

Inactivation of Vanadium Bromoperoxidase: Formation of 2-Oxohistidine[†]

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ABSTRACT: The basis of the irreversible inactivation of the vanadium bromoperoxidase (V-BrPO) isolated from the marine alga *Ascophyllum nodosum* under turnover conditions at low pH (i.e., 15 to 100 mM H₂O₂, 0.1 KBr, ca. 15 nM V-BrPO in 0.1 M citrate, pH 4) has been investigated. Inactivation under these conditions was found to produce 2-oxohistidine as identified by HPLC using electrochemical detection. Formation of 2-oxohistidine requires all the components of turnover (i.e., bromide, hydrogen peroxide, and V-BrPO) as well as low pH; inactivation does not occur nor is significant 2-oxohistidine formed in the presence of hydrogen peroxide alone. The oxidation of histidine did not occur by singlet oxygen generated by V-BrPO, because neither 2-oxohistidine nor inactivation occur under the conditions in which singlet oxygen is produced quantitatively by V-BrPO. The addition of aqueous bromine to *N*^α-benzoylhistidine at low pH formed *N*^α-benzoyl-2-oxohistidine. *cis*-Dioxovanadium(V) (VO₂⁺) in strong acid and MoO(O₂)₂(ox)²⁻ (ox²⁻ is oxalate) at pH 5, both of which are functional mimics of V-BrPO by oxidizing bromide by hydrogen peroxide, catalyzed the oxidation of *N*^α-benzoylhistidine to *N*^α-benzoyl-2-oxohistidine. Furthermore, when hypobromite was added to *N*^α-benzoylhistidine in the presence of hydrogen peroxide at neutral pH, conditions under which HOBr would react first with H₂O₂ to produce singlet oxygen, no *N*^α-benzoyl-2-oxohistidine was formed. Thus the oxidation of histidine in V-BrPO is proposed to occur via oxidized bromine species. Irreversible inactivation V-BrPO was also found to be accompanied by release of vanadium.

Oxidative stress leads to amino acid oxidation and increased levels of damaged proteins. Many amino acids are susceptible to oxidation, including, in particular, histidine. Oxidation of histidine has been shown to produce asparagine in a free radical mediated process (Stadtman, 1990). Recently, oxidation of histidine to 2-oxohistidine has been demonstrated in Cu,Zn-superoxide dismutase (Cu,Zn-SOD) (Uchida & Kawakishi, 1994). 2-Oxohistidine formation is a result of inactivation of Cu,Zn-SOD by its own reaction product, hydrogen peroxide (Hodgson & Fridovich, 1975). The mechanism of oxidation in Cu,Zn-SOD is thought to be a free radical process resulting from reduction of Cu(II) by H₂O₂ and then subsequent reduction of another equivalent of H₂O₂ by Cu(I) producing the active histidine oxidant. Several other radical generating systems (e.g., metal/H₂O₂ and metal/ascorbate) also effect oxidation of histidine to 2-oxohistidine (Uchida & Kawakishi, 1994). The use of HPLC with electrochemical detection enabled the identification of 2-oxohistidine in Cu,Zn-SOD, since 2-oxohistidine is not observed by standard amino acid analysis techniques (Uchida & Kawakishi, 1994). 2-Oxohistidine has also been detected in bovine serum albumin during the autoxidation of ascorbic acid by Cu²⁺ (Uchida & Kawakishi, 1993, 1986).

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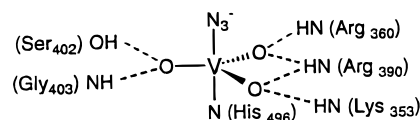


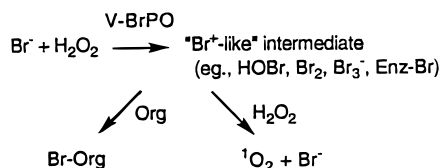
FIGURE 1: Active site of V-CIPO adapted from Messerschmidt and Wever (1996). The azide ligand results from crystallization from azide-containing buffer; preliminary structural studies on the azide-free form indicates an oxygen atom is in the apical position replacing N₃⁻.

The identification of 2-oxohistidine in inactivated Cu,Zn-SOD prompted us to investigate whether the irreversible inactivation of the marine vanadium bromoperoxidase (V-BrPO)¹ that occurs at low pH under turnover in the absence of an organic substrate is due to the oxidation of histidine to 2-oxohistidine.

V-BrPO (*Ascophyllum nodosum*) is an acidic glycoprotein containing one vanadium(V) per subunit (de Boer et al., 1986; Everett & Butler, 1989; Krenn et al., 1989). Vanadium can be removed from V-BrPO producing the inactive apoenzyme derivative, and the activity can be fully restored by the addition of vanadate to apo-(V)-BrPO (de Boer et al., 1988). While the structure of V-BrPO is not yet known, the structure of vanadium chloroperoxidase (V-CIPO), a related enzyme isolated from the fungus *Curvularia inaequalis*, shows that vanadate is coordinated to the protein by only one histidine residue, His 496, in a pentagonal bipyramidal geometry about vanadium(V) (Figure 1; Messerschmidt & Wever, 1996). Several other amino acid side chains (i.e.,

¹ Abbreviations: BCA, bicinchoninic acid; BrPO, bromoperoxidase; ECD, electrochemical detection; MCD, monochlorodimedone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PITC, phenylisothiocyanate; ¹O₂, singlet oxygen; TEA, triethanolamine; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; V-BrPO, vanadium bromoperoxidase; V-CIPO, vanadium chloroperoxidase.

