Evidence for Organic Substrate Binding to Vanadium Bromoperoxidase

Richard A. Tschirret-Guth and Alison Butler*

Department of Chemistry
University of California
Santa Barbara, California 93106

Received September 20, 1993

Vanadium-containing bromoperoxidases (V-BrPO) are unique enzymes isolated primarily from marine algae.[1-4] Halogenated natural products, including chiral halogenated terpenes[5] and indoles,[6] are abundant in marine organisms, and the biosyntheses of these products are thought to be mediated by haloperoxidases. The role of haloperoxidase in direct halogenation or in production of diffusible oxidized bromine species (HOBr, Br_2, Br_3^-) is a topic of much current interest.[1-4] V-BrPO catalyzes the peroxidative halogenation (Cl^-, Br^-, I^-) of organic substrates and the halide-assisted disproportionation of hydrogen peroxide, forming dioxygen (Scheme 1).[7-9] Under conditions under which V-BrPO reactions are usually carried out [i.e., pH 6.5 (refs 1–4 and references therein)], oxidized bromine species (e.g., HOBr, Br_2, Br_3^-, Enz-Br) cannot be detected because the reaction of these species with H_2O_2 or organic substrates is too fast. However, in the absence of an organic substrate, Br_3^- was detected under conditions (i.e., pH 5)[10] which stabilized it with respect to formation of HOBr and Br_2 or oxidation of H_2O_2. We now report the first evidence that V-BrPO can bind certain organic substrates (i.e., indoles) and that the active brominating moiety under these conditions is not an enzyme-released bromine species (e.g., HOBr, Br_2, Br_3^-).

V-BrPO catalyzes the bromination of 2-methylindole, 2-phenylindole, and phenolsulfonephthalein (phenol red) to 3-bromo-2-methylindole,[11] 3-bromo-2-phenylindole,[11] and 3′,3″,5′,5″-tetrabromophenolsulfonephthalein (bromophenol blue),[12] respectively. In a mixture of 2-methylindole and phenol red, V-BrPO preferentially brominates 2-methylindole, as shown by the lag phase in the appearance of bromophenol blue (Figure 1). The lag phase increases with increasing concentration of 2-methylindole, although the rate of bromination of phenol red remains independent of the 2-methylindole concentration. By comparison, a lag phase is not observed in the competitive bromination of2-methylindole and phenol red by HOBr (Figure 2). Under these conditions, bromination of 2-methylindole and phenol red occurs concurrently and an increase in the 2-methylindole concentration leads to a decrease in the appearance of bromophenol blue. This differential reactivity between V-BrPO and HOBr suggests that HOBr is not the active brominating species in the V-BrPO-catalyzed reactions of 2-methylindole, a situation which could arise by indole binding to V-BrPO (see below).

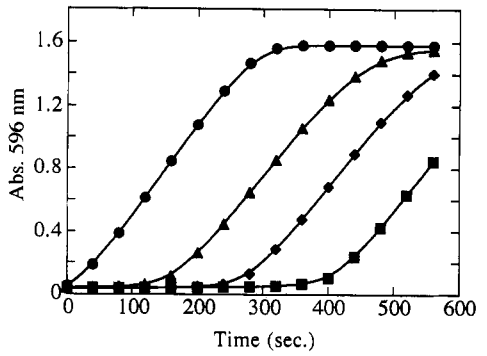


Figure 1. Time course of the bromination of phenol red by by V-BrPO as a function of 2-methylindole concentration. Concentration of 2-methylindole: ●, 0 M; ▲, 28.8 μM; ◆, 57.6 μM; ■, 86.4 μM. Conditions: 25.3 μM phenol red, 40 mM KBr, 0.416 mM H_2O_2, 2.5 nM V-BrPO in 0.08 M phosphate buffer, pH 6.5 with 20% ethanol; the reaction was initiated by the addition of V-BrPO. V-BrPO was isolated from the marine brown alga *Ascophyllum nodosum* as previously described.[1]

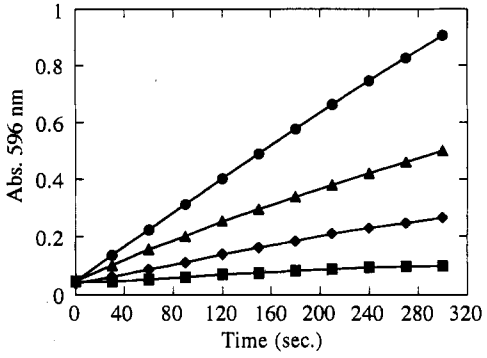
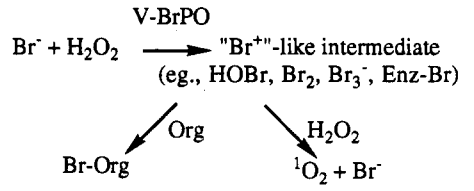


