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VANADATE ACTIVATION OF BROMOPEROXIDASE FROM Corallina officinalis

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Summary A nonheme bromoperoxidase has been purified to homogeneity from the red seaweed Corallina officinalis. Like the corresponding enzyme previously reported from C. pilulifera, this bromoperoxidase contains a significant amount of nonheme iron. However, it is vanadate ion and not iron that activates the enzyme, and maximal activity is achieved with stoichiometric vanadium incorporation. The absence of competition between vanadium and iron suggests that they occupy distinct binding sites in the protein. A correlation between vanadium content and catalytic activity indicates that less than 12 percent of the maximal activity of the enzyme can be derived from metals other than vanadium.

Introduction Biological halogenation reactions have a broad significance in environmental, medical and pharmacological terms through their importance in the biosynthesis of antibiotics and hormones. For many years, the only enzymes known to catalyze halogenation chemistry were heme-containing peroxidases.[1-3] Recently, however, a class of nonheme haloperoxidases has been identified, associated with marine algae, lichens, and some microorganisms.[4-8] Most of these have been characterized as belonging to a novel class of vanadium containing enzymes, in which V^{+5} species have been identified in the active site.[4,6,9] The bromoperoxidase from the red seaweed Corallina pilulifera is an apparent exception in that iron is the only redox active metal reported to be associated to a significant extent with the purified protein.[5] We have isolated bromoperoxidase from a closely related red seaweed, C. officinalis, and also find that iron is present in significant quantities in samples of the purified protein. However, vanadium is also present, and systematic vanadate activation reveals that only vanadium content correlates with catalytic activity. On reduction, EPR signals characteristic of V^{+4} are observed, with spectral parameters similar to those reported for bromoperoxidase from the brown seaweed Ascophyllum nodosum.[10] These observations indicate that the bromoperoxidase from red seaweed is also a

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Abbreviation: MCD, monochlorodimedone.

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vanadium enzyme, apparently containing a relatively labile active site metal ion.

Materials and Methods Coralina officinalis, obtained from Marine Biological Laboratory (Woods Hole, MA), was harvested in spring and fall of 1988 from intertidal zone habitats. On arrival in our laboratories, the seaweed was washed with deionized water and stored frozen.

Bromoperoxidase was isolated from the seaweed following a modification of a previously reported procedure for the bromoperoxidase from C. pilulifera.[11] Approximately 30 g thawed seaweed was ground in a mortar and pestle with acid-washed glass beads at 4 °C. for 20 min and then suspended in 60 mL of 0.05 M KH_2PO_4 buffer (pH 6.5). Glass beads and debris were removed by centrifugation (8,000Xg, 20 min). Ammonium sulfate was then added to the crude extract to 80% saturation, and the precipitate was collected by centrifugation (10,000Xg, 20 min). The pellet was dissolved in 40 mL 0.1 M KH_2PO_4 buffer (pH 7.0) and dialyzed overnight against 2 L of the same buffer containing 0.1 M NaCl. The dialysate was then applied to 30 mL DEAE-cellulose (Whatman DE-52) previously equilibrated with the dialysis buffer in a Buchner funnel and eluted stepwise with three bed volumes of pH 7 buffer containing 0.1, 0.3 and 1.0 M NaCl. Bromoperoxidase was eluted in the 0.3 M NaCl step. The enzyme solution from the previous step was brought to 10% saturation with ammonium sulfate and loaded on a column of phenyl Sepharose CL-4B (Pharmacia) (2.5 X 5 cm) previously equilibrated with 0.1 M buffer (pH 7) 10% saturated in ammonium sulfate. After washing with two bed volumes of the starting buffer, the column was eluted with a linear gradient from 10-0 % saturation in ammonium sulfate, affording homogenous bromoperoxidase.

Protein concentrations were determined using the method of Lowry et al.[12] using a bovine serum albumin standard and from UV absorbance measurements. Native and denaturing polyacrylamide gel electrophoresis were carried out with 7% and 10% gels, respectively, as described previously.[11] Bromoperoxidase activity was measured at 25 °C by following the bromination of monochlorodimedone.[13] Bromoperoxidase activity stain was carried out according to a previously reported procedure.[13]

Metal ion analyses were performed on a Perkin Elmer Model 703 Atomic Absorption Spectrophotometer equipped with an HGA 2200 Graphite Furnace. For vanadium analyses, standards and samples were prepared in 0.25% $Mg(NO_3)_2$ and atomized at 2800 °C in a pyrolytically coated graphite tube.

