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The novel non-heme vanadium bromoperoxidase from marine algae: phosphate inactivation

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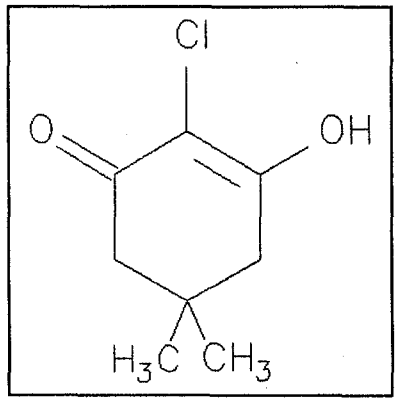
SUMMARY

Vanadium bromoperoxidase is a naturally occurring vanadium-containing enzyme isolated from marine algae. V-BrPO catalyzes the oxidation of halides by hydrogen peroxide which can result in the halogenation of organic substrates. Bromoperoxidase activity is measured by the halogenation of monochlorodimedone (2-chloro-5,5-dimethyl-1,3-dimedone, MCD). In the absence of an organic substrate, V-BrPO catalyzes the halide-assisted disproportionation of hydrogen peroxide yielding dioxygen. The dioxygen formed is in the singlet excited state ($^1O_2$). V-BrPO is quite stable to thermal denaturation and denaturation by certain organic solvents which makes V-BrPO an excellent candidate for industrial applications. The stability of V-BrPO in the presence of strong oxidants and in the presence of phosphate is reported. Incubation of V-BrPO in phosphate buffer (1–100 mM at pH 6; 2–10 mM at pH 5) inactivates the enzyme. The inactivity can be fully restored by the addition of vanadate if excess phosphate is removed. The inactivation of V-BrPO by phosphate can be prevented by the presence of H_2O_2 (4–40 mM). We are currently investigating the mechanism of V-BrPO inactivation by phosphate. V-BrPO was not inactivated by HOCl (1 mM) nor H_2O_2. In addition V-BrPO was not inactivated under turnover conditions of 1 mM H_2O_2 with 0.1–1 M Cl^- at pH 5 nor 2 mM H_2O_2 with 0.1 M Br^-.

INTRODUCTION

Vanadium bromoperoxidase was discovered in the early 1980s in the marine brown alga, *Ascophyllum nodosum* [32]. Since this time vanadium bromoperoxidase has been discovered in many other species of marine brown (Phaeophyta) seaweeds (e.g., *Ascophyllum nodosum* [10,32,34], *Macrocystis pyrifera* [2,26], *Fucus distichus* [2,26], *Laminaria saccharina* [5]), marine red seaweeds (e.g. *Ceramium rubrum* [18], *Corallina pilulifera* [19]), and a terrestrial lichen [24]. In addition vanadium-dependent bromoperoxidase activity has been detected in many other species of marine algae (e.g., *Pelvetia* [5], *Chorda* [5], etc.). Vanadium bromoperoxidase is a member of a new class of non heme-containing haloperoxidases distinct from the well known FeHeme haloperoxidases, (e.g., chloroperoxidase, bromoperoxidase and iodoperoxidase). FeHeme bromoperoxidases have also been discovered in marine organisms, including the alga *Penicillus capitatus* [22]. In addition to the widespread occurrence of bromoperoxidases in marine organisms, the existence of brominated and chlorinated compounds is nearly ubiquitous in marine organisms. One proposal is that these organisms may use such halogenated compounds in a chemical defense-type capacity [23].

