H_2O_2}$ for noncompetitive inhibition by H_2O_2 is given by eq 2,

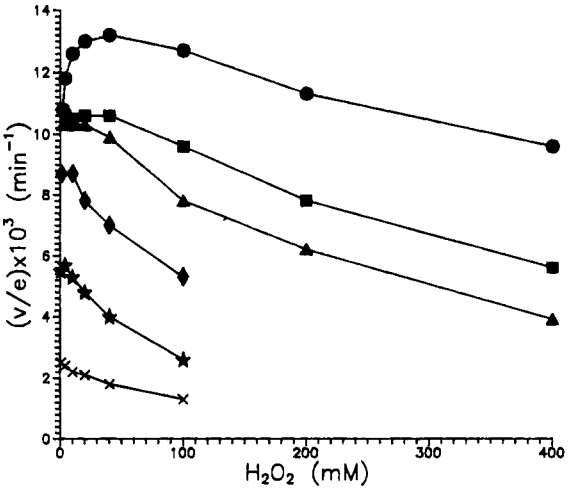


FIGURE 1: Plot of the initial steady-state rate of dioxygen formation vs hydrogen peroxide concentration at varying pH values: (●) pH 5.0, (■) pH 5.5, (▲) pH 6.0, (♦) pH 6.5, (★) pH 7.0, and (×) pH 8.0. *e* is the total enzyme concentration.

where V and K are defined by

$$V = \frac{V_{max}}{\left(1 + \frac{[H_2O_2]}{K_{ii}^{H_2O_2}}\right)\left(1 + \frac{K_m^{H_2O_2}}{[H_2O_2]}\right)} \quad (7)$$

$$K = \frac{K_m^{Br}\left(1 + \frac{[H_2O_2]}{K_{is}^{H_2O_2}}\right)}{\left(1 + \frac{[H_2O_2]}{K_{ii}^{H_2O_2}}\right)\left(1 + \frac{K_m^{H_2O_2}}{[H_2O_2]}\right)} \quad (8)$$

Thus plots of $1/v$ versus $1/[Br^-]$ at fixed $[H_2O_2]$ are linear and intersect to the left of the Y-axis. The slope of this plot is K/V:

$$\frac{K}{V} = \frac{K_m^{Br}}{V_{max}} + \frac{K_m^{Br}[H_2O_2]}{K_{is}^{H_2O_2}(V_{max})} \quad (9)$$

RESULTS AND DISCUSSION

Inhibition of V-BrPO-Catalyzed Dioxygen Formation by Hydrogen Peroxide. Addition of V-BrPO to a solution of bromide and hydrogen peroxide induces dioxygen formation via the bromide-assisted disproportionation of hydrogen peroxide (see Scheme 1). At high concentrations of hydrogen peroxide (i.e., $[H_2O_2] \gg K_m^{H_2O_2}$; Everett et al., 1990b), the rate of dioxygen formation decreases as shown in Figure 1 for pH 5.0−8.0 and at 0.1 M bromide. Under conditions of the steady-state kinetic studies (i.e., turnover for at least 10 min), the decreased rate of dioxygen formation is due to inhibition of V-BrPO (see below), although at longer turnover times and high concentration of hydrogen peroxide V-BrPO can be partially inactivated (see below).

Steady-State Mechanism of Inhibition by H_2O_2. The initial steady-state rates of dioxygen formation catalyzed by V-BrPO were investigated as a function of hydrogen peroxide concentration under conditions of inhibition (i.e., 21.7−400 mM H_2O_2), bromide concentration (5.0−54.4 mM KBr), and pH (5.5−8.0). Below pH 5.0, hydrogen peroxide inhibition is not very significant. As previously reported, double-