Vanadium activation of bromoperoxidase was accomplished by incubating the native enzyme with vanadate ion (50 uM - 3 mM). After incubation for up to 48 hours, the enzyme solutions were dialyzed extensively against 0.1 M K_2HPO_4 buffer (pH 7) to remove unbound metal.

EPR spectra were obtained on a Bruker ER300 EPR spectrometer equipped with an Oxford Instruments ESR900 continuous flow He cryostat. Optical absorption spectra were recorded on an IBM Model 9430 UV-VIS absorption spectrometer.

Results and Discussion The results of a typical purification for bromoperoxidase from C. officinalis are given in Table I. The purified enzyme obtained by this procedure gave rise to one major band (>90%) on nondenaturing electrophoresis. An additional minor band (approx. 10%) was always observed close to the major band, but both regions stained positive for bromoperoxidase activity. The purified enzyme was essentially homogeneous by SDS-polyacrylamide gel electrophoresis, showing a single major band (>95%) with a relative molecular mass (M_r) of 64,000. The purified enzyme did not show absorption bands in the 300 - 700 nm region, indicating that this bromoperoxidase does not contain heme or flavin prosthetic groups.

Table I. Purification Data for Bromoperoxidase from Corallina officinalis

Purification Stage	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)
Cell extract	186	133	0.72	100
80% $(NH_4)_2SO_4$ Precipitation	93	136	1.46	102
DE-52 Batch Elution	5.2	58	11.2	44
Phenyl Sepharose CL-4B	0.12	43	352	32

The metal content of the purified protein was determined by iron and vanadium atomic absorption spectroscopy. Like the corresponding enzyme isolated from C. pilulifera,[5] the bromoperoxidase from C. officinalis contains a significant amount of nonheme iron, varying from 0.4 - 0.8 Fe/mol in different preparations. In contrast, vanadium was reported to be absent from the C. pilulifera enzyme and was found to be present only at low levels in the native C. officinalis enzyme.

We have found that incubation of the native enzyme with vanadate salts results in an increase in specific activity. Following removal of unbound vanadium by dialysis, the vanadium content of the activated enzyme was determined by atomic absorption spectroscopy and correlated with catalytic activity as shown in Figure 1. Over a range of vanadium incorporation from 0.2 - 1.4 V/mol, a striking correlation is observed between vanadium content and specific activity. Above 1 V/mol, the vanadate ion appears to be nonspecifically bound and does not contribute further to the catalytic activity. A regression analysis of the data points below this plateau shows that maximal activity is obtained at approximately 1 V/mol. The non-zero intercept of the regression line indicates that at most 12% of the observed bromoperoxidase activity cannot be attributed to the vanadium content. This limit is reduced further if the least-squares fit is applied only to the lowest set of data points, and it appears likely that the activity depends exclusively on vanadium. The vanadium content of the native enzyme, which has never been treated with vanadium salts, is also consistent with the observed specific activity. This clearly demonstrates that vanadium is essential for the bromoperoxidase activity. In contrast, attempts to activate the enzyme using ferrous and ferric salts alone and in cysteine or EDTA chelates were unsuccessful. Both iron and vanadium are present in the samples and there is no evidence for competition for binding, which indicates that distinct

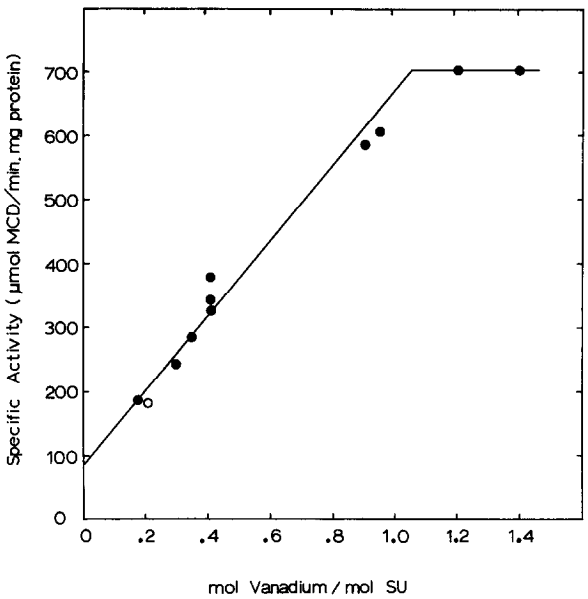

Figure 1. Correlation of vanadium content with bromoperoxidase activity. Native (O) and vanadate-treated enzyme (●) were analyzed for vanadium and assayed for bromoperoxidase activity.

binding sites exist for the two metal ions. There is no correlation between iron content and specific activity in the samples we have examined, which along with the kinetic data on low-vanadium enzyme samples demonstrates that iron alone is not sufficient for catalytic activity. However, in the absence of an enzyme sample specifically depleted in iron, the possibility remains that both Fe and V are present in the active site.

