Characterization of the Catalytic Properties of Bromoperoxidase†

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ABSTRACT: Bromoperoxidase from the marine green alga *Penicillus capitatus* is a soluble heme protein capable of catalyzing the peroxidation of a wide variety of organic and inorganic substrates. Bromoperoxidase at neutral pH has high specific activity for bromide and iodide oxidations. The V_{max} values for bromide and iodide oxidations are 5900 and 35 000 mol min^{-1} (mol of BPO)$^{-1}$, respectively. At acidic pH, bromoperoxidase catalyzes the oxidation of chloride ion. Radiolabeling experiments demonstrate the ability of the enzyme to catalyze chloride ion oxidation and insertion of chloride into the halide acceptor molecule monochlorodimedone. The rates of several reactions catalyzed by bromoperoxidase are strongly enhanced by bromide and chloride ions. The rate of molecular bromine formation is nearly twice the rate of bromination of monochlorodimedone. Thus, bromination is proposed to occur via molecular bromine rather than via an enzyme-bound intermediate. The pH–rate profiles for bromide and chloride ion oxidations provide new insights for possible control mechanisms for halide ion peroxidations.

Bromoperoxidase purified from the marine alga *Penicillus capitatus* is a heme-containing peroxidase that shares many spectral and catalytic properties with other plant and mammalian peroxidases. The spectra of the oxidized and reduced derivatives of bromoperoxidase closely resemble the spectra of those peroxidases which have a proximal histidine ligand to the heme iron. In the presence of peroxides, bromoperoxidase forms the two classical peroxidase intermediates, compounds I and II, which contain 2 and 1 oxidizing equiv above native enzyme. These intermediates are capable of proceding through the one-electron donor peroxidative cycle shown in eq 1–4.

$$\text{native enzyme} + \text{ROOH} \rightarrow \text{compound I} + \text{ROH} \quad (1)$$

$$\text{compound I} + \text{AH} \rightarrow \text{compound II} + \text{A}^{\bullet} \quad (2)$$

$$\text{compound II} + \text{AH} \rightarrow \text{native enzyme} + \text{H}_2\text{O} + \text{A}^{\bullet} \quad (3)$$

$$2\text{A}^{\bullet} \rightarrow \text{products} \quad (4)$$

Unlike one-electron donors, substrates such as hydrogen peroxide, sulfite, ethanol, formic acid, and halide ions donate two electrons directly to compound I. These reactions produce native enzyme without generating detectable levels of compound II. One of the distinguishing features between the various peroxidase enzymes is the difference in their abilities to catalyze the peroxidation of the two-electron substrates. With regard to the halide ions, only myleoperoxidase, eosinophil peroxidase, and various chloroperoxidase enzymes have thus far been shown to be able to catalyze the oxidation of chloride as well as bromide and iodide ions (Harrison & Schultz, 1976; Stelmaszynska & Zgliczynski, 1974; Morris & Hager, 1966b; Wever et al., 1981; Liu et al., 1987; Weisner et al., 1986). Evidence presented in this paper shows that bromoperoxidase purified from *P. capitatus* is also capable of catalyzing chloride oxidation at acid pH values. A previous study (Manthey & Hager, 1981) failed to detect chlorination activity with bromoperoxidase because the pH optimum for bromination, the preferred halide substrate for bromoperoxidase, is pH 6. In contrast, the pH optimum for chlorination by bromoperoxidase is pH 3.7. The enzyme does not catalyze detectable levels of chlorination at neutral pH and does not catalyze detectable bromination at pH 3.

Materials and Methods

Enzyme Preparation. The purification of bromoperoxidase was performed as previously reported (Manthey & Hager, 1981; Manthey et al., 1984). All enzyme preparations had *RZ* values of 0.80 or greater. Enzyme concentrations were based on heme content, using an extinction coefficient of 106 000 M^{-1} cm^{-1} at 412 nm.

2-Thiouracil Oxidation. Assays monitoring the oxidation of 2-thiouracil were based on those previously described (Morris & Hager, 1966a). Turnover rates were calculated by using an extinction coefficient of 12 000 M^{-1} cm^{-1} at 278 nm as previously reported (Elion et al., 1946).