Scheme 1



Lys 353, Arg 360, Arg 390, Ser 402) and the amide nitrogen of Gly 403 are hydrogen bonded to the vanadate oxygen atoms, stabilizing vanadate coordination to the protein. His 404, present in the active site channel in close proximity to the vanadate ion, is thought to function in acid/base catalysis; it must be protonated for H_2O_2 to bind to V-BrPO (van Schijndel et al., 1994). While the full sequence of V-BrPO (*A. nodosum*) is not known, there is sequence similarity between V-CIPO and V-BrPO including the regions containing the histidine ligand, His 496, the acid/base histidine, His 404 in the active site channel, and four of the five the amino acids which hydrogen bond to the vanadate oxygen atoms (Messerschmidt & Wever, 1996; Vilter, 1995). Thus the vanadium binding site and the overall protein structure should be very similar for both enzymes.

V-BrPO catalyzes the oxidation of bromide, chloride, and iodide by hydrogen peroxide (Butler & Walker, 1993). The oxidized halogen intermediate can halogenate an appropriate organic substrate or oxidize a second equivalent of hydrogen peroxide producing dioxygen (Scheme 1; Everett & Butler, 1989; Everett et al., 1990a). In the case of the peroxidative oxidation of bromide catalyzed by V-BrPO, the dioxygen produced has been shown to be in the singlet excited state (${}^1\Delta_g$; Scheme 1; Everett et al., 1990b). Singlet oxygen is formed quantitatively above neutral pH and does not cause inactivation of V-BrPO (Everett et al., 1990b).

Steady-state kinetic analyses of V-BrPO show that both of the substrates, bromide and hydrogen peroxide, are also noncompetitive inhibitors of V-BrPO (Everett et al., 1990a; Soedjak et al., 1995). An ionizable group with a pK_a between 6.5 and 7 is involved in H_2O_2 inhibition (Soedjak et al., 1995), which is suggestive of histidine and possibly the analogous histidine to His 404 in V-CIPO. The inhibition by hydrogen peroxide is fully reversible at short reaction times (<10 min) between pH 5.5 and 8.0 (Soedjak et al., 1995). At longer reaction times significant inactivation occurs; however, this inactivation is fully reversed on addition of vanadate to the inactivated enzyme. An irreversible inactivation occurs at pH 4–5 (100 mM H_2O_2 , 100 mM KBr, 4.5 nM V-BrPO in 0.1 M citrate buffer), which is not reversed by addition of vanadate (Soedjak et al., 1995). The nature of this low pH irreversible inactivation is the subject of the present investigation.

MATERIALS AND METHODS

Bromoperoxidase Preparation. Vanadium bromoperoxidase was isolated from *A. nodosum* collected at Kornwerderzand, Holland in 1990. The isolation procedure described previously (Everett et al., 1990b) was modified as follows: Cross-linked polyvinylpyrrolidone (1.5%) was included in the initial extraction of V-BrPO in 0.2 M Tris buffer, pH 8.3. In some preparations Sephadex G-100 was used in place of Sephacryl S-200. The ammonium sulfate and ethanolic precipitation steps and the concanavalin A column were omitted. For some experiments (i.e., amino acid analyses),

V-BrPO was purified by electroelution. Here V-BrPO samples were run on a native 8% Bio-Rad MiniProtein II polyacrylamide gel using 50 mM Tris-HCl, pH 8.3, as the running buffer. The edges of the gel were stained for peroxidase activity with *o*-dianisidine (Vilter & Glombitza, 1983), and the remaining unstained gel bands corresponding to V-BrPO were excised. The enzyme was eluted on a Bio-Rad Model 422 Electroeluter with 50 mM Tris, pH 8.3, using a 60 mA current for 3–5 h. Reducing SDS-PAGE showed one major band (>95%) at MW 67 000. The enzyme stock solution was stored in 0.1 M Tris buffer, pH 8.3, 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^- and 2 mM H_2O_2 , in 0.1 M phosphate, pH 6.0. The change in extinction coefficient between MCD and Br-MCD is 19 900 $\text{cm}^{-1} \text{M}^{-1}$ above pH 5 (Hager et al., 1966). The specific activity of the purified V-BrPO was 115–125 μmol brominated per min per mg of V-BrPO (units/mg), varying between different isolations.

H_2O_2 Inactivation of V-BrPO. V-BrPO was inactivated by a modified version of a procedure initially described by Soedjak (1991). Typically, 15 nM V-BrPO was incubated with 100 mM H_2O_2 and 100 mM KBr in 50 or 100 mM citrate buffer at pH 4.0 for 6 min, at which time the turnover was quenched by the addition of an AODISK.² In control reactions either the H_2O_2 or the KBr was excluded from the incubation solution. The reacted solutions were then washed with doubly distilled H_2O by ultrafiltration [i.e., a stirred cell with a YM30 membrane (Amicon, Inc.), Centriprep 10 or 30 (Amicon, Inc.), or Centricon 30 (Amicon, Inc.)] to remove remaining hydrogen peroxide and salts. Washed enzyme solutions were then lyophilized (SpeedVac Concentrator SVC-100H, Savant Inc.).