Reactivity and mechanism. V-BrPO catalyzes the bromination of a variety of organic substrates, including monochlorodimedone (2-chloro-5,5-dimethyl-1,3-dimedone, MCD) (Scheme 1), a cyclic β-diketone. With the exception of MCD and possibly other β-diketone and β-keto-acid moieties, very few organic substrates are efficiently brominated. For example V-BrPO can catalyze the formation of 1,3,5-tribromophenol, 5-bromocytosine and



Monochlorodimedone (MCD)
Scheme 1

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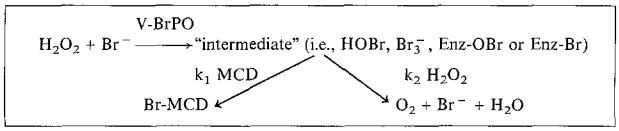
5-bromouracil, however, the reaction is not stoichiometric with hydrogen peroxide consumption, since much more than one equivalent of hydrogen peroxide is consumed per equivalent of brominated product produced [10]. In the absence of an organic substrate, V-BrPO catalyzes the formation of dioxygen in a reaction best described as a 'bromide-assisted disproportionation of hydrogen peroxide' or bromide-assisted catalatic activity [10]. We have established that both of these reactions proceed via the formation of a common intermediate [11], although the identity of the intermediate as enzyme-bound or released under physiological conditions has not been established unambiguously (see Scheme 2). MCD bromination and dioxygen formation are competitive processes (i.e., $k_1[MCD]$ vs. $k_2[H_2O_2]$) that fully account for the fate of the intermediate under most conditions [11].



Scheme 2

Recently we have shown that the dioxygen produced in the bromide-assisted disproportionation of hydrogen peroxide is in the singlet excited state [12]. The singlet dioxygen was identified by the characteristic emission at 1268 nm resulting form the decay of $^1O_2$ ($^1\Delta_g$) to $^3O_2$ ($^3\Sigma_g^-$), the effect of specific singlet oxygen quenchers and the solvent isotope effect on the singlet oxygen intensity and lifetime [12]. Singlet oxygen is produced in near stoichiometric yield (i.e., 80%) by V-BrPO, without inactivating V-BrPO. One striking feature of V-BrPO is its *exceptional* stability.

Properties of vanadium bromoperoxidase. All of the vanadium bromoperoxidase enzymes isolated so far are remarkably similar in their physical characteristics. The V-BrPOs are acidic (i.e., p*I* = 4) glycoproteins [2,20,26] which bind ca. one equivalent of vanadium per subunit (M.W. ca. 65 000/subunit) [6]. The vanadium ion can be removed from the V-BrPOs by dialysis against 1–10 mM EDTA in citrate-phosphate buffer, pH 3.8, producing the inactive apo-enzyme derivatives [2,6,26,32]. The activity can be fully restored by the addition of vanadate ($H_2VO_4^-/HVO_4^{2-}$) to the apo-(V)-BrPOs [2,8,26], thus demonstrating that enzyme-bound vanadium is required for catalytic activity. As isolated, the V-BrPOs contain a substoichiometric vanadium/subunit ratio (i.e. ca. 0.4), however, the vanadium/subunit stoichiometry can be increased to 1/1 upon addition of vanadate [2,8,26]. The increased binding of vanadate is also reflected in an increased specific activity up to a vanadium/subunit stoichiometry of 1/1 [2,8,26]. Moreover, the amino acid composition of the vanadium bromoperoxidases has been shown to be very similar [35].

Applicability of vanadium bromoperoxidase. Potential commercial applications of vanadium bromoperoxidase demand efficient utilization of reactants and an extended operational stability of the enzyme. These criteria are not met by fungal chloroperoxidase, myeloperoxidase (a chloroperoxidase) or lactoperoxidase (a bromoperoxidase), because these FeHeme enzymes are inactivated during turnover conditions [1,12]. On the other hand, the non heme chloroperoxidase from *Curvularia inaequalis* is very stable [21]. V-BrPO is also a thermally robust enzyme. The activity of V-BrPO is not affected by incubation at 50 °C for several hours [34]. At 70 °C, 38% of the bromoperoxidase activity remains after 1 h [34]. V-BrPO retains full activity under turnover conditions for three weeks at room temperature [7]. V-BrPO also displays an exceptional stability in many organic solvents [7,9]. The bromoperoxidase activity of V-BrPO in 60% acetone (for 40 days), 60% ethanol, 60% methanol or a mixture of 47% butanol/40% ethanol in water is reported to be identical to the activity of V-BrPO stored in water [7,9]. A slight increase in bromoperoxidase activity was observed in the presence of 1-propanol, Tween-80, cholate, or Triton X-100 [34]. In fact one application of the stability in organic solvents has been the encapsulation of V-BrPO in reverse micelles; the preliminary results indicate the bromoperoxidase activity increased by two-fold [13]. V-BrPO is also quite resistant to denaturation by guanidine hydrochloride [31].