Rate Comparisons for Tribromide Formation and Monochlorodimedone Bromination. Rates of monochlorodimedone (MCD)[1] bromination were measured by the decrease in absorbance at 278 nm as a function of time using a stopped-flow spectrophotometer (Gibson & Milnes, 1964). The changes in the MCD concentrations were calculated by using the extinction coefficient of 12 200 M^{-1} cm^{-1} at 278 nm (Morris & Hager, 1966b). The spectrophotometer was interfaced with a DEC LSI-11 computer for data analysis. In separate experiments, rates of tribromide (Br_3^-) formation were measured as an increase in absorbance at 267 nm in the millisecond time range. The concentrations of oxidized bromine species (Br_2 + Br_3^-) were calculated by using the extinction coefficient for Br_3^- at 267 nm and the value of the equilibrium constant for the formation of Br_3^- from Br_2 and Br^-. These values are 3.64 × 10^4 M^{-1} cm^{-1} and 14.4 M^{-1}, respectively (Libby et al., 1982). Hydrolysis of Br_2 and Br_3^- was negligible under the assay conditions (Liebhafsky, 1939, 1935). The reaction conditions were identical for the two sets of assays. Molar concentrations of Br^- were used to maximize the levels of Br_3^- formation. The reactions were run in potassium citrate buffers at pH 4.2 in order to minimize the level of enzyme inactivation seen at higher pH. Hydrogen peroxide concentrations were measured

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[1] Abbrevations: MCD, monochlorodimedone; BPO, bromoperoxidase.

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by I_3^- formation (Cotton & Dunford, 1973). This technique also was used to measure bromine concentrations since bromine effectively oxidizes iodide to I_3^-.

Rate Measurements of Single Electron Donor Substrate Peroxidation Reactions. The peroxidation of pyrogallol was measured as an increase in 430-nm absorbance. The reaction mixtures contained 2.7 mL of buffer having an ionic strength of 0.20, 3.5 μmol of hydrogen peroxide, appropriate levels of bromoperoxidase, and 5.0 μmol of pyrogallol. Assay mixtures for guaiacol oxidations measured at 470 nm were the same except for the substitution of 0.10 mL of a guaiacol-saturated water solution for the pyrogallol. The rates of hydroquinone peroxidations were measured by the decrease in absorbance at 288 nm. Assay mixtures contained 2.7 mL of buffer, 0.2 μmol of hydroquinone, 0.4 μmol of hydrogen peroxide, and appropriate levels of bromoperoxidase. The buffers used for the peroxidation reactions were potassium citrate buffer in the pH range 3.5–6.2 and potassium phosphate in the pH range 6.2–7.8.

MCD Chlorination. The rate of MCD chlorination was measured in an identical manner as for MCD bromination. The reaction mixtures included 2.7 mL of 0.10 M potassium citrate buffer, pH 3.7, approximately 1.0 nmol of bromoperoxidase, 0.78 μmol of hydrogen peroxide, 0.21 μmol of MCD, and 0.3 mmol of KCl. Enzymatic synthesis of [^{36}Cl]dichlorodimedone was carried out in a 4-mL reaction mixture containing 0.10 M potassium citrate buffer, pH 4.2, 0.62 μmol of MCD, 15 μmol of sodium [^{36}Cl]chloride (2.5×10^5 μCi/mol), 1.5 μmol of hydrogen peroxide, and bromoperoxidase. At various time points during the reaction, 200-μL aliquots of the reaction mixture were removed and mixed with approximately 1 mL of ether. The ether layers were subsequently removed and dried under a stream of nitrogen. The dried extracts containing [^{36}Cl]dichlorodimedone were dissolved in 20 μL of ethanol and spotted on silica gel TLC plates.

Separation of Dichlorodimedone and MCD by Thin-Layer Chromatography. Authentic MCD, dichlorodimedone, and the ether extracts obtained from experimental reaction mixtures were applied to silica gel thin-layer chromatography plates. The plates were developed by using a mobile phase of ethyl acetate/hexane/glacial acetic acid (60:40:2) (Hager et al., 1966). Under these conditions, the R_f values were 0.68 for MCD and 0.82 for dichlorodimedone. Unlabeled standards were visualized by using a long-wavelength ultraviolet lamp and by iodine adsorption. Radiolabeled spots were visualized by developing autoradiograms of the silica gel plates.

Results

Bromoperoxidase catalyzes a wide range of peroxidative reactions which can be classified into three general groups. The primary class of reactions involves halide ion oxidations and the associated reactions accompanying molecular halogen formation. The second and third groups of reactions involve catalatic O_2 evolution from hydrogen peroxide and free radical peroxidations, respectively. The presence of halide ions strongly affects the rates of the catalase-type reactions. The pH–rate dependencies have been measured to detect the presence of catalytically important amino acid residues near the enzyme active site.

