Characterization of Nonheme Iron and Reaction Mechanism of Bromoperoxidase in Corallina pilulifera*

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The properties of the nonheme iron of bromoperoxidase from Corallina pilulifera were studied. The enzyme lost its activity when reduced with formamidine-sulfonic acid and recovered it when oxidized by air. Incubation of the enzyme with ferric or ferrous ion-chelating agents indicated that its nonheme iron was ferric. Analyses of circular dichroism and proton NMR spectra suggested that the ferric iron tightly bound to cysteine, histidine, or tyrosine residues of the enzyme. The enzyme catalyzed Br\(^{-}\)-dependent catalase reactions to yield 1 mol of O\(_2\) from 2 mol of H\(_2\)O\(_2\). No O\(_2\) evolution was observed when bromination reaction of monochlorodimedone occurred. From these results, together with previous knowledge of this enzyme, it was concluded that it activated bromide anion (Br\(^{-}\)) to bromonium cation (Br\(^{+}\)) using one molecule of H\(_2\)O\(_2\), and this Br\(^{-}\)OH\(^{-}\) formed at the active site then decomposed another H\(_2\)O\(_2\) to yield O\(_2\) in the absence of halogen acceptors (substrate). When substrate was present in the reaction mixture, it and H\(_2\)O\(_2\) competitively reacted with the reaction intermediate (Br\(^{+}\)OH\(^{-}\)) to give brominated products.

Bromoperoxidase has been found in and isolated from several marine algae and microorganisms such as Rhodoma (1), Ripleophalus (2), Penicillus (2, 3), Corallina and Amphiroa (4), and Pseudomonas aureofaciens (5). It has been found that the enzyme of Corallina pilulifera is a new type of haloperoxidase containing nonheme iron as a prosthetic group (6, 7) (NH type), instead of the usual heme iron (H type), which is seen in chloroperoxidase of Caldarriomyces fumago (EC 1.11.1.10) (8–10), bromoperoxidases of Rhodoma (1), Ripleophalus (2), Penicillus (2, 3), and Pseudomonas (5), as well as in iodoperoxidase (EC 1.11.1.8) (11). Recently, another nonheme (NH type) bromoperoxidase has been reported in a brown algae, Ascophyllum nodosum, which contains vanadium as a prosthetic group (12, 13).

The detailed properties of the enzyme of C. pilulifera were reported in previous papers (6, 7), but those of the enzyme’s nonheme iron were not clear. Elucidation of its valence state and ligand properties is important in understanding the halogenation mechanism of the enzyme.

The halogenation intermediate of the enzyme is a halonium cation (X\(^{+}\)), not a radical (4, 7, 14). It has been found that the bromination reaction of the enzyme occurs at its active site (14). On the other hand, bromination by the H-type chloroperoxidase of the fungus C. fumago is caused by the release of molecular halogen into the reaction mixture (10, 14, 15). In addition, no catalase and only slight peroxidase activities exhibited by bromoperoxidase of C. pilulifera (7) imply that its reaction mechanism is quite different from that of H-type haloperoxidase.

This work describes the halogenation mechanism of nonheme bromoperoxidase of C. pilulifera and clarifies the differences between the reaction mechanisms of heme and nonheme haloperoxidases.

MATERIALS AND METHODS

Collection of Algae and Enzyme Purification—C. pilulifera (Corallinaceae, Rhodophyta) was collected from shallow water (0.5–1.0 m deep) on the shores of Takahama (Fukui Prefecture), Japan, in April 1985, and frozen at −20°C until use. The enzyme was purified from crude extracts of C. pilulifera as described in the previous paper (6).

Characterization of the Purified Enzyme—The properties of the purified enzyme were analyzed according to the methods described in previous papers (7, 16) and compared with those of enzyme purified from the same algae collected at a different site (Shirahama, Wakayama Prefecture, Japan). Enzyme purified from algal samples collected at Takahama was used throughout this study.

Enzyme Assay—Bromoperoxidase activity was assayed by measurement of the change in absorbance at 290 nm caused by the conversion of monochlorodimedone to monobromomonochlorodimedone (4). Catalase activity was measured by the modified method of Beers and Sizer (17), whose detailed reaction conditions were described in the previous paper (4). Peroxidase activity was measured with o-dianisidine at 25°C (18). O\(_2\) concentration in the reaction mixture was monitored with a Beckman Fieldib oxygen analyzer equipped with a Beckman 39533 polarographic oxygen sensor. Before analysis, the reaction mixture was saturated with nitrogen gas, and the reaction was carried out in a closed system with constant stirring by a magnetic stirrer. Throughout this report, 1 unit of enzyme activity is equal to the amount of enzyme that converted 1 μmol of substrate in 1 min at 25°C.