The FeHeme haloperoxidases are far less stable. FeHeme chloroperoxidase (*C. fumago*) is an order of magnitude less resistant to inactivation by HOBr than is V-BrPO [9]. Myeloperoxidase [1] and lactoperoxidase [12] are partially inactivated by incubation with hydrogen peroxide and lactoperoxidase is fully inactivated under turnover conditions in the absence of an organic acceptor [12]. V-BrPO does not lose activity upon incubation in high concentrations of hydrogen peroxide [28]. V-BrPO is not inactivated by oxidized bromine species or singlet oxygen which are produced during turnover in the absence of an organic acceptor [12]. In addition non heme chloroperoxidase (*C. inaequalis*) is also resistant to denaturation by hydrogen peroxide [21].

Recently we have shown that V-BrPO can catalyze the chlorination of certain organic substrates (e.g., MCD, phenol red, taurine) or form dioxygen in the absence of an organic substrate [27]. With the discovery of chlorination activity of V-BrPO, we can now address the origin of chlorinated marine natural products. Many chlorinated natural products have important applications as drugs in the pharmaceutical industries. A carbon-chlorine bond is significantly stronger than a carbon-bromine bond which

makes chlorinated compounds more attractive as drugs. Thus, with the discovery of chloroperoxidase activity of the vanadium enzyme, its commercial viability is greatly increased. Most significantly, however, the stability of V-BrPO (which should now possibly be named "vanadium haloperoxidase", but which will still be referred to as V-BrPO throughout this paper), is far superior to the FeHeme chloroperoxidase and myeloperoxidase.

We have further investigated the stability of vanadium bromoperoxidase under a variety of conditions. We report herein on the effects of strong oxidants as well as the effect of phosphate on the bromoperoxidase activity of V-BrPO.

MATERIALS AND METHODS

Bromoperoxidase preparation. Vanadium bromoperoxidase was isolated from *Ascophyllum nodosum* collected at Kornwerderzand, Holland in April 1986 and 1989. The isolation procedure has been described previously [12]. The enzyme stock solution was stored in 50 mM Hepes (*N*-2-hydroxyethylpiperazine *N'*-2-ethane sulfonic acid), pH 7.0, or water at 0 °C or 4 °C. The specific activity for the bromination of MCD is ca. 112–120 U/mg in a 0.1 M phosphate assay buffer, pH 6.5 containing 0.1 M KBr, 2 mM H_2O_2, 50 μM MCD, 0.2 M Na_2SO_4. The specific activity for V-BrPO is usually given at pH 6.5 at room temperature even though the pH optimum depends on the conditions (e.g., hydrogen peroxide and bromide concentrations [11,34].

Bromoperoxidase activity measurements. The standard assay for determining the specific bromoperoxidase activity is the conversion of 2-chloro-5,5-dimethyl-1,3-dimedone (monochlorodimedone, MCD) to 2-bromo-2-chloro-5,5,-dimethyl-1,3-dimedone (Br-MCD) [16]. The reaction is followed spectrophotometrically at 290 nm, i.e., the λ_{max} of MCD. The specific activity is expressed in units/mg (U/mg) which is defined as the μmol of MCD brominated per min per mg of bromoperoxidase. The difference in extinction coefficients, $\Delta\varepsilon$, between MCD and Br-MCD at 290 nm is 19900 M^{-1} cm^{-1}, pH > 4. Under the conditions described below, aqueous vanadate and adventitiously bound vanadium(V) do not have BrPO activity [2,26].