Our results on vanadate activation of C. officinalis bromoperoxidase may help to clarify the previously reported absence of vanadium in the related enzyme from C. pilulifera.[5] In the latter case, the relatively low activity of the purified enzyme (26 μmol/min·mg protein) is consistent with incorporation of less than 0.04 V/mol, assuming the same maximal activity. At such low levels of vanadium, the sensitivity of the metal analysis may not have been sufficient to detect this minority species. The effect of vanadate in activation of the enzyme from C. pulilifera appears not to have been examined.[5]

Previous EPR studies of the vanadium bromoperoxidase from the brown seaweed Ascophyllum nodosum have led to the characterization of a distinctive EPR spectrum for the reduced enzyme.[10] In contrast, no EPR signals were observed for the enzyme from C. pilulifera.[14] For the bromoperoxidase from C. officinalis, we observe no signal for the native enzyme even after incorporation of essentially stoichiometric vanadium to fully activate the enzyme. On reduction with sodium dithionite, however, the characteristic EPR spectrum of the vanadyl ion is observed (Figure 2). This spectrum in many

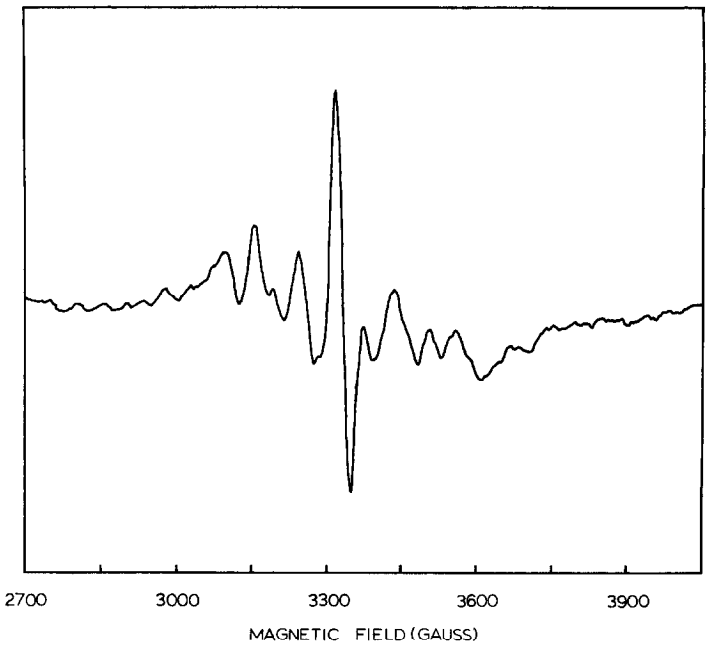

Figure 2. Low temperature EPR spectrum for dithionite-reduced Corallina officinalis bromoperoxidase. Instrument parameters: temperature 6 K, frequency 9.44 GHz, power 10 μW, modulation amplitude 10 G.

ways resembles that previously reported for the bromoperoxidase from brown seaweed.[10] Precise values of spin Hamiltonian parameters have been difficult to obtain from this spectrum, but spectral simulation suggests a significant rhombicity of vanadium hyperfine interactions. Further spectroscopic studies on nonheme bromoperoxidase are currently in progress.

Conclusions The combined metal ion analyses and activity measurements characterize the bromoperoxidase from C. officinalis as a vanadium enzyme. The native enzyme contains relatively low levels of vanadium but it is possible to incorporate stoichiometric metal by incubation of the enzyme with vanadate salts, thus producing enzyme with the highest specific activity. The correlation between catalytic activity and vanadium content clearly indicates a catalytic role for vanadium in this enzyme, while the significance of the nonheme iron which is present in these enzyme samples is less clear. EPR data suggest that the vanadium is bound in a higher oxidation state complex which appears to be characteristic of the growing class of nonheme vanadium haloperoxidases.

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