Figure 2. Bromination of phenol red by HOBr as a function of 2-methylindole concentration. Concentration of 2-methylindole: ●, 0 M; ▲, 28.8 μM; ◆, 57.6 μM; ■, 86.4 μM. Conditions: 25.37 μM phenol red, 40 mM KBr, 0.416 mM H_2O_2, in 0.08 M phosphate buffer, pH 6.5 with 20% ethanol. Ten aliquots of 10 μL each of a 3.63 mM NaOBr stock solution were added successively at 30-s intervals to the initial 1-mL reaction solution, and the absorbance was measured 20 s after each addition. The stock solution of NaOBr was prepared by dilution of bromine vapors into 0.07 M NaOH and standardized by I_3^- formation (λ_{max} = 353 nm, ϵ = 26 000 M^{-1} cm^{-1} determined in 0.1 M acetate, pH 4.5 with 0.1 M KI). The slight curvature is due to the dilution from addition of NaOBr.

Scheme 1



Further evidence that the enzyme-catalyzed bromination of indole is not mediated by enzyme-released HOBr comes from a comparison of the rate of V-BrPO-catalyzed bromide-assisted disproportionation of hydrogen peroxide (forming O_2) in the presence and absence of 2-methylindole *versus* the rate of oxidation of H_2O_2 by HOBr (forming O_2) in the presence and absence of 2-methylindole. The normalized rate of V-BrPO-catalyzed O_2

* To whom correspondence should be addressed.

(1) Butler, A.; Walker, J. V. *Chem. Rev.* **1993**, *93*, 1937–1944.
(2) Butler, A. In *Bioinorganic Catalysis*; Reedijk, J., Ed.; Marcel Dekker: New York, 1992; pp 425–445.
(3) Wever, R.; Kustin, K. *Adv. Inorg. Chem.* **1990**, *35*, 81–115.
(4) Wever, R.; Krenn, B. E. In *Vanadium in Biological Systems*; Chasteen, N. D., Ed.; Kluwer, Academic Publishers: Dordrecht, The Netherlands 1990; pp 81–98.
(5) Faulkner, D. J. *Nat. Prod. Rep.* **1984**, *1*, 551–598. Faulkner, D. J. *Nat. Prod. Rep.* **1986**, *3*, 1–33. Faulkner, D. J. *Nat. Prod. Rep.* **1987**, *4*, 539–576. Faulkner, D. J. *Nat. Prod. Rep.* **1988**, *5*, 613–663. Faulkner, D. J. *Nat. Prod. Rep.* **1990**, *7*, 269–309. Faulkner, D. J. *Nat. Prod. Rep.* **1991**, *8*, 97–147. Faulkner, D. J. *Nat. Prod. Rep.* **1992**, *9*, 323–364. Faulkner, D. J. *Nat. Prod. Rep.* **1993**, *10*, 497–539 and references therein.
(6) Norton, R. S.; Wells, R. J. *J. Am. Chem. Soc.* **1982**, *104*, 3628–3635.
(7) Everett, R. R.; Kanofsky, J. R.; Butler, A. *J. Biol. Chem.* **1990**, *265*, 4908–4914.
(8) Soedjak, H. S.; Butler, A. *Inorg. Chem.* **1990**, *29*, 5015–5017.
(9) Everett, R. R.; Soedjak, H. S.; Butler, A. *J. Biol. Chem.* **1990**, *265*, 15671–15679.
(10) de Boer, E.; Wever, R. *J. Biol. Chem.* **1988**, *263*, 12326–12332.
(11) 3-Bromo-2-methylindole and 3-bromo-2-phenylindole were identified by mass spectral analyses and ^1H and ^{13}C NMR. The absorbance change between 2-methylindole and 3-bromo-2-methylindole is too small to allow measurement of the rate of bromination of 2-methylindole spectrophotometrically.
(12) Neidleman, S. L.; Geigert, J. *Biohalogenation*; Ellis Horwood Ltd Press: Chichester, 1986.

0002-7863/94/1516-0411$04.50/0

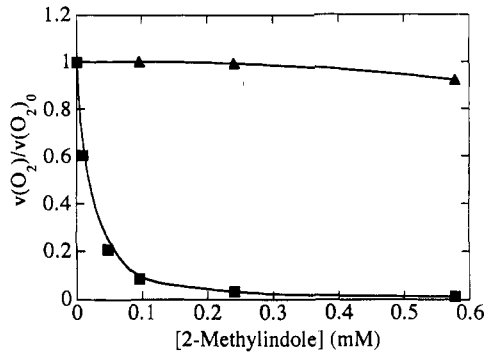


Figure 3. Normalized rate of dioxygen formation in the presence of 2-methylindole. ■, V-BrPO; ▲, HOBr. Conditions: 40 mM KBr, 10 mM H_2O_2, in 0.08 M phosphate buffer, pH 6.5 with 20% ethanol. In the enzyme reactions, V-BrPO was 9.7 nM; under these conditions and in the absence of O_2, the rate of O_2 formation was 49 μM/min. In the HOBr reactions, HOBr was added by syringe pump at a rate of 31 μM/min. O_2 formation was monitored with a YSI oxygen probe.