Catalase Activity of Bromoperoxidase. Bromoperoxidase, like chloroperoxidase, catalyzes the classic catalase reaction with the formation of O_2 and water from two molecules of hydrogen peroxide. This reaction is termed the catalatic reaction. Values for the second-order rate constants of the catalatic reactions of bromoperoxidase were constant between pH 4.2 and 9.9. The average value of k_2 for bromoperoxidase, 2.42×10^{-3} M^{-1} s^{-1}, is approximately only 0.1% the rate observed with catalase (Chance, 1952) and 10% the rate observed with chloroperoxidase (Thomas et al., 1970a). Enzyme inactivation occurred during the later portions of the bromoperoxidase assays; hence, only initial portions of the time courses were used for the determination of the k_1 values.

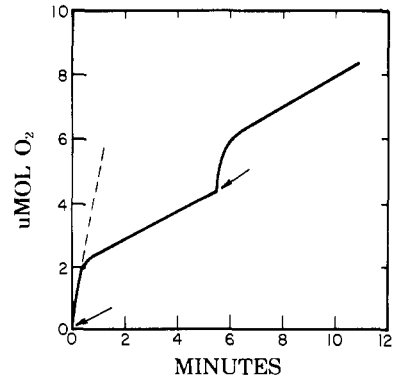

FIGURE 1: Bromide-enhanced oxygen evolution. The curves show the formation of O_2 as a function of time for the biphasic Br^--enhanced catalatic reaction of bromoperoxidase. The reaction mixture contained 150 μmol of potassium citrate buffer, pH 5.57, 4.5 μmol of hydrogen peroxide, 15 μmol of KBr, and 30 pmol of bromoperoxidase in a total volume of 1.5 mL. Arrows indicate the addition of the first and second aliquots of bromoperoxidase.

Halide-Enhanced Catalatic Reaction. Addition of either Br^- or Cl^- greatly enhances the catalatic reaction rates of bromoperoxidase. The rate enhancement with Br^- is greatest at neutral pH and decreases rapidly at acidic pH. In contrast, Cl^- enhancement is greatest at acidic pH and decreases with increased pH (data not shown). At low Br^- concentrations, the Br^--enhanced catalatic reactions of bromoperoxidase are biphasic, whereas at high Br^- concentrations the reaction time courses are no longer biphasic, but rather show approximately exponentially decreasing initial rates. A typical time course for the biphasic reaction at low Br^- concentrations is presented in Figure 1. Addition of a second aliquot of enzyme to the reaction during the slow phase produces a new burst of activity. In contrast, addition of peroxide to the reaction during the slow phase produced only a slight amount of additional O_2 evolution. The slow phase can be accounted for by enzyme inactivation during the catalatic reaction.

Similar biphasic reaction time courses are also observed for bromoperoxidase-catalyzed peroxidation of single electron donors. In these cases, the biphasic time courses are attributed to the accumulation of steady-state levels of a catalytically inactive compound III species (Manthey & Hager, 1985). A combination of chemical inactivation by bromine compounds and accumulation of compound III may account for the biphasic kinetics in the above Br^--enhanced catalatic reactions.

When the catalatic reaction was carried in the presence of high concentrations of bromide (1 M), transient high concentrations of Br_3^- were detected. Under these conditions, rates of formation of oxygen from hydrogen peroxide decrease exponentially. The optical spectrum of the Br_3^- matched the spectrum previously reported (Libby et al., 1982). Relatively stable levels of Br_3^- were formed at lower pH in citrate buffer.

These results are consistent with the conclusion that bromoperoxidase oxidizes bromide ion to molecular bromine as the primary product in the catalatic reaction. In the presence of high concentrations of bromide, molecular bromide is rapidly converted to tribromide ion which reacts with a second molecule of hydrogen peroxide to generate dioxygen. The kinetics of oxygen evolution in this reaction are indistinguishable from the kinetics of the catalase reaction. However,