Instrumental Analysis—Proton nuclear magnetic resonance (HNMR) spectra were recorded at 300 MHz on a Nicolet NT-300 spectrometer equipped with a 1250 computer system. For recordings of paramagnetically shifted proton NMR spectra, typically 50K transients were accumulated to obtain the Fourier transformed spectrum with 8K data points and 6.0-μs 90° pulse. The enzyme sample was dialyzed against 5 mM potassium phosphate buffer (pH 7.0) before measurement.

Electron spin resonance (ESR) spectra were recorded at room temperature in a 10-mm light-path cuvette with a JASCO J-500 C spectropolarimeter equipped with an electromagnet (1.5 T).

1. N. Itoh, A. K. M. Q. Hasan, Y. Izumi, and H. Yamada, manuscript in preparation.  
2. The abbreviation used is: CD, circular dichroism.
Characterization of Nonheme Iron and Reaction Mechanism of Bromoperoxidase

Chemicals—Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt) was purchased from Wako Pure Chemical Industries, Japan, and formamidinesulfonic acid from Aldrich. NaOBr solution was prepared by dissolving 120 mg of Br₂ in 3 ml of 1 N NaOH, followed by dilution to 100 ml with distilled water.

RESULTS

Differences in Enzyme Properties of Algae Collected from Different Sites—The enzyme purified from C. pilulifera collected at Takahama (Japan Sea) was identical to that from Shirahama (Pacific Ocean) in the following ways: elution patterns of DEAE-Sepharose and Sepharose 6B chromatographies, Kᵥ values for KBr and H₂O₂, optimum pH, electrophoretic mobilities on disc gel and sodium dodecyl sulfate-slab gels, and immunological properties (16). However, the contents of iron and magnesium, which affected the specific activities, absorption spectra, and dry weight extinction coefficients (E₅₃₀ nm⁻¹), were found to be different, as shown in Table I Miniprint.

Valency State of Nonheme Iron of the Enzyme—Incubation of the native enzyme with 2 mM formamidinesulfonic acid, a mild reducing agent, for about 2 h under anaerobic conditions, caused the enzyme’s complete inactivation. When it was reoxidized thereafter by bubbling with air, its activity returned almost to the original level (Fig. 1, Miniprint). Addition of 2 mM sodium citrate caused a similar change, but enzyme activity was not restored by oxidation with air.

When the enzyme was incubated with 2 mM Tiron which is a chelating agent specific for ferric iron (19), changes in spectra of the enzyme were observed, which indicated the formation of a chelated complex (Fig. 2a, Miniprint). Absorbance at 450 nm was increased, with a concomitant decrease in enzyme activity (Fig. 2b, Miniprint).

Incubation of the enzyme with a ferrous ion chelating agent, o-phenanthroline, had no effect on the enzyme activity, but incubation with o-phenanthroline under reducing conditions completely inhibited it. These data showed that nonheme iron of bromoperoxidase was ferric.

Instrumental Analyses of the Enzyme—As described in the previous paper, the enzyme’s nonheme iron tightly bound to the polypeptide residues, and no decrease in iron content was observed after prolonged incubation with EDTA or o-phenanthroline (7). It was also found that the enzyme contained no acid-labile sulfide (7). In the CD spectra of native and reduced enzyme samples, there were no characteristic peaks corresponding to [2Fe-2S] and [4Fe-4S] clusters, which are common in nonheme iron proteins (Fig. 3, Miniprint) (20, 21).

These spectra were rather similar to that of rubredoxin, whose nonheme iron forms complexes with sulfur donor ligands of 4 cysteine residues (22).

Only a broad peak at 22.3 ppm, caused by protons in paramagnetic ferric iron field was observed in proton NMR spectrum of the enzyme. Further information concerning the ferric ion ligands could not be obtained from the proton NMR spectrum because of the enzyme’s high molecular weight (790,000).

No ESR-active ferric ions were observed in native enzyme under the conditions tested (77 K). Addition of 2 mM H₂O₂ with or without 20 mM KBr to the enzyme caused no change in the ESR spectrum.