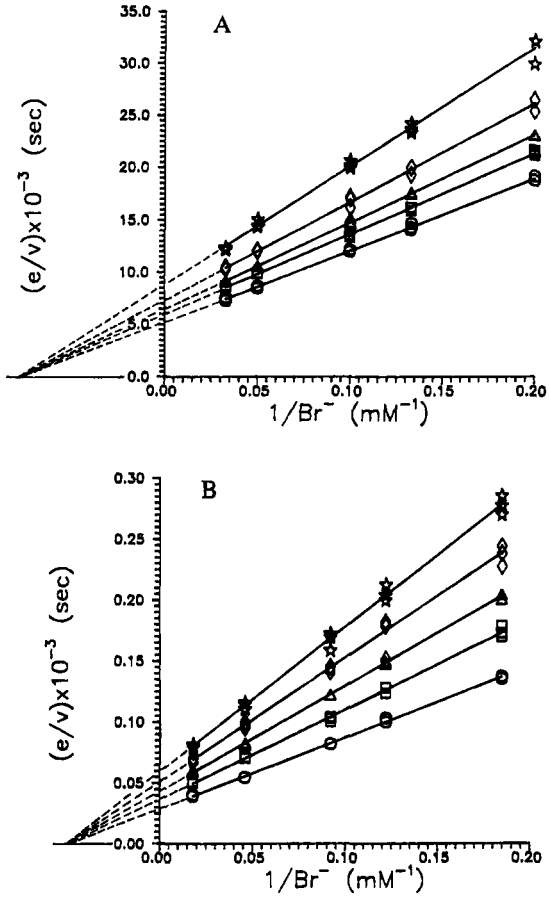


FIGURE 2: Primary double-reciprocal plot of the rate of dioxygen formation as a function of bromide concentration at fixed, high hydrogen peroxide concentration. (A) pH 5.5. In the order of increasing slope (○) 120 mM H_2O_2, (□) 180 mM H_2O_2, (△) 220 mM H_2O_2, (◇) 300 mM H_2O_2, and (☆) 400 mM H_2O_2. (B) pH 8.0. In the order of increasing slope (○) 21.7 mM H_2O_2, (□) 43.5 mM H_2O_2, (△) 65.2 mM H_2O_2, (◇) 87.0 mM H_2O_2 and (☆) 108.7 mM H_2O_2.

reciprocal plots of the initial steady-state rates of dioxygen formation versus bromide concentration at low hydrogen peroxide concentration (0.1−1.0 mM) produce a set of approximately parallel lines, which is most consistent with a ping-pong mechanism (eqs 1−5 of Materials and Methods) (Everett et al., 1990b; de Boer & Wever, 1988), although this mechanism cannot be rigorously tested because the bromide-assisted disproportionation of hydrogen peroxide reaction is not reversible. At higher fixed concentrations of hydrogen peroxide, the double-reciprocal plots produce a set of intersecting lines, which is shown in Figure 2 for pH 5.5 and 8.0. The intersection point of the double-reciprocal plots is on the *X*-axis dividing the second and third quadrants, showing that hydrogen peroxide is a noncompetitive inhibitor of V-BrPO (see eqs 2, 7, and 8 of Materials and Methods). Double-reciprocal plots of the steady-state kinetic data obtained at pH 6.0, 6.5, and 7.0 also intersect on the *X*-axis to the left of the *Y*-axis (data not shown). Table 1 shows a comparison of the values of the competitive term, $K_{is}^{H_2O_2}$, and the uncompetitive term, $K_{ii}^{H_2O_2}$, as a function of pH characterizing the bromide-assisted disproportionation of hydrogen peroxide. The inhibition constants were obtained from the best fit to Cleland's NONCOMP program (Cleland, 1979) (eq 6 of Materials and Methods).[2] For all pH conditions, the values of $K_{is}^{H_2O_2}$ and $K_{ii}^{H_2O_2}$ are identical within experimental error, indicating that H_2O_2 inhibition is not affected by bromide binding. At higher pH values,

Table 1: Kinetic Parameters for V-BrPO-Catalyzed Bromide-Assisted Disproportionation of Hydrogen Peroxide under Conditions of Inhibition by Hydrogen Peroxide[a]

pH	$K_{is}^{H_2O_2}$ [b] (mM)	$K_{ii}^{H_2O_2}$ [b] (mM)	$\sigma_{noncomp}$ (mM)	σ_{comp} (mM)	K_{is}^{comp} (mM)
5.5	309 (33)	316 (27)	0.056	0.24	45 (18)
6.0	206 (19)	240 (19)	0.070	0.32	45 (15)
6.5	101 (7)	104 (5)	0.046	0.29	20 (6)
7.0	67 (7)	63 (5)	0.041	0.95	−1530 (5180)
8.0	62 (4)	66 (4)	0.010	0.065	10 (5)

[a] The values of $K_{is}^{H_2O_2}$, $K_{ii}^{H_2O_2}$, and $\sigma_{noncomp}$ were obtained from fits to the NONCOMP program; the values of K_{is}^{comp} and σ_{comp} were obtained from fits to the COMP program (Cleland, 1979). The number in parentheses is the standard deviation. [b] The values of $K_{is}^{H_2O_2}$ and $K_{ii}^{H_2O_2}$ at pH 5.5−6.5 are ca. 10^3 larger than $K_m^{H_2O_2}$. The $K_m^{H_2O_2}$ values are [pH 5.25, 230 μM; pH 5.91, 244 μM; pH 6.52, 94 μM] (Everett et al., 1990b).