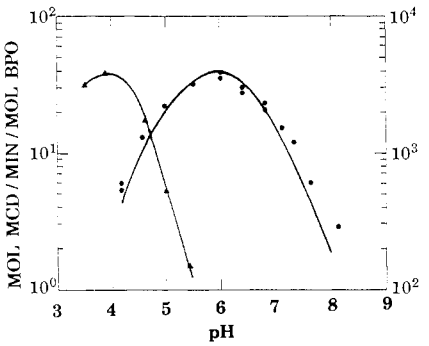

FIGURE 2: pH–rate profiles for MCD bromination and chlorination. The curves plot the rate of MCD bromination (●) and chlorination (▲) as a function of pH. The reaction mixtures contained 2.7 mL of buffer, 0.24 μmol of monochlorodimedone, 0.39 μmol of hydrogen peroxide, 300 μmol of KBr or KCl, and appropriate amounts of bromoperoxidase. All buffers were 150 μmol of potassium citrate in the pH range 3.5–6.2 and 150 μmol of potassium phosphate in the pH range 6.2–7.8. The left and right y axes relate respectively to chlorination and bromination activity.

at low bromide ion concentrations, the bromide oxidation product is hypobromous acid (or hypobromate, depending on the pH). Hypobromous acid either could be formed directly as the initial oxidation product or could arise from the hydrolysis of molecular bromine (eq 5).

$$Br_2 + H_2O \rightarrow HBrO + H^+ + Br^- \quad (5)$$

Hypobromous acid is a strong oxidant, both of hydrogen peroxide (eq 6) and of the enzyme. Thus, the fast phase of

$$HBrO + H_2O_2 \rightarrow H^+ + Br^- + O_2 + H_2O \quad (6)$$

the reaction shown in Figure 1 probably represents an initial rapid pulse of hypobromous acid formation. Hypobromous acid in turn is rapidly utilized to generate O_2 from hydrogen peroxide or possibly reacts with amino acid residues at the enzyme active site. By the end of the initial fast phase, all the hypobromate has been reduced, dioxygen has been formed, and a considerable amount of enzyme inactivation has occurred. No details concerning the chemical inactivation of bromoperoxidase in these reactions are known.

pH–Rate Profile of MCD Bromination. The steady-state pH–rate profile of MCD bromination and chlorination catalyzed by bromoperoxidase is shown in Figure 2. The pH optimum for bromination at high levels of bromide ion occurs at pH 6.0. The kinetic data fit eq 7 which describes the

$$V = \frac{V_{max}}{1 + [H^+]/K_a + K_b/[H^+]} \quad (7)$$

presence of two catalytically important ionizations (Alberty & Massey, 1954). The curve shows that for optimal activity, protonation of an ionization group with a pK_a of 6.4 and the deprotonation of a more acidic group near pK_a 5.3 are required. The value for the maximum reaction velocity, V_{max}, is defined as 5900 mol of MCD min^{-1} (mol of BPO)$^{-1}$. Values for the kinetic and ionization contants were derived by assigning values in the pH–rate equations to generate best-fit curves.

MCD is readily brominated by Br_3^-. Since bromoperoxidase rapidly catalyzes tribromide ion formation in the presence of high levels of Br^- and hydrogen peroxide, stopped-flow measurements were conducted to compare the relative rates of MCD bromination and Br_3^- formation. Stopped-flow measurements allow rate measurements to be made prior to any significant level of compound III accumulation. Compound III has previously been shown to be a catalytically inactive intermediate that is generated by bromoperoxidase in the presence of peroxide and MCD (Manthey & Hager, 1985). The relative rates measured for Br_3^- formation and MCD bromination were 1.74×10^4 and 9.3×10^3 mol min^{-1} (mol of BPO)$^{-1}$, respectively. The measurements also showed that the pH–rate profiles for the two reactions were parallel at acidic pH (data not shown). These results are consistent with Br_3^- as the actual brominating species for MCD, instead of MCD bromination proceeding by an enzyme-bound Br^+ intermediate.

Synthesis of Dichlorodimedone. As shown in a previous section, bromoperoxidase is capable of using Cl^- as a cofactor in the catalatic reaction. In order to determine whether or not bromoperoxidase actually catalyzes the oxidation of Cl^-, the MCD halogenation reaction was run in the presence of chloride ion. The initial experiments were carried out at pH 6, the pH optimum for bromination. These assays gave negative results. However, when the MCD reaction was carried out at pH 3, the assays showed a time-dependent decrease in MCD absorbance at 278 nm. This result suggests Cl^- oxidation and subsequent MCD halogenation. As shown in Figure 2, the rate of MCD chlorination is greatest at acidic pH. The slope of the pH–rate curve shows that the rate is controlled by a single ionization at low pH. MCD has an enol–enolate ionization at pH 3.0, and it is possible that the pH dependence of the MCD chlorination reaction relects the ionization state of MCD (Hewson & Hager, 1979).