Preparation of 2-Oxohistidine and N^α -Benzoyl-2-oxohistidine for Use as Standards: Method A. 2-Oxohistidine was prepared as previously described (Uchida & Kawakishi, 1986). In this procedure, N^α -benzoylhistidine (e.g., 0.5–5.0 mM) was oxidized by reaction with ca. 50 μM copper sulfate and ca. 5 mM ascorbate in 0.1 M phosphate buffer, pH 7, overnight at 37 °C (Uchida & Kawakishi, 1986). The reaction mixture was evaporated to dryness, and the solid was extracted with ca. 1 mL of methanol. The methanolic solution was then dried down, giving N^α -benzoyl-2-oxohistidine. Hydrolysis of N^α -benzoyl-2-oxohistidine, to remove the benzoyl derivatization, was achieved by dissolving the solid (e.g., 0.5–1.0 mg) in 300 μL of 6 M HCl. The hydrolysis solution was placed in an ampule, deaerated with argon, flame sealed, and heated in an oven at 110 °C overnight as described below. The hydrolyzed sample was then dried down giving 2-oxohistidine, which was used as a standard for HPLC electrochemical detection (HPLC-ECD; see below). Unhydrolyzed N^α -benzoyl-2-oxohistidine could also be detected electrochemically using the same electrochemical conditions used to detect 2-oxohistidine (see Figure 4); however, N^α -benzoylhistidine is not observable electrochemically (Uchida & Kawakishi, 1993). The 2-oxohistidine

² AODISK Neutralizer (Ciba Vision) is a platinum-coated plastic disk that catalyzes the disproportionation of hydrogen peroxide.

moiety has been shown to be stable to the acid hydrolysis conditions required to remove the *N*^α-benzoyl derivatization (Uchida & Kawakishi, 1993).

Method B. *N*^α-Benzoyl-2-oxohistidine was also prepared by reaction of 0.5–5.0 mM *N*^α-benzoylhistidine with 0.25–5 equiv of HOBr (made by dissolving bromine vapor in aqueous NaOH). Reactions were carried out in the presence of 0.1 M KBr and in either 0.1 M HClO₄, 0.1 M HCl, 0.1 M citrate buffer, pH 4.0, 0.1 M phosphate buffer, pH 5.7, or 0.1 M phosphate buffer, pH 7.2. *N*^α-Benzoyl-2-oxohistidine was detected by HPLC-ECD as described above (e.g., Figure 4). When collection of the product was necessary, the reaction mixture was lyophilized. *N*^α-Benzoyl-2-oxohistidine was then extracted into methanol and dried down via rotary evaporation. *N*^α-Benzoyl-2-oxohistidine was also formed by reaction of HOBr with *N*^α-benzoylhistidine in strong acid (0.05 M HClO₄), demonstrating further that the 2-oxo moiety is stable in acid (see Supporting Information).

Amino Acid Analyses. Hydrolysis of the enzyme samples (i.e., turnover-inactivated V-BrPO or native V-BrPO) was achieved by dissolving ca. 200 μg in ca. 300 μL of 6.0 M HCl in sealable ampules and deaerating with argon for approximately 2 min. The deaerated ampules were then flame sealed and placed in an oven at 110 °C overnight. On completion of hydrolysis, the ampules were broken open and the contents evaporated to dryness (SpeedVac Concentrator, Savant Inc.).

The procedure for quantitative amino acid analysis was carried out by a modification of Edman's method as originally described by Ebert (1986). The hydrolyzed protein samples were dried down then redissolved twice in H₂O and redried to remove residual HCl. The samples were then dissolved in 5 μL of 50% ethanol. To this solution was added 10 μL of phenylisothiocyanate (PITC) derivatization buffer. The derivatization buffer is composed of a 90% mixture of 7:1:2 ethanol/PITC/triethanolamine (TEA), respectively, in H₂O. Derivatization was allowed to proceed for at least 10 min, at which time the samples were dried down.

The derivatized amino acids were separated by HPLC using a stepwise gradient modified from that described by Ebert (1986). Solvent A was 2.75 mL of TEA in 1 L of 50 mM sodium acetate set to pH 6.4 with phosphoric acid, and solvent B was 50% solvent A, 40% acetonitrile, and 10% methanol. The samples were run on a Waters HPLC system with UV monitoring at 254 nm (the absorbance maximum for PITC derivatives). Amino acids were separated using a Spherisorb ODS-2 column or a YMC ODS-AQ C-18 column; both columns were heated to 37 °C with a water jacket. Samples were dissolved in 150 μL of solvent A and filtered with 0.2 μm centrifuge filters.

HPLC Electrochemical Detection. 2-Oxohistidine was analyzed by HPLC electrochemical detection (HPLC-ECD) using a Waters 464 electrochemical detector equipped with a glassy carbon electrode. Detection was run in the DC mode at a potential of 850 mV (Uchida & Kawakishi, 1993). Underivatized protein hydrolysis samples were run over either a Spherisorb ODS-2 column (Phase Separations, Ltd.) or a YMC ODS-AQ C-18 column (YMC, Inc.). Amino acid separation was achieved under isocratic elution conditions of 50 mM NaCl, 0.1% TFA, and 20% methanol at 0.7 mL/min for the Spherisorb column and 1.0 mL/min for the YMC column. The protein hydrolysate samples were dissolved

in the elution solvent or water and filtered through 0.2 μm filters prior to injection. Injection volumes were typically 100–200 μL. The presence of 2-oxohistidine in the enzyme was identified by comparison of retention time to a standard of 2-oxohistidine. *N*^α-Benzoylhistidine samples (e.g., *N*^α-benzoylhistidine, *N*^α-2-oxobenzoylhistidine, and 2-oxohistidine) were also monitored spectrophotometrically (typically 230 nm). Under our protein hydrolysate conditions, 2-oxohistidine could not be detected spectrophotometrically because it was not present in high enough concentration.