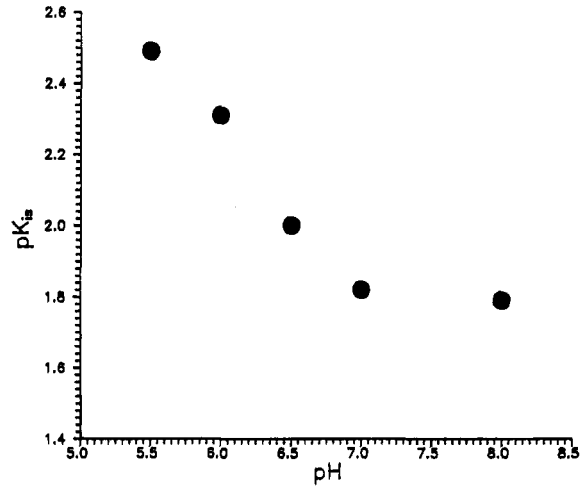


FIGURE 3: Plot of pK_{is} versus pH. (A lot of pK_{ii} versus pH is similar.)

hydrogen peroxide is clearly a much stronger inhibitor of V-BrPO than at lower pH values. A plot of pK_{is} (or pK_{ii}) versus pH (Figure 3; Dixon & Webb, 1964) shows that an ionizable group with a pK_a between 6.5 and 7 is involved in the inhibition. Histidine and vanadium(V)-coordinated water (pK_a ca. 6.3; Bonadies & Carrano, 1986; Giacomelli et al., 1982) are possible candidates. Aqueous solutions of vanadate readily coordinate 1 or 2 equiv of hydrogen peroxide. The pK_a of vanadium(V)-bound peroxide or bound hydroperoxide is not known; however, it is likely *much* lower than 6.5−7.0 since at neutral pH only the oxodiperoxovanadium(V) anion, $VO(O_2)_2^-$, exists in aqueous solution; although, one must be aware that the effect of the ligand environment of V-BrPO on the pK_a of a bound peroxide or hydroperoxide is not known. The equilibrium constant for diperoxovanadate formation is much larger at higher pH than at low pH (Harrison & Howarth, 1985). Thus, it may also be possible that the mechanism of inhibition involves diperoxide coordination to V-BrPO.[3]

Reversibility of Inhibition. The reversibility of hydrogen peroxide inhibition was investigated under the conditions of the initial steady-state kinetic experiments at pH 6.0 and 8.0. Reversible noncompetitive inhibition by hydrogen peroxide

[2] Kinetic data were also fitted to a rate law for competitive H_2O_2 inhibition using Cleland's COMP program (Cleland, 1979); however, judging from the 4−23-fold lower σ values and significantly lower errors in the kinetic parameters (Table 1), the fit was much better to the rate law for noncompetitive inhibition by H_2O_2.

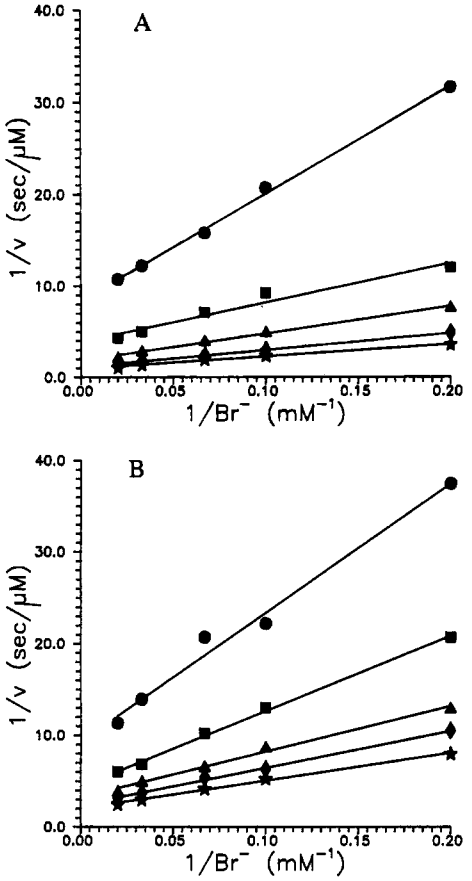


FIGURE 4: Primary double-reciprocal plot of the rate of dioxygen formation as a function of bromide concentration at specific V-BrPO concentrations. (A) 4 mM H_2O_2, pH 6.0. In the order of increasing slope (★) 6 nM V-BrPO, (♦) 4.5 nM V-BrPO, (▲) 3.0 nM V-BrPO, (■) 1.5 nM V-BrPO and (●) 0.6 nM V-BrPO. (B) 400 mM H_2O_2, pH 6.0. In the order of increasing slope (★) 7.5 nM V-BrPO, (♦) 6.0 nM V-BrPO, (▲) 4.5 nM V-BrPO, (■) 3.0 nM V-BrPO, and (●) 1.5 nM V-BrPO.