In order to unequivocally demonstrate the ability of bromoperoxidase to catalyze the chlorination of MCD, the reaction was run in the presence of $^{36}Cl^-$. Aliquots of MCD halogenation reaction mixtures containing $^{36}Cl^-$ were removed at various time points and immediately extracted with ether. The extracts were dried and quantitatively applied to silica gel TLC plates. The results in lanes 0–4 show the $^{36}Cl^-$ incorporation into MCD in a time-dependent fashion (Figure 3). Reaction rates decreased with time; thus, there is little apparent difference in product levels at the later time points. Dichlorodimedone standards migrated to the same position as the enzyme-generated product. The source of $^{36}Cl^-$ at the origin remains in question. It is speculated that free $^{36}Cl^-$ and perhaps traces of oxidized dimedone products contribute to the radioactivity at the origin. Lane 6 contains a 3-fold larger aliquot compared to lane 5. The results show that at low pH, bromoperoxidase catalyzes Cl^- oxidation and the subsequent insertion of a chlorine atom into a nucleophilic acceptor molecule.

Halide-Dependent Oxidation of 2-Thiouracil. In addition to MCD halogenation, bromoperoxidase catalyzes a second class of halide ion dependent reactions. These reactions involve sulfhydryl oxidations and have been studied by monitoring the oxidation of the sulfhydryl group of 2-thiouracil. Figure 4 shows the pH–rate profiles of 2-thiouracil oxidations catalyzed in the presence of either Br^- or Cl^-. Both curves fit eq 8 where

$$V = \frac{V_{max}}{1 + K_a/[H^+]} \quad (8)$$

the maximal velocities occur at slightly acidic pH. The profiles for the two halides have different pK_a values. The pK_a values for the Br^-- and Cl^--enhanced reactions are 6.1 and 4.2, respectively. The V_{max} for the Br^--enhanced reaction is approximately 300 times faster than the value for the Cl^--enhanced reaction.

Triiodide Formation. The pH–rate profile of triiodide, I_3^-, formation catalyzed by bromoperoxidase indicates that iodide oxidation is analogous to bromide ion utilization. The rate equation that fits the iodide data is the same equation that describes the kinetics of MCD bromination. Like the MCD

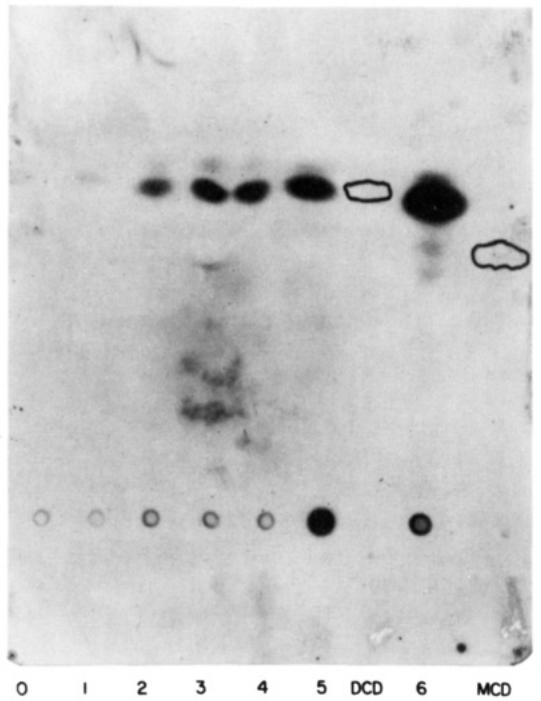

FIGURE 3: Detection of [^{36}Cl]dichlorodimedone. The experimental details for the formation of [^{36}Cl]dichlorodimedone are given under Materials and Methods. Ether extracts of the MCD chlorination reactions catalyzed by bromoperoxidase were developed on a silica gel TLC plate. Lanes 1–5 correspond to aliquots taken at increasing time points during the reaction. Lanes labeled DCD and MCD show the migration of authentic dichlorodimedone and MCD. Lane 6 shows the chromatography of an ether extract of the reaction mixture after the reaction reached completion. The plates were developed by using a mobile phase of ethyl acetate/hexane/glacial acetic acid (60:40:2). Radiolabeled spots were visualized by autoradiography.