Atomic Absorption Analyses. The ability of H₂O₂-inactivated V-BrPO to bind vanadium(V) was examined by atomic absorption spectrometry (AAS). Samples of V-BrPO (40 μg) which had been inactivated under the low pH turnover conditions described above were washed with 0.1 M Tris buffer, pH 8.3, by ultrafiltration (Centricon 30; Amicon, Inc.). Approximately 50 μM ammonium vanadate was then added to the washed and concentrated solution of inactivated V-BrPO and allowed to incubate overnight. The incubated solution was washed thoroughly by ultrafiltration and tested for vanadium content by AAS. AAS was performed on a Perkin Elmer SpectraAA furnace atomic absorption spectrometer using a standards additions procedure with 250 ppb vanadium standard. Samples (10 μL each) were autoinjected for analysis.

General Reagents and Procedures. The concentration of H₂O₂ was determined spectrophotometrically by the formation of triiodide (I₃⁻) (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 and *N*^α-benzoylhistidine were purchased from Sigma. Cross-linked polyvinylpyrrolidone was purchased from Aldrich. All other chemicals were Reagent grade.

RESULTS AND DISCUSSION

Electrochemical Detection of 2-Oxohistidine in Hydrolyzed Samples of the Low-pH-Turnover-Inactivated V-BrPO. We have shown previously that V-BrPO is irreversibly inactivated under turnover at pH 4 in the absence of organic substrates. To investigate whether 2-oxohistidine was formed, samples of V-BrPO which had been inactivated under turnover conditions at pH 4 (0.1 M KBr and 0.1 M H₂O₂ in 0.1 M citrate buffer for ca. 5 min; see Materials and Methods; Soedjak et al., 1995) and hydrolyzed *in vacuo* overnight in 6 M HCl (see Materials and Methods) were analyzed by electrochemical detection on HPLC (HPLC-ECD). HPLC-ECD analysis demonstrated that 2-oxohistidine is formed during the low pH inactivation of V-BrPO (Figure 2b), by comparison to the retention time of an authentic sample of 2-oxohistidine (Figure 2a), which had been prepared as described in the literature (Uchida & Kawakishi, 1986). When V-BrPO is incubated under identical conditions except in the absence of KBr (i.e., 0.1 M H₂O₂ in 0.1 M citrate, pH 4, for ca. 5 min), then hydrolyzed and analyzed by HPLC-ECD, a small signal at the same retention time as 2-oxohistidine may be present (Figure 2c), but this signal is not appreciably above the noise level, demonstrating that H₂O₂ does not oxidize His directly under these condi-

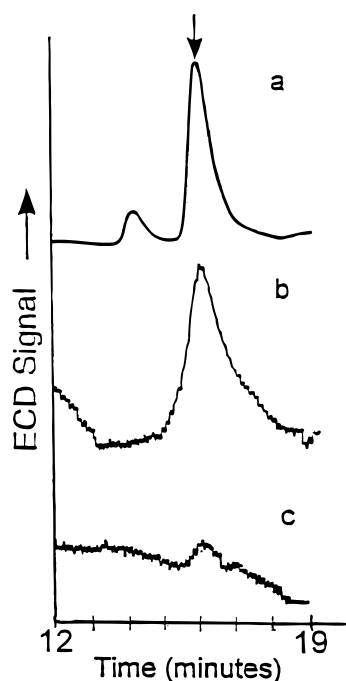


FIGURE 2: HPLC-electrochemical detection of 2-oxohistidine in turnover-inactivated V-BrPO at low pH. (a) Standard of 2-oxohistidine; (b) hydrolyzed, turnover-inactivated V-BrPO (see Materials and Methods for inactivation conditions); (c) hydrolyzed, native V-BrPO after incubation in 0.1 M H_2O_2 /0.1 M citrate, pH 4, for ca. 5 min. The arrow indicates the peak for 2-oxohistidine. The conditions for the HPLC-ECD are described in Materials and Methods, using the Spherisorb ODS-2 (Phase Separations, Ltd.) column.

tions.³ On the other hand, when V-BrPO is incubated with 0.1 M KBr under the same conditions, but, in the absence of hydrogen peroxide, no 2-oxohistidine was formed (data not shown), demonstrating that 2-oxohistidine is not present in native V-BrPO. Thus the formation of 2-oxohistidine requires the presence of both hydrogen peroxide and bromide.

Investigation of the effect of pH on the formation of 2-oxohistidine (Figure 3) shows that 2-oxohistidine is formed at pH 4 (Figure 3a), but little, if any, is formed at pH 5.7 (Figure 3b) and pH 7.2 (Figure 3c). These results follow the pH trend for irreversible inactivation of V-BrPO which is only observed at pH 4 and 5 (Soedjak et al., 1995). Thus in addition to the requirement of bromide and hydrogen peroxide, a low pH is required for the formation of significant 2-oxohistidine.