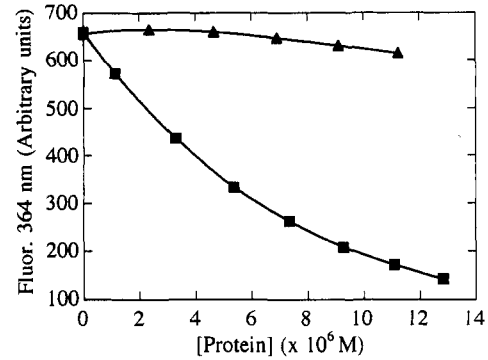


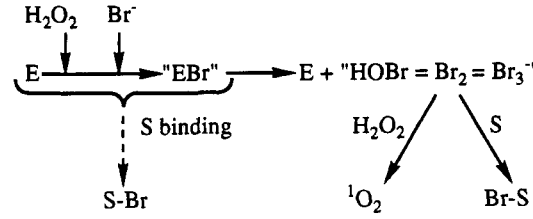
Figure 4. Fluorescence quenching of 2-phenylindole by vanadium bromoperoxidase. ■, V-BrPO; ▲, bovine serum albumin; $\lambda_{excitation}$, 314 nm using a Perkin-Elmer LS50 fluorimeter. The experiment was carried out at 21 °C by addition of 20-μL aliquots of a 97 μM V-BrPO stock solution or 20-μL aliquots of a 202 μM BSA stock solution to 1.7 mL of 0.57 μM 2-phenylindole in 0.1 M tris buffer, pH 8.13.

formation in the presence and absence of 2-methylindole [i.e., $v(O_2)/v(O_2)_0$, where $v(O_2)_0$ is the initial rate of O_2 formation in the absence of organic substrate] decreases as the concentration of 2-methylindole increases (Figure 3), showing that indole bromination is favored over H_2O_2 oxidation. To compare the enzymatic reaction to that using HOBr, hypobromite was added to the H_2O_2 solution by syringe pump at a rate adjusted to match the activity of V-BrPO. In distinct contrast to V-BrPO, the normalized rate of O_2 formation from the oxidation of H_2O_2 by HOBr in the presence and absence of 2-methylindole is constant [$v(O_2)/v(O_2)_0 \cong 1$] as the concentration of 2-methylindole is increased (Figure 3), indicating that HOBr preferentially oxidizes H_2O_2 and that enzyme reaction does not occur by released HOBr. The same kinetic behavior is observed for 2-phenylindole (data not shown).

Indole binding to V-BrPO is further indicated by quenching of the fluorescence of 2-phenylindole by V-BrPO (Figure 4). By contrast, bovine serum albumin, a protein of molecular weight similar to that of V-BrPO, did not quench the fluorescence of 2-phenylindole (Figure 4). Apo-V-BrPO also quenches the fluorescence of 2-phenylindole, indicating that indole binding does not require active-site bound vanadium.

In summary, these results show that the bromination reactivity of V-BrPO toward substituted indoles is not consistent with enzyme-released HOBr. These results are also the first demonstration that organic substrates can bind to V-BrPO from both competitive kinetic results and fluorescence quenching results. A mechanistic scheme for V-BrPO involving substrate binding is proposed in Scheme 2; V-BrPO binds H_2O_2 and Br^-, leading to a putative "enzyme-bound" or "enzyme-trapped" brominating

Scheme 2



moiety, EBr, which in the absence of an indole releases HOBr (or other bromine species, e.g., Br_2, Br_3^-). When indole is present, it binds to V-BrPO, preventing release of an oxidized bromine species and leading to indole bromination. The present studies do not address whether O_2 formation in the presence of the indole could arise from reaction of the substrate-bound-EBr species with H_2O_2 or only by reaction of the released species with H_2O_2. We are extending these studies to investigate how the nature of other organic substrates directs the reactivity of V-BrPO.

Acknowledgment. A.B. is grateful for support from the NSF (DMB90-18025), and the A. P. Sloan Foundation. This work is funded in part by a grant to A.B. from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under Grant No. NA 89AA-D-SG138, Project No. R/MP-53 through the California Sea Grant College, and in part by the California State Resources Agency. R.T.-G. is a Sea Grant Trainee. We thank J. T. Groves, Princeton University, for useful discussions.