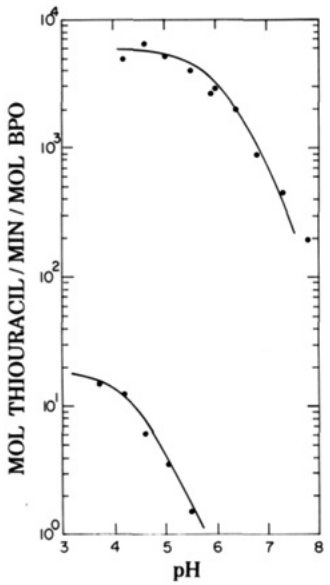

FIGURE 4: pH–rate profile for the halide-dependent oxidation of 2-thiouracil. The upper curve shows the data for the Br^--dependent oxidation of 2-thiouracil whereas the lower curve shows the results for the Cl^--dependent oxidation reaction. The reaction mixtures contained 2.7 mL of buffer prepared as described in Figure 2, 100 μL of a saturated 2-thiouracil solution, 1 μmol of hydrogen peroxide, 170 pmol of bromoperoxidase, and either 75 μmol of KBr or 300 μmol of KCl.

bromination reaction, protonation of a group with a pK_a value of approximately 5.2 inhibits I_3^- formation, whereas ionization of a more basic group with a pK_a near 7.4 is needed for maximal activity. The V_{max} equals 3.5×10^4 mol of I_3^- min^{-1} (mol of BPO)$^{-1}$. This value is approximately 6 times the turnover number found for the MCD bromination reaction.

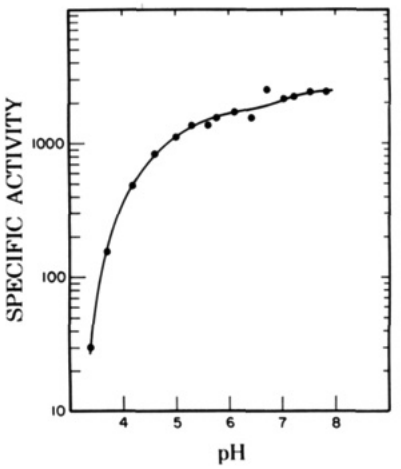

FIGURE 5: pH–rate profile for hydroquinone peroxidation. Oxidation of hydroquinone was measured as a loss in absorbance at 288 nm. Specific activity is defined as 1 μmol of substrate utilized min^{-1} (mol of BPO)$^{-1}$. The reaction mixture contained 2.7 mL of buffer prepared as described in Figure 2, 77 pmol of bromoperoxidase, 0.2 μmol of hydroquinone, and 0.40 μmol of hydrogen peroxide.

Peroxidase Activity of Bromoperoxidase. The third class of peroxidase reactions catalyzed by bromoperoxidase is the single electron oxidation of phenolic electron donors. These reactions were characterized by examining the peroxidation of pyrogallol, guaiacol, and hydroquinone. Figure 5 shows the pH–rate profile of the peroxidation of hydroquinone in the presence of bromoperoxidase and hydrogen peroxide. All three electron donors mentioned above show similar broad pH optima between pH 4.5 and 8 (data not shown). The time courses for these assays show biphasic behavior where an initial very rapid reaction occurs before it is possible to start recording the spectral measurements. The recorded activities reflect the much slower linear rates which follow the initial rapid rate. This biphasic reaction involving single electron donors is attributed to the accumulation of inactive compound III under these experimental conditions (Manthey & Hager, 1985). The specific activities for the bromoperoxidase-catalyzed peroxidations of hydroquinone, pyrogallol, and guaiacol are 1500, 4200, and 166 min^{-1}, respectively.

Halide Binding to Bromoperoxidase. The pH–rate profiles that have been presented in the previous sections have demonstrated the presence of several catalytically important ionizations associated with the bromoperoxidase molecule. Further studies were done to characterize the role of these ionizations in controlling reaction rates and halide ion binding at the active site. These experiments were done by monitoring changes in the Soret absorption band which accompany halide ion binding to the enzyme. The pH titrations of bromoperoxidase in unbuffered solutions show the presence of three ionizations which produce changes in the Soret optical spectrum. A high-pH ionization with a pK_a greater than 8.5 produces only a small change in the Soret band absorbance (data not shown). Two other ionizations occurred at lower pH, and a plot of the pK_a values of these ionizations as a function of Br^- concentration is shown in Figure 6. The ionization with a pK_a of 6.6 produced only a small change in the Soret band absorbance. The value of this ionization is independent of the Br^- concentration. However, the value of the most acidic ionization constant is dependent on the Br^- concentration. The pK_a of this group ranged from less than 5.0 to 6.4 as the Br^- concentration increased from 10 to 130 mM. The Soret band difference spectrum of the acidic form