Br⁻-dependent Catalase Reaction of the Enzyme—Catalase activity of the enzyme was assayed by spectrophotometric measurement of the decrease in H₂O₂ concentration at 240 nm and oxygen electrode measurement of the formation of O₂. The enzyme showed no catalase activity in the absence of bromide. However, in the presence of bromide it decomposed 2 mol of H₂O₂ to give 1 mol of O₂ as shown in Table II, Miniprint. In the presence of monochlorodimedone, a good acceptor of bromonium cations, no O₂ formation was observed. The specific activity of the Br⁻-dependent catalase reaction was just twice that of the bromination of monochlorodimedone. The Kᵥ values for KBr and H₂O₂ of both reactions were identical. Therefore, it was thought that the enzyme’s Br⁻-dependent catalase and bromination reactions would proceed by the same mechanism.

Catalase Reaction of the Enzyme in the Presence of Monochlorodimedone—The rate of O₂ formation from H₂O₂ was carefully measured at different monochlorodimedone concentrations (Fig. 4, Miniprint). It was found that O₂ evolution occurred only after the complete consumption of monochlorodimedone, indicating that monochlorodimedone was brominated preferably by the bromination intermediate (Br⁺). After the consumption of monochlorodimedone, the bromination intermediate was then able to act on H₂O₂.

Comparison of the Enzyme Bromination Reaction with the NaOBr Reaction—NaOBr (HOBr) is known to oxidize H₂O₂ to yield O₂ as follows: NaOBr + H₂O₂ → NaBr + O₂ + H₂O (23-26). Supposing that the reaction intermediate of bromoperoxidase were NaOBr (HOBr), and that it was released into the reaction mixture, then the enzymic and chemical reactions of NaOBr should show the same rate of O₂ evolution in the presence of several halogen acceptors. Bromoperoxidase of C. pilulifera catalyzes the bromination of not only monochlorodimedone but also of many other organic compounds, such as substituted phenols (4), heterocycles (14), and substituted alkenes. We compared the O₂ formation rate of the enzyme reaction with that of the NaOBr reaction in the presence of either monochlorodimedone, phenol, or cytosine. The rate of feeding NaOBr solution into the reaction mixture was adjusted until the O₂ formation rate was the same as in the enzymic reaction in the absence of halogen acceptor. As shown in Fig. 5, Miniprint, in the NaOBr reaction, O₂ formation was observed at high concentrations of monochlorodimedone (1.25 mM), a result completely different from that obtained with the enzymic reaction (Fig. 4, Miniprint). The rates of O₂ formation in the enzymic reaction in the presence of phenol or cytosine also differed from those of the NaOBr reaction. Thus, it was obvious that the enzyme possessed some affinity for monochlorodimedone and cytosine. These data suggested that bromination reaction of the enzyme was not due to the release of NaOBr or molecular bromine into the reaction mixture, which was in accord with previous results (14).

Kinetics of O₂ Formation by the Enzyme Reaction in the Presence of Phenol—The effects of the presence of the halogen acceptor, phenol, on the kinetics of O₂ formation were examined in detail (Fig. 6, Miniprint). The kinetic data indicated that phenol’s inhibition of O₂ formation was competitive, with a Kᵥ value calculated from the plot data to be 3.8 × 10⁻⁴ M. The results revealed that H₂O₂ and phenol competitively reacted with the bromination intermediate formed at the enzyme’s active site.

DISCUSSION

Bromoperoxidase of C. pilulifera is an interesting haloperoxidase which possesses a nonheme iron as a prosthetic group.
As described in the previous paper (7), the enzyme isolated from *C. pilulifera* collected at Shirahama (Pacific Ocean, temperate sea) contained 2.3 ± 0.2 iron atoms and 1.6 ± 0.1 magnesium atoms/molecule of dodecameric enzyme. In this study, enzyme was again purified to homogeneity from algae at a different collection site, Takahama (Japan Sea, temperate sea). It was found that the newly purified enzyme differed from the previous one in its iron and magnesium contents (14 ± 0.5 iron and 0.7 ± 0.1 magnesium atoms/molecule of enzyme), but the other physicochemical and immunological properties (16) were identical. It was speculated that the algal growing conditions, e.g. growing season, day light, tide movement, mineral and nutrient compositions, and temperature of the sea water, probably affected the metal content of the enzyme, resulting in different enzyme activities. Seasonal changes in bromoperoxidase activity has been reported in *Rhodomela larix* (1). Ferric ions were found to be essential for the enzyme activity of *C. pilulifera*. However, the reason for the low specific activity of the Takahama enzyme in spite of the high ferric ion content is obscure (Table I). Compared with the Shirahama enzyme, the magnesium content of that from Takahama was low, so, magnesium ions may play a very important role in conformational maintenance of the enzyme, which in turn would affect its specific activity. Other factors resulting in low enzyme specific activity such as protease digestion of the enzyme toward the end of the growing season are possibilities. We have not yet checked the seasonal variations of algal bromoperoxidase activity at any one collection site.