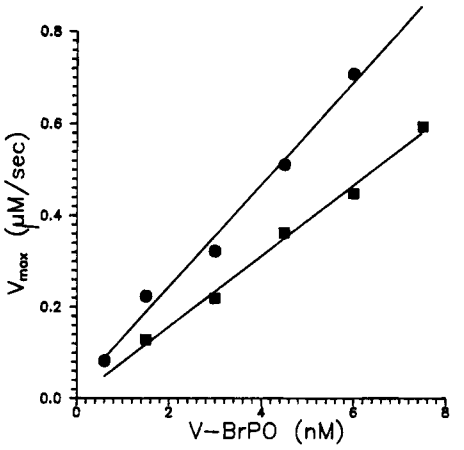


FIGURE 5: Plot of V_{max} versus V-BrPO concentration at pH 6.0: (●) 4 mM H_2O_2 and (■) 400 mM H_2O_2.

Table 2: Stoichiometry of H_2O_2 Consumed in Bromination of MCD[a]

pH	H_2O_2 (μM)	MCD (μM)	$[MCD]_{reacted}$ [b] (μM)	ratio $[H_2O_2]/[MCD]_{reacted}$
5.0	50	75	47.0 (0.3)[c]	1.06
	75	100	70.3 (1.1)	1.07
6.0	50	75	49.4 (0.5	1.01
	75	100	74.2 (0.7)	1.01
6.5[d]	25	100	25	1.00
	50	100	49	1.02
	75	100	72	1.04
7.0	50	75	39.5 (0.4)	1.26
	75	100	55.7 (1.0)	1.35
8.0	50	75	23.5 (0.4)	2.13
	75	100	32.7 (0.5)	2.29

[a] The reactions were run in 0.1 M citrate buffer at pH 5.0 containing 20 mM bromide and 0.1 M phosphate buffer, pH 6.0, containing 50 mM bromide or 0.1 M phosphate at pH 6.5, 7.0, and 8.0 containing 100 mM bromide. The V-BrPO concentration was 100 nM; the ionic strength was 0.97M. [b] The concentration of MCD reacted was calculated from the decrease in absorbance at 290 nm (see Materials and Methods). [c] The value in parentheses is the standard deviation. [d] A single determination.

can be distinguished from irreversible inactivation by hydrogen peroxide from plots of V_{max} versus enzyme concentration (Segel, 1975). The slope of the plot for a reversible noncompetitive inhibitor will be less than the control (i.e., uninhibiting $[H_2O_2]$), and both plots will intersect the origin. The slope of the plot for an irreversible inhibitor will be the same as the control, but will intersect the X-axis at the value of the enzyme concentration that is irreversibly inactivated. V_{max} was determined from the slope (see eq 9 of Materials and Methods) of double-reciprocal plots of the initial rate of dioxygen formation versus bromide concentration (5−50 mM) as a function of V-BrPO concentration at 4 mM (uninhibiting) and 400 mM (inhibiting) hydrogen peroxide, respectively, at pH 6 (see Figure 4). The replot of V_{max} versus the V-BrPO concentration (see Figure 5) shows that both sets of data intersect the origin and that the slope of the plot at 400 mM H_2O_2 is smaller than at 4 mM H_2O_2. Thus, V-BrPO is not irreversibly inactivated under the conditions of the steady-state kinetic experiments at pH 6.0. The reversibility of H_2O_2 inhibition was also established at pH 8 under conditions of 20−100 mM bromide at 2 mM H_2O_2 (uninhibiting) and 100 mM H_2O_2 (inhibiting) (data not shown). Under some conditions, partial inactivation of V-BrPO is observed at much longer turnover times (i.e., >60 min) (see below).