The effect of the concentration of H_2O_2 on the inactivation of V-BrPO was investigated (Table 1). Turnover of 15 nM V-BrPO with equimolar H_2O_2 (15 nM) resulted in ca. 40% overall irreversible inactivation of V-BrPO, whereas turnover in the presence of excess H_2O_2 (i.e., 100 nM and 100 mM) resulted in nearly complete inactivation. In the case of the equimolar H_2O_2 /V-BrPO experiment, the activity loss which was recovered by vanadate addition was the result of citrate displacement of bound vanadium after the H_2O_2 had been consumed (Soedjak et al., 1991).

Organic Substrate Protection of V-BrPO Against Turnover Inactivation. Turnover of V-BrPO at pH 4 in the presence of an organic substrate [i.e., monochlorodimedone (MCD)

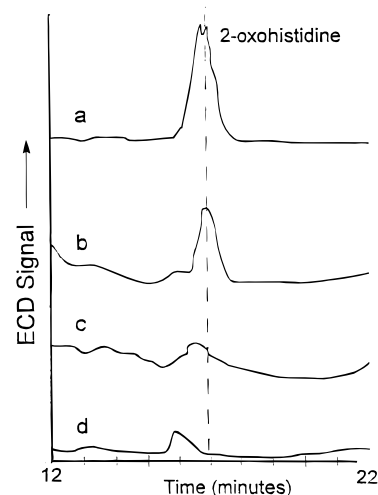


FIGURE 3: Effect of pH on the formation of 2-oxohistidine during turnover of V-BrPO. The turnover conditions were 15 nM V-BrPO, 100 mM H_2O_2 , and 100 mM KBr in (a) 0.1 M citrate, pH 4.0, (b) 0.1 M phosphate, pH 5.7, (c) 0.1 M phosphate, pH 7.2, and (d) 0.1 M phosphate, pH 8.0, for 6 min. The Spherisorb ODS-2 (Phase Separations, Ltd.) HPLC column was used. The dashed line indicates the position of 2-oxohistidine. A Logitech Scanman 256 was used to scan (using the FotoTouch Color program) and digitize (using the "Un-Scan-It" program) the HPLC-ECD traces.

Table 1: Effect of Hydrogen Peroxide Concentration on the Turnover Inactivation of V-BrPO at pH 4

$[\text{H}_2\text{O}_2]$	specific activity after turnover at pH 4 ^a (units/mg)	specific activity after addition of vanadate to the turnover sample, pH 4 ^b (units/mg)	percent remaining activity
0	125	150	(120)
15 nM	44	87	58
100 nM	14	14	9
100 mM	2.5	5	1.6

^a Reaction conditions: 15 nM V-BrPO was incubated for 10 h in 0.1 M citrate buffer, pH 4.0, containing 0.1 M KBr and the specified concentration of H_2O_2 . ^b After incubation of V-BrPO under the turnover conditions described in footnote a, the remaining H_2O_2 was removed by ultrafiltration (washing with 0.1 M Tris buffer pH 8.3) and ammonium vanadate (final concentration 50 μM) was added to the samples under conditions of 0.1 M Tris buffer, pH 8.3, and allowed to incubate for 3 h. The bromoperoxidase activity was measured using the standard MCD assay conditions (see Materials and Methods).

or *N*^α-benzoylhistidine] was found to protect V-BrPO against inactivation or 2-oxohistidine formation. These experiments were carried out by comparison of the specific activity of V-BrPO after turnover of 15 nM V-BrPO in a reaction solution of 0.1 M H_2O_2 , 0.1 M KBr, and 0.5 mM MCD in 0.1 M citrate, pH 4, for 5 min. The specific bromoperoxidase activity of V-BrPO in the presence of MCD was 90 units/mg compared to a specific activity of 115 units/mg for native V-BrPO. Under the same conditions but in the absence of MCD, V-BrPO was completely inactivated. After turnover of V-BrPO for 20 min in the MCD-containing reaction solution, at which time all the of MCD has been brominated, the V-BrPO sample was similarly completely inactivated. Thus in the presence of MCD, V-BrPO catalyzes the bromination of MCD without significant inactivation of V-BrPO; in addition, 2-oxohistidine was not formed during turnover in the presence of MCD (data not shown). However, once MCD is consumed, V-BrPO is inactivated at low pH.

N^α-Benzoylhistidine was also investigated as a substrate protector of V-BrPO inactivation. When 5 nM V-BrPO was

³ The oxidation of a small amount of histidine in this sample may be due to the presence of a small amount of bromide in the enzyme sample as isolated (Vilter, 1995).