Bromoperoxidase of *C. pilulifera* rapidly lost its activity during reduction by formamidinesulfinic acid but recovered it following subsequent oxidation by air (Fig. 1). This suggested that the valency state of nonheme iron of the enzyme was trivalent (Fe(III)). Tests on the enzyme’s complex formation, using Tiron and o-phenanthroline supported this suggestion. However, the reason why the loss of activity which was plotted in Fig. 2 did not parallel the change in the absorption spectrum is obscure. We speculated that the interaction between the enzyme’s ferric ion and Tiron is weak, so the Tiron-treated enzyme recovers its activity under the assay conditions, in the presence of H2O2 and KBr. Inability to detect ESR active ferric ions was probably due to the rapid relaxation time at 77K of the enzyme’s nonheme iron electrons. Tiron is known to form chelated complexes with free ferric ions in a short time (19); Kojima et al. (27) reported that pyrocatechase, an enzyme which contains Fe(III), lost its activity in about 60 min when incubated with Tiron. Thus, the slow rate of complex formation of bromoperoxidase with Tiron (Fig. 2) suggested that the enzyme’s ferric ions were strongly bound to the polypeptide residues at the active site, which prohibited easy complex formation with metal-chelating agents. That the enzyme did not lose its activity following prolonged incubation with EDTA or the treatment with 6 M urea for several hours is in accord with this proposal. The enzyme has been found to contain no acid-labile sulfide (7), a finding which was supported by our analysis data of CD spectrum of the enzyme. The similarity of the CD spectra of the enzyme to ferric-rubredoxin implied that ferric ion directly bound to 4 cysteine residues of the enzyme. The results of amino acid analysis of the enzyme, which found 4 cysteine residues/enzyme subunit (7), are not in conflict with this speculation. We failed to measure the enzyme’s resonance Raman spectrum because the purified enzyme was contaminated with a covalently bound red substance. This red dye interfered with resonance Raman measurements because of its fluorescence. Thus, the complete ligand properties of the ferric ion of the

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**Fig. 7.** Comparison of reaction mechanisms of NH-type bromoperoxidase of *C. pilulifera* (a) and H-type chloroperoxidase of *C. furmago* (b).
enzyme have not yet been clarified. Other possible ferric ion ligands are tyrosine phenolate and histidine imidazole anions.

Iodoperoxidase and chloroperoxidase are known to show the halide-dependent catalase activity yielding singlet oxygen, \( \text{O}_2^* \) (24-26), but they show catalytic activities even in the absence of halide. Thus, this is the first report of an absolutely \( \text{Br}^- \)-dependent catalase reaction. The chemical equivalence of \( \text{H}_2\text{O}_2 \) consumption and \( \text{O}_2 \) formation in the \( \text{Br}^- \)-dependent catalase reaction suggested that one molecule of \( \text{H}_2\text{O}_2 \) was used in the oxidation of \( \text{Br}^- \) to \( \text{Br}^+ \), the latter then reacting with another molecule of \( \text{H}_2\text{O}_2 \) to yield one molecule of \( \text{O}_2 \). This proposal is based on the observations that the halogenation intermediate of \( C. \) pilulifera bromoperoxidase was a halonium cation (\( \text{X}^+ \)), and that hypobromous acid (\( \text{Br}^+\text{OH}^- \)) catalyzed the oxidation of \( \text{H}_2\text{O}_2 \) to \( \text{O}_2 \). However, in the presence of the halogen acceptor, monochlorodimedone, the active intermediate (\( \text{Br}^+\text{OH}^- \)) was used only in the bromination of that, and no detectable \( \text{O}_2 \) was produced (Table II and Fig. 4). In addition, the bromination rate of monochlorodimedone was the same as that of \( \text{O}_2 \) formation in \( \text{Br}^- \)-dependent catalase reaction. Therefore, it was concluded that the enzyme bromination proceeded as shown in the formula in Fig. 7a.

On the other hand, the differences in the \( \text{O}_2 \) formation rates between the HOBr chemical and enzymatic reactions in the presence of halogen acceptors showed that the bromoperoxidase-catalyzed bromination occurred at the enzyme’s active site and was not dependent on release of molecular bromine or HOBr into the reaction mixture. The result was in fair agreement with the previous study’s findings that no formation of tribromide ions was observed in the reaction mixture (14). On this point, \( C. \) pilulifera nonheme bromoperoxidase halogenation is quite different from the usual heme haloperoxidases. The results in Fig. 5 also showed that the enzyme had some affinity toward the halogen acceptors, resulting in different rates of bromination from those shown by the NaOBr reaction.