Stoichiometry of H_2O_2 Consumption and Origin of Oxygen Atoms in Dioxygen. It has been established previously that the stoichiometry of hydrogen peroxide consumption to dioxygen produced is 2 at pH 6.5 in the bromide-assisted H_2O_2 disproportionation catalyzed by V-BrPO (Everett et al., 1990a). The stoichiometry of H_2O_2 consumption in the MCD bromination reaction and in the bromide-assisted H_2O_2 disproportionation at other pH values has not been reported however. Table 2 shows the stoichiometry of H_2O_2 consumed during MCD bromination under conditions of limiting

[3] If one supposes that the mechanism of the bromide-assisted disproportionation of hydrogen peroxide is as follows:

$$H_2O_2 + Br^- \overset{enz}{\rightleftharpoons} H_2O + OBr^-$$

$$OBr^- + H_2O_2 \rightarrow O_2 + H_2O + Br^-$$

then one might think that HO_2^- could compete with OBr^- by binding to V-BrPO in the reverse enzymatic reaction, leading to inhibition of V-BrPO. This is unlikely for several reasons. The observed pH dependence of inhibition (i.e., an ionizable proton with a pK_a of 6.5−7) is not consistent with the pK_a of H_2O_2 (pK_a 11.6) or HOBr (pK_a 8.7). We have tried to test the reversibility of the enzymatic reaction. We have added HOBr to V-BrPO and looked for dioxygen formation with an O_2 electrode, because any H_2O_2 produced in this reverse reaction would reduce excess HOBr immediately. No dioxygen was detected under conditions of 0.11−11 mM deoxygenated OBr^-/HOBr in 0.1 M phosphate, pH 8, containing 0.2 M Na_2SO_4 to which 30 nM V-BrPO (final concentration) was added. Moreover, for the above reason, it is also not possible to test for released HOBr.

Table 3: Stoichiometry of Dioxygen Produced in Bromide-Assisted Disproportionation of Hydrogen Peroxide[a]

pH	$[H_2O_2]$ (mM)	yield of O_2 (mM)	ratio $[H_2O_2]/[O_2]$
4.0	0.25	0.125	2.0
5.0	0.25	0.13	1.9
6.0	0.25	0.14	1.8
7.0	0.25	0.14	1.8
8.0	0.25	0.12	2.1

[a] The reaction conditions are the same as those described in Table 2, except that the V-BrPO concentration was 6 nM.

Table 4: Mass Spectral Analysis of Origin of Oxygen Atoms in Dioxygen Produced by Bromide-Assisted Disproportionation of Hydrogen Peroxide[a]

$[Br^-]$ (mM)	$[H_2{}^{16}O_2]$ (mM)	$[H_2{}^{18}O_2]$ (mM)	$H_2{}^{18}O_2/H_2{}^{16}O_2$	$^{18,18}O_2/^{16,16}O_2$
100	30.6	31.9	1.04	1.06
100	21.3	42.9	2.01	1.98
1	31.9	28.8	0.90	0.85

[a] The reactions were run in 0.1 M phosphate pH 6 for 3 h at 100 mM bromide and for 6 h at 1 mM bromide. The V-BrPO concentration was 60 nM. When $H_2{}^{18}O_2$ is used solely, $^{18,18}O_2$ is produced exclusively.

H_2O_2 and excess MCD. At the end of the MCD bromination reaction (determined spectrophotometrically), 1 equiv of MCD was reacted per equivalent of hydrogen peroxide consumed (i.e., consistent with Scheme 1) in the reactions performed at pH 5.0−6.5. At pH 7.0 and 8.0, the ratio of H_2O_2 consumed to MCD reacted is greater than 1. At these higher pH values, the bromide-assisted disproportionation of hydrogen peroxide is far more competitive with MCD bromination for lower concentrations of hydrogen peroxide and for a given MCD concentration (Everett et al., 1990a). Thus, the greater ratio of $[H_2O_2]/[MCD]_{reacted}$ at pH 7.0 and 8.0 reflects the concomitantly occurring bromide-assisted catalatic reaction.

The stoichiometry of H_2O_2 consumed per equivalent of dioxygen produced is 2 at pH 4.0−8.0, as shown in Table 3. The stoichiometry is consistent with the overall reaction of hydrogen peroxide disproportionation: $2H_2O_2 \rightarrow O_2 + 2H_2O$.

The origin of the oxygen atoms in the dioxygen produced by the bromide-assisted disproportionation of hydrogen peroxide was investigated by mass spectral analysis of the dioxygen produced from ^{18}O-labeled hydrogen peroxide. The mass spectrum shows that only $^{16,16}O_2$ (MW 32 g/mol) and $^{18,18}O_2$ (MW 36 g/mol) are formed and that $^{16,18}O_2$ (MW 34 g/mol) is not formed in the V-BrPO catalyzed reaction of mixtures of $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$. The ratio of $^{18,18}O_2/^{16,16}O_2$ corrected for the background is equal to the ratio $H_2{}^{18}O_2/H_2{}^{16}O_2$ at the start of the reaction (Table 4). The absence of $^{16,18}O_2$ demonstrates that scrambling does not occur. Thus, we can conclude that both oxygen atoms in dioxygen originate from the same molecule of hydrogen peroxide. This result is consistent with singlet oxygen formation since its formation in the haloperoxidase systems must arise from a two-electron oxidation of hydrogen peroxide (Kanofsky, 1989a,b).