Inactivation of V-BrPO by phosphate and protection of inactivation by hydrogen peroxide. V-BrPO (0.13–8.0 μM) was incubated in phosphate buffer (1–100 mM at pH 6.0 or 2–10 mM at pH 5.0) for various time periods (10–48 h) in the absence or presence of hydrogen peroxide (4–40 mM) in a total volume of 2 ml. After specific incubation times, V-BrPO was washed extensively (i.e., at least 5 concentration and dilution cycles) by ultrafiltration using a Centricon 30 (Amicon, Inc.) with doubly distilled water to remove phosphate and hydrogen peroxide. Ammonium vanadate was then added (less than 1 mM) and left to react for 5–8 h before ultrafiltration with doubly distilled water to wash out excess vanadate. The protein concentration was redetermined (see below) and the activity remeasured by the standard bromoperoxidase assay.

Preparation and characterization of the chloramine derivative of ammonia. Ammonia (10 mM) was incubated in 5 mM acetate, pH 5, in the presence of 0.1 M Cl^-, 4 mM H_2O_2 and 0.5 μM V-BrPO. The reaction was monitored spectrophotometrically at the absorption maximum (i.e., 240 nm for NH_2Cl) until the maximum absorbance was reached (i.e., 2.5 h). Catalase (10 μg/ml final concentration was then added to consume the remaining hydrogen peroxide. The chloramine product was obtained free of V-BrPO and catalase by ultrafiltration (Centricon 30). The UV spectra of the chloramine were referenced to equimolar ammonia in the same buffers containing 0.1 M chloride.

General reagents and procedures. The concentration of H_2O_2 (30% aqueous solution, Fisher Scientific) was determined spectrophotometrically by the formation of triiodide (I_3^-) [4]. Protein concentrations were determined by the bicinchoninic acid assay (BCA) [25], with reagents purchased from Pierce Chemical Co. The protein standard was albumin (Pierce). MCD was prepared as reported in the literature [15] or purchased from Sigma. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

Inactivation of V-BrPO by phosphate

When V-BrPO is incubated in 0.1 M phosphate buffer at pH 6 for 14 h, the bromoperoxidase activity is reduced by approximately 85% (see Table 1). The bromoperoxidase activity can be fully restored by the addition of vanadate. The extent of inactivation in a given time period depends on the phosphate concentration, with increased inactivation occurring at an overall higher phosphate concentration (Table 1). The extent of inactivation also increases with the length of incubation, as shown in Table 1 (e.g., for 10 mM and 20 mM phosphate).

When phosphate is removed from the inactivated samples of V-BrPO by ultrafiltration, the inactivated V-BrPO derivative can be fully reactivated by the addition of vanadate (see last column of Table 1). Full reactivation occurs only in the absence of appreciable phosphate, molybdate and arsenate concentrations [6,8,31]. Thus, the inactivation of V-BrPO in phosphate buffer is likely due to a phosphate-induced displacement of BrPO-bound vanadate.

The standard conditions reported to fully deplete vanadium in vanadium bromoperoxidase are 0.1 M phos-

TABLE 1

Effect of phosphate on bromoperoxidase activity of V-BrPO in the absence of hydrogen peroxide

$[P_i]$ mM[a] pH 6.0	[V-BrPO] μM	Incubation time (h)	Specific[b,c] activity	Specific + V(V) addition
0	0	0	110 (6)	112 (4)
100	0.36	14	17 (1)	104 (3)
100	0.36	10	22 (0)	112 (6)
1	0.19	14	85 (0)	95 (1)
5	0.19	14	93 (1)	106 (1)
10	0.19	14	59 (1)	96 (0)
10	0.13	14	56 (1)	107 (4)
20	0.13	14	48 (0)	106 (3)
30	0.13	14	47 (0)	110 (0)
40	0.13	14	57 (0)	106 (4)
50	0.13	14	32 (1)	112 (4)
10	0.13	24	45 (1)	100 (2)
20	0.13	24	35 (0)	99 (2)
30	0.13	24	38 (1)	100 (2)
40	0.13	24	47 (1)	100 (1)
50	0.13	24	17 (1)	104 (2)
5	8.0	48	38 (6)	121 (2)
10	8.0	48	28 (3)	111 (1)
2 (pH 5)	8.0	48	51 (4)	116 (3)
5 (pH 5)	8.0	48	27 (1)	118 (8)
10 (pH 5)	8.0	48	19 (1)	116 (4)