As shown in Fig. 6, \( \text{O}_2 \) formation by bromoperoxidase catalatic reaction was competitively inhibited by phenol, indicating that both phenol and \( \text{H}_2\text{O}_2 \) reacted on the same bromination intermediate. Lack of formation of \( \text{O}_2 \) in the presence of monochlorodimedone (Fig. 4) was probably due to a high enzyme affinity toward it. In the previous paper, we showed that inhibition of monochlorodimedone bromination by fluoride ions was uncompetitive over a concentration range of fluoride ions from 1.25 to 5.0 mM (7). This phenomenon can easily be understood from the enzyme’s reaction mechanism. Fluoride ions probably bind to the \( \text{Br}^+\text{OH}^- \) intermediate to give \( \text{BrF}_3 \). The reactivity of \( \text{BrF}_3 \) as an electrophile is lower than that of \( \text{Br}^+\text{OH}^- \) because of its high binding energy.

The reaction mechanism of nonheme (NH type) bromoperoxidase of \( C. \) pilulifera is summarized in Fig. 7a and compared with that of heme (H type) chloroperoxidase of \( C. \) funago (Fig. 7b). The characteristic of the bromoperoxidase reaction in terms of no catalase reaction and minimal peroxidase activity are clearly explained by its reaction mechanism.

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Characterization of Nonheme Iron and Reaction Mechanism of Bromoperoxidase

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RESULTS

Table 1. Differences in the properties of bromoperoxidases from the different collection sites.

| Properties               | Pinkrum | Takahama |
|--------------------------|---------|----------|
| Specific activity (μkat/mg protein) | 26.3    | 4.9      |
| Iron content (μmol/mg enzyme)   | 2.3 ± 0.2 | 14.6 ± 5.5 |
| Spectrum content (μmol/100 mg)  | 1.6 ± 0.1 | 0.7 ± 0.1 |
| Activity (μmol/mg enzyme)       | 15     | 2.05     |
| Wavelength (nm)              | 280     | 318      |

(a) Data of Ishii et al. (1).

Figure 1. Effect of ammonium thiocyanate on enzyme activity.

The enzyme sample (1.1 mg protein) in 50 mM potassium phosphate buffer, pH 8.0, was amonothiocyanate incubated with 0 mM ammonium thiocyanate at 25°C. After 2 h, 8% was passed through the sample to reoxidize the enzyme.

Figure 2. Enzyme spectrophotometric activity changes following incubation with Triton X-100 (1% v/v) and chloroform-d (for 10 min).

Figure 3. Spectra of native and reduced enzymes. The spectra were recorded in a 1-cm light path cuvette at room temperature.

Table 2. Bromoperoxidase-catalyzed reactions of G. pullulans.

| Reaction                          | Specific activity (μkat/mg protein) | kcat [M] |
|-----------------------------------|-------------------------------------|---------|
| Catalase activity (Br⁻)           | 0                                   | 1.0 ± 10² |
| Br⁻-dependent catalase activity   | 10.1                                | 1.0 ± 10² |
| (H₂O₂ decomposition) (for Br⁻)    | 1.1 ± 10⁻⁴                          |         |
| (O₂ evolution)                    | 10⁻⁴                                |         |
| Bromination of monochlorodimedone | 4.9                                 | 1.0 ± 10² |
| (O₂ evolution)                    | 0.2 ± 10⁻³                          |         |
| Peroxidase activity (μkat/mg protein) | 0.0007                |         |

Figure 4. Effect of monochlorodimedone on the Br⁻-dependent catalase reaction of the enzyme. The evolution rate of O₂ was monitored in the same manner as described in Table 2.
Characterization of Nonheme Iron and Reaction Mechanism of Bromoperoxidase

Figure 5. Comparison of the O₂ evolution rates by enzymatic and NaOBr reactions in the presence of some halogen acceptors. The enzyme reaction was done as described in Table 2. In the case of NaOBr reaction, NaOBr solution was injected into the reaction mixture without enzyme at a flow rate of 67 µl/min.

Figure 6. Double-reciprocal plots of O₂ formation rate against H₂O₂ concentration in the presence of various concentrations of phenol. The reaction was performed as described in Table 2.