Competition between MCD Bromination and Bromide-Assisted Disproportionation of Hydrogen Peroxide. The stoichiometry experiments of hydrogen peroxide consumption clearly show that the dioxygen formation reaction

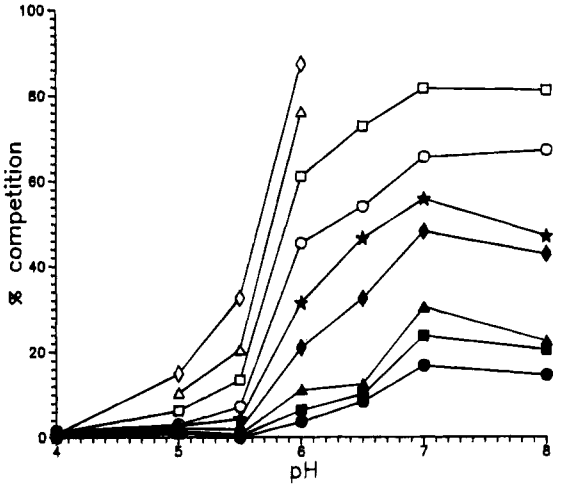


FIGURE 6: Plot of percent competition of the rate of dioxygen formation during MCD bromination versus pH at varying H_2O_2 concentration. Percent competition is defined as the ratio of the rate of dioxygen formation during MCD (50 μM) bromination to the rate of dioxygen formation in the absence of MCD. In the order of increasing H_2O_2 concentration: (●) 1 mM, (■) 2 mM, (▲) 4 mM, (♦) 10 mM, (★) 20 mM, (○) 40 mM, (□) 100 mM, (△) 200 mM, and (◇) 400 mM.

competes with MCD bromination at 75 or 100 μM MCD and at 50 or 75 μM H_2O_2 at pH 7.0 and 8.0 (Table 2). Our previous investigations indicated that, at higher H_2O_2 concentration, the competition becomes much more pronounced since the rate of dioxygen formation during MCD bromination increases relative to the rate of MCD bromination [Table 2 in Everett et al. (1990b)]. We have further investigated the competition of dioxygen formation during MCD bromination as a function of H_2O_2 concentration and pH (Figure 6). In all cases, the sum of the rate of dioxygen formation during MCD bromination and the rate of MCD bromination is equal to the rate of dioxygen formation in the absence of MCD [Table 2 in Everett et al. (1990b) for pH 6.5; data at other pH's not shown (Soedjak, 1991)], which is consistent with the fate of the intermediate being controlled by $k_1[MCD]$ and $k_2[H_2O_2]$ (Everett et al., 1990b). The percent competition, which is the ratio (times 100) of the rates of dioxygen formation during MCD bromination and dioxygen formation in the absence of MCD, increases at higher H_2O_2 concentration. The competition also increases dramatically at higher pH. The reaction pathway must reflect the effects of pH on the nature of the oxidized halide intermediate (see Scheme 1). Bray and Livingston (1928) have shown that the rate of reduction of H_2O_2 by bromine is

$$-\frac{d[H_2O_2]}{dt} = \frac{k[Br_2][H_2O_2]}{[Br^-][H^+]}$$

The inverse dependence on bromide and acid concentration arises from the rapid hydrolysis of bromine, which leads to an equilibrium mixture of hypobromous acid (HOBr), bromine (Br_2), and tribromide (Br_3^-). At lower acid concentration, the formation of $HOBr/OBr^-$ is favored. Thus, the results of Bray and Livingston (1928) show that HOBr is reduced more rapidly by H_2O_2 than Br_2 or Br_3^-. The pH dependence of the percent competition of the dioxygen formation reaction catalyzed by V-BrPO is consistent with the results of Bray and Livingston. However, the rate of hydrogen peroxide reduction of other oxidized