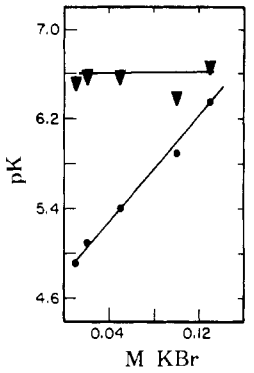

FIGURE 6: Plot of pK_a values as a function of bromide ion concentration. Purified bromoperoxidase was dialyzed against unbuffered solutions of various concentrations of KBr. Titrations were carried out with 0.10 N HCl or H_2SO_4 solutions. The more alkaline ionization (▼), which is independent of KBr concentration, and the acidic ionization (●), which is KBr concentration dependent, are plotted as a function of the KBr concentration.

of bromoperoxidase in the absence of halide ions clearly contrasts with the difference spectrum observed for the low-pH bromide–bromoperoxidase complex. The latter complex shows a 2–3-nm red shift in the Soret band. These results compare closely with the pH properties of the Soret band absorbance of other peroxidases such as myeloperoxidase and chloroperoxidase (Stelmaszynska & Zgliczynski, 1974; Thomas et al., 1970b). Other peroxidases such as lactoperoxidase and hog intestinal peroxidase show no difference in the Soret band spectra of the acid and acid–halide complexes (Kimura & Yamazaki, 1978).

Similar difference spectra were measured for the Cl^- and I^- complexes of bromoperoxidase. The pK_a values determined for bromoperoxidase in 0.10 M KI and KCl are 6.05 and 6.1, respectively. The similarity of these values to the value of 5.9 determined for bromoperoxidase in the presence of 0.10 M KBr strongly suggests that these halide ions bind to the same site on the enzyme. Furthermore, these results show that halide ion binding to the enzyme controls the ionization properties of essential amino acid residues which influence the heme active site.

Discussion

A summary of the kinetic data and the identification of the important ionization constants in the various bromoperoxidase reactions is given in Table I. A comparison of the reaction rates and the pH–rate profiles of Br_3^- formation, the MCD bromination reaction, and the Br^--enhanced catalatic reaction suggests the involvement of molecular bromine as an intermediate in all three of these reactions. The comparison shows that the rate of Br_3^- formation is faster than the rate of MCD bromination. This finding provides kinetic evidence that the electrophilic bromination reaction proceeds via the intermediate formation of Br_3^-. In the catalatic reaction, the transient accumulation of Br_3^- can be detected. Since Br_3^- rapidly decomposes hydrogen peroxide nonenzymatically to molecular oxygen and water, the transient accumulation of Br_3^- would be consistent with it being an active intermediate. It has not been determined whether the chlorination reactions catalyzed by bromoperoxidase proceed via Cl_2 or by an enzyme-bound intermediate. There are experimental difficulties in solving this problem. These difficulties arise from the relatively slow rate of the enzymatic chlorination reaction and the instability of Cl_2 in the aqueous reaction mixtures.

The analysis of the pH–rate profiles of the bromination, iodination, and chlorination reactions catalyzed by bromoperoxidase yields information concerning the presence of important active-site ionizations. The kinetic data of the various halide-dependent reactions summarized in Table I show the presence of an acidic group with a pK_a value of 5.3. Protonation of this group inhibits the bromination and iodination reactions. The pH-dependent spectral shift of the Soret band also detects the presence of a similar ionizing group. The linear dependence of the value of this ionization constant with halide ion concentration demonstrates that this ionization is linked to halide ion binding. It is proposed that this ionization reflects the presence of a distal histidine residue, which upon protonation contributes to the halide ion binding site near the heme center. At a single halide ion concentration, 0.10 M, all three halide ion substrates produce similar halide ion complexes with bromoperoxidase. Since the halide ion binding properties of Br^- and Cl^- are identical at this site, halide ion binding cannot explain the different pH–rate profiles observed for the halogenation of MCD with these two halide ions. Also, if it is assumed that the ability to oxidize Cl^- is due to the enhanced oxidation strength of compound I at acidic pH, then it would be expected that Br^- oxidation also would be energetically more favorable at low pH. This is certainly not the case. Thus, there must be additional control mechanisms involved in the regulation of halide ion oxidations by bromoperoxidase.