[a] $[P_i]$ is the total phosphate concentration.
[b] The values in parentheses are the standard deviation.
[c] The specific activity was determined under the standard assay conditions (see MATERIALS AND METHODS).

phate-citrate buffer, pH 3.8, with 10 mM EDTA. In fact attempts to remove vanadium from V-BrPO in 0.1 M citrate buffer, pH 3.8, with 10 mM EDTA but without phosphate resulted in only a partial decrease of the specific activity. Atomic absorption measurements confirmed that vanadium was still bound to the enzyme (data not shown). Thus even at low pH and in the presence of a chelator, i.e., conditions which effectively remove many other metal ions from proteins, not all of the vanadium is removed. The reason that phosphate is required to fully remove vanadium(V) from V-BrPO is not understood, yet. Phosphate may act to coordinate vanadium(V) thereby liberating vanadate, or phosphate may be required to bind in place of vanadium(V). Either possibility is reasonable. Polyoxophosphate-vanadate species are well known in aqueous solution. Moreover, pyrophosphate has been shown to assist iron(III) removal from ferric-transferrin [17]. Pyrophosphate is thought to coordinate to the transferrin-bound ferric ion, thereby assisting its release from the protein. On the other hand, the size, bond lengths, geometry, pK_as, and charge of vanadate are nearly identical to phosphate. Consequently, vanadate is often used as a phosphate mimic, as in the inhibition of many phosphate metabolizing enzymes [3].

Phosphate is a commonly used buffer in biochemical investigations. In fact the isolation procedure for the bromoperoxidase from the marine red alga, *Corallina pilulifera*, employed 50 mM phosphate, pH 6.5 [36]. The specific activity of the purified enzyme was very low. The activity could be increased significantly by addition of vanadate [19]. Because iron was also associated with the enzyme and very little, if any, vanadium was detected in the initial studies, this enzyme was originally thought to be an iron enzyme, although, it is now considered to be a vanadium enzyme [19]. The reported isolation procedure of V-BrPO (*A. nodosum*) also uses phosphate as an elution buffer in the chromatographic purification steps [34]. Thus the less than unit stoichiometry of vanadium/subunit may arise from phosphate displacement of bound vanadium, during the purification procedure.

TABLE 2

Protection of phosphate inactivation of V-BrPO by hydrogen peroxide

$[H_2O_2]$ mM	$[P_i]$ mM pH 6.0	[V-BrPO] μM	Incubation time (h)	Specific activity	Specific activity + V(V) addition
0	100	0.36	10	22 (0)	112 (6)
4	100	0.36	10	127 (7)	119 (5)
10	100	0.36	10	114 (1)	111 (1)
10	100	0.36	14	102 (1)	125 (6)
40	100	0.36	14	93 (0)	105 (2)

See caption for Table 1 for further details.

Protection of phosphate inactivation of V-BrPO by hydrogen peroxide

The inactivation of V-BrPO by phosphate at pH 6 can be prevented by the addition of hydrogen peroxide during the incubation (see Table 2). Whereas incubation of V-BrPO in 0.1 M phosphate at pH 6 for 10 h produces a ca. 80% drop in the bromoperoxidase activity, no inactivation occurs upon incubation of the same mixture with 4–40 mM hydrogen peroxide (Table 2). Clearly hydrogen peroxide protects V-BrPO from inactivation. One can envision that protection occurs by binding of hydrogen peroxide to BrPO-bound vanadium(V), thereby preventing coordination by phosphate or EDTA.