Table 5: V-BrPO Inactivation at High H_2O_2 Concentrations and Long Reaction Times and Reactivation of V-BrPO by Vanadate[a]

pH	% BrPO activity after H_2O_2 incubation[b] (non turnover; no Br^-)	% BrPO activity after H_2O_2 + Br^- turnover[b]	% BrPO activity after addition of vanadate to turnover-incubated sample[b,c]
4.0	86 (92)[d]	4	4
5.0	100	15	26
		35	41
5.5	100	35	92
6.0	100	50	80
		51	100
6.5	92 (100)[d]	66	85
		80	100
7.0	97 (100)[d]	67	94
		76[e]	100
8.0	100	88	100
		81	100

[a] The reaction solutions contained 0.1 M KBr, V-BrPO (4.5 nM at pH 4.0–6.5, 9 nM at pH 7.0, 18 nM at pH 8.0), and 100 mM H_2O_2 in 0.1 M citrate buffer pH 4.0–5.5 or 0.1 M phosphate buffer pH 6.0–8.0 (25 mL total volume). After 3 min, ferrous ammonium sulfate was added to a final concentration of 40 μM, and the reaction solution was concentrated by ultrafiltration (Centriprep 30, Amicon Inc.). The reaction solution was washed extensively with doubly distilled water (i.e., >5 concentration–dilution cycles). The protein concentration was redetermined by the BCA assay, and the specific activity was redetermined under the standard bromoperoxidase conditions. [b] The percent activity is the ratio of the bromoperoxidase activity of the incubated sample to the activity of the control, which is V-BrPO incubated under identical reactions without H_2O_2. The bromoperoxidase activity was determined under the standard bromoperoxidase assay conditions. [c] After ultrafiltration to remove iron, NH_4VO_3 was added (*ca.* μM). Excess vanadate was washed out with water by ultrafiltration. [d] The value in parentheses is the percent activity after addition of vanadate. [e] Incubation in 40 mM H_2O_2.

bromine species such as bromamines or other possible enzyme-bound intermediates and the pH dependencies are not known. Moreover, the dependence of the rate of MCD bromination on the nature of the brominating reagent (HOBr, Br_2, or Br_3^-) has not been reported.

Electrophilic versus Radical Bromination. To determine whether the initial product of bromide oxidation catalyzed by V-BrPO is oxidized by one or two electrons, the product of bromination of 2,3-dimethoxytoluene (DMT) was determined. DMT reacts with bromine radical ($Br^\bullet$) to give 2,3-dimethoxybenzyl bromide and with Br^+ to give ring-substituted bromo-2,3-dimethoxytoluene (Volhardt, 1987; Clague & Butler, 1995). Only a single product, bromo-2,3-dimethoxytoluene, was observed at pH 6.5 and pH 4 under the following conditions: 4.5 nM V-BrPO, 10 mM or 100 mM H_2O_2, 0.1 M KBr, and 6 mM DMT, in 0.1 M Hepes buffer, pH 6.5/10% EtOH, and 4.5 nM V-BrPO, 10 mM or 100 mM H_2O_2, 0.1 M KBr, and 6 mM DMT, in 0.1 M citrate buffer, pH 4/10% EtOH. Thus, bromination occurs by an electrophilic mechanism and not a radical mechanism. At pH 8, under conditions of 10 mM or 100 mM H_2O_2 in 0.1 M Tris buffer, neither product was observed because all of the H_2O_2 is consumed in the bromide-assisted disproportionation of H_2O_2.

Inactivation of V-BrPO. As established above, H_2O_2 is a noncompetitive inhibitor of V-BrPO, when the steady-state kinetics are observed during the initial portion of the reaction (e.g., first 10 min at the highest concentrations of H_2O_2). If the reaction proceeds for longer times, the specific activity of V-BrPO decreases. The activity loss was investigated under conditions where unreacted H_2O_2 could be destroyed at specific time intervals by the addition of ferrous ammonium sulfate. After iron addition, the reaction solution was washed extensively with water by ultrafiltration, and the specific activity of the enzyme sample was redetermined (Table 5). At pH 4.0 and 5.0, the inactivation of V-BrPO is rapid and irreversible. The inactivation is independent of H_2O_2 concentration since little or no inhibition occurs below pH 5.5 (data not shown). At pH 6.0, V-BrPO is inactivated by approximately 50%; however, the inactivation can be substantially reversed by the addition of vanadate (Table 5). At pH 6.5–8.0, the percent inactivation is even less than at pH 4.0–6.0, and the activity can be fully restored by the addition of vanadate (Table 5). Turnover is required for inactivation at all pH values since incubation of V-BrPO with 0.1 M H_2O_2 does not cause much, if any, inactivation (Table 5). The increase in activity upon the addition of vanadate could suggest that protein-bound vanadium can be released under turnover with very high H_2O_2 concentration forming the apoenzyme derivative. It is also conceivable that an inactive form of protein-bound vanadium is generated (e.g., reduced), which is reversed by the addition of aqueous vanadate (e.g., oxidized to the active V(V) state). It has been reported that VO^{2+}-BrPO is an inactive derivative (de Boer et al., 1988).