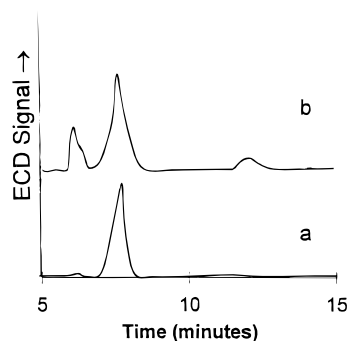


FIGURE 4: HPLC-ECD of N^α -benzoylhistidine oxidation by HOBr and Cu(II)-ascorbate. (a) HOBr oxidation product of N^α -benzoylhistidine; (b) authentic N^α -benzoyl-2-oxohistidine prepared by Cu(II) ascorbate oxidation of N^α -benzoylhistidine. The YMC ODS-AQ C-18 (YMC, Inc.) HPLC column was used. The data were digitized as described in the legend to Figure 3.

incubated for 15 min with 0.1 M H_2O_2 and 0.1 M KBr, in 0.1 M citrate buffer, pH 4.0, with 0.2 mM N^α -benzoylhistidine or without N^α -benzoylhistidine, the specific bromoperoxidase activities were 76 and 19 units/mg, respectively, compared to the native V-BrPO sample before incubation, which had a specific activity of 115 units/mg. The reaction in the presence of N^α -benzoylhistidine was also accompanied by the formation of N^α -benzoyl-2-oxohistidine (see Supporting Information). Thus N^α -benzoylhistidine provides partial substrate protection of V-BrPO. The protection occurs because N^α -benzoylhistidine is oxidized to N^α -benzoyl-2-oxohistidine over V-BrPO-histidine oxidation.

Reaction of Hypobromite with N^α -Benzoylhistidine. The possible role of oxidized bromine species in the V-BrPO-catalyzed oxidation of histidine was investigated by reaction of hypobromite with N^α -benzoylhistidine. Hypobromite (i.e., 1 μ mol from a stock solution of 10–20 mM OBr^- in 0.1 M NaOH) was added to 1 μ mol of N^α -benzoylhistidine in 0.1 M citrate buffer, pH 4. (When hypobromite is diluted into citrate buffer, pH 4, a rapid equilibration occurs producing a mixture of HOBr, Br_2 , and Br_3^-). HPLC-ECD analysis shows that the retention time of the product of the HOBr reaction with N^α -benzoylhistidine (Figure 4a) is the same as that for N^α -benzoyl-2-oxohistidine prepared by the method of Uchida and Kawakishi (1986) (Figure 4b), indicating that HOBr oxidation forms N^α -benzoyl-2-oxohistidine. The FAB-MS of the HOBr oxidation product gave a MW of 276 g/m ($M+1$), consistent with formation of N^α -benzoyl-2-oxohistidine. The 1H NMR of this product shows only one imidazolone proton at 5.6 ppm. [The other resonances are as follows: methylene, 3.2 ppm (d); C_α , 4.45 ppm (m); CONH, 8.3 ppm (s); PhCO, 7–8 ppm region.]

The pH dependence of the HOBr oxidation of N^α -benzoylhistidine shows that N^α -benzoyl-2-oxohistidine is formed when the reaction is carried out at pH 4 and 5.7 (Figure 5, panels a and b, respectively), suggesting that oxidized bromine species produced by V-BrPO in the enzyme reaction mediates histidine oxidation. By contrast, HPLC-ECD did not show formation of N^α -benzoyl-2-oxohistidine when HOBr was reacted with N^α -benzoylhistidine above pH 6 (i.e., pH 7.2 phosphate buffer as shown in Figure 5c or pH 6.5 Hepes buffer and pH 8.0 phosphate buffer).

Because V-BrPO catalyzes the formation of singlet oxygen at neutral pH during the peroxidative oxidation of bromide

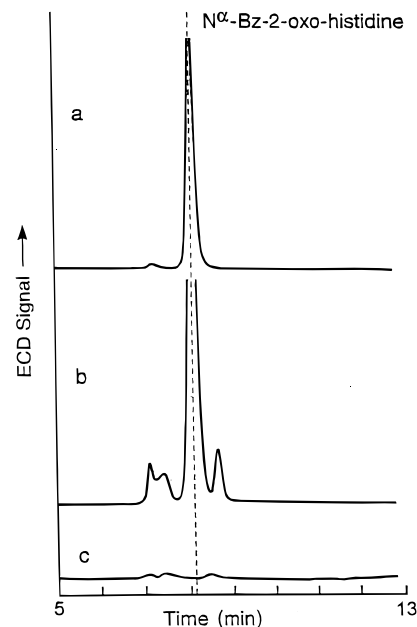


FIGURE 5: Effect of pH on the formation of N^α -benzoyl-2-oxohistidine in the reaction of HOBr with N^α -benzoylhistidine. Reaction conditions: 1 mM OBr^- was added to 1.0 mM N^α -benzoylhistidine in (a) 0.1 M citrate, pH 4.0, (b) 0.1 M phosphate, pH 5.7, and (c) 0.1 M phosphate, pH 7.2. The reaction time was 10 min. The YMC ODS-AQ C-18 (YMC, Inc.) HPLC column was used. The dashed vertical line indicates the peak for N^α -benzoyl-2-oxohistidine.

in the absence of an organic substrate (Everett et al., 1990a), the oxidation of histidine by singlet oxygen was investigated. The reaction of 2.6 mM N^α -benzoylhistidine in 0.1 M phosphate buffer, pH 7.2, containing 8 mM H_2O_2 with 2.6 mM HOBr, i.e., conditions in which singlet oxygen would be produced by the oxidation of H_2O_2 by HOBr (Everett et al., 1990a), did not show production of 2-oxohistidine. Thus, even under stoichiometric conditions, singlet oxygen does not oxidize N^α -benzoylhistidine to the 2-oxo derivative.