Table I: Catalytic Properties of Bromoperoxidase

reaction	pK_a	pK_b	V_{max} or k_1
chloride-dependent oxidation of 2-thiouracil		4.5	18
bromide-dependent oxidation of 2-thiouracil		6.0	5800
monochlorodimedone chlorination		3	1400
monochlorodimedone bromination	5.3	6.4	5900
compound I formation with hydrogen peroxide	5.3		2.1×10^7 M^{-1} s^{-1}
halide-free catalase activity			2.4×10^4 M^{-1} s^{-1}
I_3^- formation	5.2	7.5	3.5×10^4
Br_3^- formation	5.3		1.3×10^4 (pH 4.6)
hydroquinone peroxidation			1200 (pH 6.8)
pyrogallol peroxidation			4200 (pH 6.8)

Bromoperoxidase from *P. capitatus* represents the first hemoprotein purified from marine algae capable of catalyzing both Cl^- and Br^- oxidation reactions. Thus, this enzyme and other peroxidase enzymes with similar activity provide a mechanism for the insertion of both bromine and chlorine into marine halometabolites. Although bromine is the most commonly occurring halide in the marine natural products, a significant number of marine halometabolites also contain chlorine (Siuda & DeBernardis, 1973). Neidleman and Geigert have proposed a nonenzymatic mechanism for the chlorination of marine natural products (Neidleman & Geigert, 1983). Their mechanism describes the nucleophilic addition of Cl^- to a carbonium ion intermediate produced from an electrophilic attack by a Br^+ species on a carbon–carbon double bond. An inspection of chlorinated marine natural products identifies compounds where chlorine and bromine atoms are located on adjacent carbon atoms and thus could be formed by this mechanism. There are, however, a large number of marine natural products which contain an isolated chlorine atom. The occurrence of these chlorometabolites suggests a chloride insertion reaction which proceeds via electrophilic enzyme chlorinating intermediates generated by peroxidative reactions.

Registry No. BPO, 69279-19-2; MCD, 7298-89-7; Cl^-, 16887-00-6; Br^-, 24959-67-9; I^-, 20461-54-5; 2-thiouracil, 141-90-2; pyrogallol, 87-66-1; hydroquinone, 123-31-9.

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Characterization of the Cytochrome *c* Oxidase in Isolated and Purified Plasma Membranes from the Cyanobacterium *Anacystis nidulans*†

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ABSTRACT: Functionally intact plasma membranes were isolated from the cyanobacterium (blue-green alga) *Anacystis nidulans* through French pressure cell extrusion of lysozyme/EDTA-treated cells, separated from thylakoid membranes by discontinuous sucrose density gradient centrifugation, and purified by repeated recentrifugation. Origin and identity of the chlorophyll-free plasma membrane fraction were confirmed by labeling of intact cells with impermeant protein markers, [^{35}S]diazobenzenesulfonate and fluorescamine, prior to membrane isolation. Rates of oxidation of reduced horse heart cytochrome *c* by purified plasma and thylakoid membranes were 90 and 2 nmol min^{-1} (mg of protein)$^{-1}$, respectively. The cytochrome oxidase in isolated plasma membranes was identified as a copper-containing aa_3-type enzyme from the properties of its redox-active and EDTA-resistant Cu^{2+} ESR signal, the characteristic inhibition profile, reduced minus oxidized difference spectra, carbon monoxide difference spectra, photoaction and photodissociation spectra of the CO-inhibited enzyme, and immunological cross-reaction of two subunits of the enzyme with antibodies against subunits I and II, and the holoenzyme, of *Paracoccus denitrificans* aa_3-type cytochrome oxidase. The data presented are the first comprehensive evidence for the occurrence of aa_3-type cytochrome oxidase in the plasma membrane of a cyanobacterium similar to the corresponding mitochondrial enzyme (EC 1.9.3.1).

Cyanobacteria (blue-green algae) or their immediate ancestors were the first organisms that carried out oxygen-releasing, plant-type photosynthesis in a hitherto essentially anaerobic biosphere (Stanier & Cohen-Bazire, 1977; Broda, 1975). At the same time the oxygen-producing cyanobacteria must have been among the first to cope with free oxygen, a well-known cell poison to strict anaerobes (Morris, 1975), eventually by some type of aerobic respiration making use of preformed photosynthetic electron-transport pathways (Broda & Peschek, 1979). In fact, common electron carriers occur in photosynthesis and respiration of both anoxygenic phototrophic bacteria (Baccarini-Melandri & Zannoni, 1978) and oxygenic cyanobacteria (Aoki & Katoh, 1982; Peschek & Schmetterer, 1982). It is not surprising, therefore, that the cyanobacteria have become the largest and ecologically most successful group of prokaryotes (Stanier & Cohen-Bazire,

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