Several possibilities may explain the irreversible inactivation of V-BrPO that occurs at low pH. For example, a critical amino acid residue may be oxidized, such as a histidine residue. Hydrogen peroxide irreversibly inactivates Cu,Zn-superoxide dismutase in the presence of high concentrations of H_2O_2 (Hodgson & Fridovich, 1975), through the oxidation of a histidine forming 2-oxohistidine (Uchida & Kawakishi, 1994). In this system, His-118, which is a ligand to Cu in the bovine enzyme, is oxidized quantitatively; other histidine residues are also partially oxidized. Alternatively, vanadate is known to oxidize serine (Mogel & McFadden, 1989) and to cleave peptides upon photolysis (Grammar et al., 1988); however, our preliminary studies do not indicate that V-BrPO is cleaved (Meister and Butler, work in progress). The nature of the inactive, modified V-BrPO derivative is under investigation.

CONCLUSIONS

In this study, we have established that hydrogen peroxide is a noncompetitive inhibitor of V-BrPO (Figure 2). The equivalence of $K_{is}^{H_2O_2}$ and $K_{ii}^{H_2O_2}$ (Table 1) indicates that H_2O_2 inhibition is not affected by bromide binding. Hydrogen peroxide is a stronger inhibitor at high pH, which may reflect the larger formation constants of diperoxovanadate complexes at high pH versus low pH. The inhibition by hydrogen peroxide is completely reversible under the condi-

tions of the steady-state kinetic experiments (Figure 5) (i.e., pH 5.5−8.0; <10 min at high [H_2O_2]). On the other hand, irreversible inactivation of V-BrPO occurs during turnover at pH 4.0 and 5.0 at high hydrogen peroxide conditions. At higher pH values, significant inactivation also occurs at longer reaction times, although the inactivation is fully reversed by treatment with vanadate (Table 5). The inverse relationship between inactivation and inhibition as a function of pH shows that H_2O_2 inhibition protects V-BrPO from inactivation. We have also demonstrated for the first time that each oxygen atom in dioxygen originates from the same molecule of hydrogen peroxide (Table 4) and that the V-BrPO-catalyzed bromination reaction is electrophilic and not a radical process. These results are consistent with previous results on the mechanism of singlet oxygen formation (Everett et al., 1990a; Kanofsky, 1989a,b) and prior ^{18}O isotopic labeling studies of the reaction of oxidized halogen species with H_2O_2 (Cahill & Taube, 1952).

Under certain conditions, the rate of MCD bromination is also inhibited by hydrogen peroxide [Table 2 in Everett et al. (1990b)], contrary to previous reports (Wever & Kustin, 1990; de Boer & Wever, 1988; Wever et al., 1985). The decrease in the rate of MCD bromination at increasing hydrogen peroxide concentration is greater than the decrease in the rate of dioxygen formation in the absence of MCD. The apparent greater inhibition of MCD bromination by H_2O_2 reflects the fact that a significant fraction of the hydrogen peroxide is consumed in the bromide-assisted catalytic reaction, producing dioxygen (i.e., the $k_2[H_2O_2]$ pathway, Scheme 1), as established by stoichiometric (Table 2) and kinetic (Figure 6) results. At all pH values investigated (i.e., pH 4.0−8.0), the rate of dioxygen formation during MCD bromination actually increases as the H_2O_2 concentration is increased over a range in which H_2O_2 inhibition is observed in the absence of MCD [Table 2 in Everett et al. (1990b)]. This apparent lack of H_2O_2 inhibition reflects the fact that inhibition can only be observed in the rate limiting-steps that are involved in the production of the common intermediate (Scheme 1). The fate of the intermediate is determined by the relative value of $k_2[H_2O_2]$ versus k_1[MCD], and both of these reactions are much faster than the formation of the intermediate. Even at very high H_2O_2 concentration, the sum of the rates of dioxygen formation during MCD bromination and MCD bromination is equal to the rate of dioxygen formation in the absence of MCD, which accounts for the total inhibition. Clearly, hydrogen peroxide inhibition of MCD bromination is complicated by the competing dioxygen formation reaction. Thus, the bromide-assisted disproportionation of hydrogen peroxide is a more reliable reaction to investigate the mechanism of H_2O_2 inhibition of V-BrPO.

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BI951126P