Bromination, oxidation, and oxidative degradation of imidazoles by aqueous bromine are well known (Boulton & Coller, 1974; Schmir & Cohen, 1965; Stensio et al., 1973). On the other hand, investigations of the bromination and oxidation of coordinated imidazoles are surprisingly limited. One notable exception is the thorough work on the bromination and oxidation of imidazoles coordinated to pentamminecobalt(III) (Blackman et al., 1991a,b). Reaction of excess bromine in acetate or phosphate, pH 4–6, with $RImH^+$ ($R = (NH_3)_5Co$; ImH is imidazole) results in the formation of $R(\text{parabanate})^{2+}$ (parabanate is imidazolidine-2,4,5-trione; Blackman et al., 1991a). The reaction was shown to proceed via the formation of $R(4,5-Br_2ImH)^{3+}$ and $R(2,4,5-Br_3ImH)^{2+}$. Addition of 1 equiv of bromine to $R(2,4,5-Br_3ImH)^{2+}$ in acetate buffer produced $R(\text{parabanate})^{2+}$, quantitatively; Blackman et al. (1991a) propose a mechanism in which additional bromination at C-2 leads to spontaneous bromide hydrolysis. Thus, in the presence of excess aqueous bromine, as in the case of enzymatic oxidation of bromide by hydrogen peroxide, oxidation of the histidyl imidazolium moiety occurs first by bromination followed by subsequent bromide hydrolysis.

Reactivity of V-BrPO Mimics. The ability of *cis*-dioxovanadium(V) (VO_2^+), a well-established functional mimic of V-BrPO in strong acid solution (Clague & Butler, 1995),

to catalyze the oxidation of N^α -benzoylhistidine was also investigated. N^α -Benzoylhistidine (1.0 or 5.0 mM) was reacted with 5.0 mM H_2O_2 , 0.2 M KBr, and 0.4 mM NH_4VO_3 in 0.05 M $HClO_4$ for 1 h. Under these conditions, cis - VO_2^+ catalyzes the formation of oxidized bromine species (i.e., Br_3^- , Br_2 , HOBr; Clague & Butler, 1995). HPLC-ECD results showed the formation of N^α -benzoyl-2-oxohistidine (see Supporting Information). cis - VO_2^+ only functions in strongly acidic media. Thus any vanadate ($HVO_4^{2-}/H_2VO_4^-$) released from inactivation of V-BrPO at pH 4 (see below) will not catalyze the oxidation of bromide by hydrogen peroxide (Clague & Butler, 1995). In addition to cis - VO_2^+ , $MoO(O_2)_2(ox)^{2-}$ (ox^{2-} is oxalate), another functional mimic of V-BrPO (Meister & Butler, 1994; Reynolds et al., 1994), also catalyzes the bromoperoxidative oxidation of N^α -benzoylhistidine forming N^α -benzoyl-2-oxohistidine (see Supporting Information); the $MoO(O_2)_2(ox)^{2-}$ catalysis was carried out under conditions of 1.5 mM N^α -benzoylhistidine, 5.0 mM H_2O_2 , 0.1 M KBr, and 1.5 mM $MoO(O_2)_2(ox)^{2-}$ in 90 mM oxalate buffer, pH 5, for 3 h.

Vanadium Binding to Turnover-Inactivated V-BrPO. Upon inactivation of V-BrPO under turnover conditions, the inactivated samples and controls (i.e., V-BrPO incubated with 0.1 M KBr in 0.1 M citrate, pH 4.0, without H_2O_2) were washed with 0.1 M Tris buffer, pH 8.3. Ammonium vanadate (50 μ M final concentration) was added to each sample and allowed to incubate overnight. The samples were then washed by ultrafiltration (i.e., five cycles of washing with water to remove excess vanadium), dried down, and then redissolved in 250 μ L of H_2O . Atomic absorption analysis demonstrated that, after reincubation with vanadate, the inactivated sample contained 57% less vanadium than the control.⁴ Thus oxidation of histidine in V-BrPO affects vanadate coordination. The basis of the reduced vanadium binding capacity is somewhat surprising considering that oxidized imidazoles, including those oxidized at the 2-position, are known to coordinate to transition metals [e.g., $(NH_3)_5Co(imidazolidine-2,4,5-trione)^{2+}$; Blackman et al., 1991a,b]. On the other hand, histidine oxidation could lead to significant conformational changes in the protein which could affect vanadate coordination.

Amino Acid Analysis of Turnover-Inactivated V-BrPO. Standard Edman's amino acid analysis was also carried out on turnover-inactivated V-BrPO (i.e., 15 nM V-BrPO, 100 mM H_2O_2 , 100 mM KBr, and 0.1 M citrate buffer at pH 4 for 5 min). Approximately 70–74% of the histidine in this sample was lost compared to the control (i.e., incubation of 15 nM V-BrPO, 100 mM KBr, in 0.1 M citrate, pH 4, in the absence of H_2O_2). 2-Oxohistidine formation was not quantitative with histidine loss in turnover-inactivated V-BrPO because further reaction of 2-oxohistidine leads to other, as yet, unidentified products.

Tyrosine (70–100%) may also be consumed during the inactivation of V-BrPO, possibly due to formation of Br-Tyr or Br_2 -Tyr (Knight et al., 1975; Knight & Welch, 1978; McElvany et al., 1980; Manthey et al., 1984); the fate

of Tyr in turnover-inactivated V-BrPO is under further investigation. No new peaks in the amino acid analysis of turnover inactivated V-BrPO were detected using phenylisothiocyanate (PITC) derivatization. Attempts to derivatize 2-oxohistidine have not been successful (Uchida & Kawakishi, 1993).

The Effect of Peroxidative Chloride Oxidation on Activity of V-BrPO. We attempted to measure the turnover inactivation of V-BrPO and formation of 2-oxohistidine with chloride as the halide source instead of bromide. The specific bromoperoxidase activity of V-BrPO measured after incubation under conditions of 0.1 or 1.0 M KCl, 0.1 M H_2O_2 , and 15 nM V-BrPO in 90 mM citrate, pH 4.0, for 20 min and subsequent removal of unreacted Cl^- and H_2O_2 by ultrafiltration was compared to that of a control in which V-BrPO was incubated in 90 mM citrate, pH 4, for 20 min without chloride or hydrogen peroxide. The specific activities for the "turnover" sample (at 0.1 M Cl^-) and the control sample were 73 and 77 units/mg, respectively, compared with a specific activity of the starting enzyme of 115 units/mg, suggesting that the loss of activity was not due to turnover since the activity of both the sample and the control decreased similarly. (The bromoperoxidase activity was measured under the standard conditions as described in Materials and Methods.) The concentration of H_2O_2 measured after 20 min showed that none of the H_2O_2 had been consumed, confirming that the loss in activity was not due to turnover. After 1 h the specific activities for the "turnover" sample and the control sample had decreased further to 53 and 58 units/mg, respectively, and the concentration of hydrogen peroxide was still 0.1 M. The activities of both samples were restored to 90 units/mg upon addition of vanadate. Thus, under these conditions peroxidative chloride oxidation does not occur.⁵ The inactivation that was observed is likely due to loss of vanadium. Such inactivation is not apparent during the peroxidative bromide oxidation experiments because the bromoperoxidase activity is *much* higher than the chloroperoxidase activity and much higher than the rate of loss of vanadium. In addition, as might be anticipated from the lack of inactivation of V-BrPO, 2-oxohistidine was not detected under the incubation conditions described above (i.e., 15 nM V-BrPO, 0.1 M KCl, and 0.1 M H_2O_2 in 90 mM citrate, pH 4.0, for 20 min).

CONCLUSION

We have shown that 2-oxohistidine is formed during the irreversible inactivation of V-BrPO which occurs at low pH (i.e., <pH 5). Formation of 2-oxohistidine requires all the components of turnover (i.e., bromide, hydrogen peroxide, and V-BrPO) as well as low pH; inactivation does not occur nor is significant 2-oxohistidine formed in the presence of hydrogen peroxide alone. Thus, unlike Cu,Zn-SOD, in which inactivation and 2-oxohistidine formation occur on incubation of the enzyme with hydrogen peroxide at neutral pH (Uchida & Kawakishi, 1994), V-BrPO requires all of the turnover components (i.e., Br^- , H_2O_2 , and V-BrPO) and

⁴ If one assumes the MW of V-BrPO (*A. nodosum*) is 97 000, as determined by ultracentrifugation (Tromp et al., 1990), then the vanadium content is 1.0 V per subunit in the native V-BrPO. The 57% loss of vanadium corresponds to 0.43 V per subunit. If one takes the MW to be 65 000 as indicated by SDS-PAGE on reduced, denatured V-BrPO, the vanadium content is 0.67 per subunit in native enzyme and 0.29 per subunit in the inactivated sample.

⁵ V-BrPO does catalyze the oxidation of chloride by hydrogen peroxide under conditions of high enzyme concentration (Soedjak & Butler, 1991). However, at high enzyme concentrations, both the specific bromoperoxidase activity of V-BrPO and the inactivation of V-BrPO by peroxidative bromide oxidation are reduced, presumably due to aggregation of V-BrPO.

a low pH. The reaction of hypobromite with *N*^α-benzoyl-histidine at low pH forms *N*^α-benzoyl-2-oxohistidine. Thus the oxidation of histidine in V-BrPO likely occurs by an initial bromination process. On the basis of the results of imidazole oxidation in the pentamminecobalt(III) complex by aqueous bromine (Blackman, 1991a), further oxidation would lead to the 2-oxo product. The oxidation of histidine in the V-BrPO system further differs from the Cu,Zn-SOD system in that the former is an electrophilic process mediated by Br⁺, whereas the later is a radical process (Uchida & Kawakishi, 1989). The oxidation of histidine does not occur by singlet oxygen in V-BrPO, because neither 2-oxohistidine nor inactivation occur under the conditions in which singlet oxygen is produced quantitatively by V-BrPO (i.e., neutral pH or above; Everett et al., 1990a). Furthermore, when hypobromite was added to *N*^α-benzoylhistidine in the presence of hydrogen peroxide at neutral pH, conditions under which HOBr would react first with H₂O₂ to produce singlet oxygen, no 2-oxohistidine was formed.

We are continuing our investigations to address further questions about the mechanism of irreversible inactivation of V-BrPO at low pH. In particular, the identification of the specific histidines in V-BrPO that are oxidized, as well as the basis of the reduced vanadium binding affinity, awaits elucidation of the full sequence of V-BrPO from *A. nodosum*. Investigations into the bromination or oxidation of other amino acid residues (e.g., Tyr) in V-BrPO are also in progress.

SUPPORTING INFORMATION AVAILABLE

Four figures showing HPLC-ECD determinations of the formation of *N*^α-benzoyl-2-oxohistidine under various conditions (5 pages). Ordering information is given on any current masthead page.

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