The conditions of the standard bromoperoxidase assay for V-BrPO [34] are 0.1 M phosphate buffer, pH 6.5, 0.1 M bromide, 0.2 M sodium sulfate and 2 mM hydrogen peroxide. Inactivation of V-BrPO is not usually observed under these conditions for two reasons. First, the assays usually contain sufficient V-BrPO so that the MCD is consumed quite rapidly (i.e., less than 3 min), whereas the time scale for phosphate inactivation is much slower at pH 6.5. Second, hydrogen peroxide protects V-BrPO against phosphate inactivation. Nevertheless, caution must still be exercised that V-BrPO is not allowed to incubate in phosphate buffer for long periods of time before initiation of the enzyme-catalyzed reaction by addition of hydrogen peroxide.

Investigation of the effect of oxidants on the stability of V-BrPO

We have been interested in the effect of a variety of oxidants that are relevant to the haloperoxidase reaction

TABLE 3

Effect of oxidants of bromoperoxidase activity of V-BrPO

System	pH	Incubation time (h)	Relative specific activity[a]	Reference
V-BrPO + 2 mM H_2O_2 (in 0.1 M phosphate buffer)	6.0, 6.5	1–3	1	This work
V-BrPO + 2 mM H_2O_2 + 0.1 M Br^- (in 0.1 M phosphate buffer)	6.0, 6.5	1	1	This work, 12
V-BrPO + 1 mM H_2O_2 + 0.1–1 M Cl^- (0.1 M citrate buffer)	5.0	1	1	This work
V-BrPO + 1 mM HOCl[b]	water	2	1	This work
FeHeme-ClPO + 2 mM H_2O_2	6.0	1	0.94	12
FeHeme-ClPO + 2 mM H_2O_2 + 0.1 M Br^-	6.0	1	0.47	12
LactoPO + 2 mM H_2O_2	6.0	1	0.82	12
LactoPO + 2 mM H_2O_2 + 0.1 M Br^-	6.0	1	0	12

[a] Ratio of specific bromoperoxidase activity (standard conditions; see MATERIALS AND METHODS) of V-BrPO after incubation under the described conditions to the specific bromoperoxidase activity before incubation.

[b] HOCl was washed out of the reaction mixture by ultrafiltration (Amicon) with doubly distilled water. The protein concentration and enzyme activity were redetermined.

on the stability of V-BrPO. Incubation of V-BrPO with hydrogen peroxide alone does not cause any inactivation (see Table 3). Under turnover conditions in the absence of an organic halogen acceptor, V-BrPO is not inactivated by turnover of multiple aliquots of 2 mM hydrogen peroxide [12] (Table 3). These are conditions that produce oxidized bromide species and singlet oxygen. We have now shown that V-BrPO is not inactivated by hypochlorous acid (Table 3). Moreover, under turnover conditions with chloride, a reaction that produces oxidized chloride species and presumably singlet oxygen, V-BrPO does not lose any activity (Table 3). Chloramines, which are also strong oxidants, do not inactivate V-BrPO. In fact V-BrPO can catalyze the formation of chloramines [27], as shown in Fig. 1 for the formation of monochloroammonia species. This chloramine was identified by (1) its UV absorption maximum at 240 nm, in agreement with the literature values [14,37], and (2) by its ability to oxidize iodide, forming triiodide (λ_{max} 353 nm) [29], bromide, forming tribromide (λ_{max} 267 nm), and 5-thio-2-nitrobenzoic acid (λ_{max} 412 nm) [30] forming 5,5'-dithiobis-(2-nitrobenzoic acid). In addition, V-BrPO has been shown to use peracetic acid as an oxidant to catalyze the formation of stable monobromoamines from primary amines [26]. Although tertiary amines reportedly do not form N-bromo derivatives in aqueous solution [35],

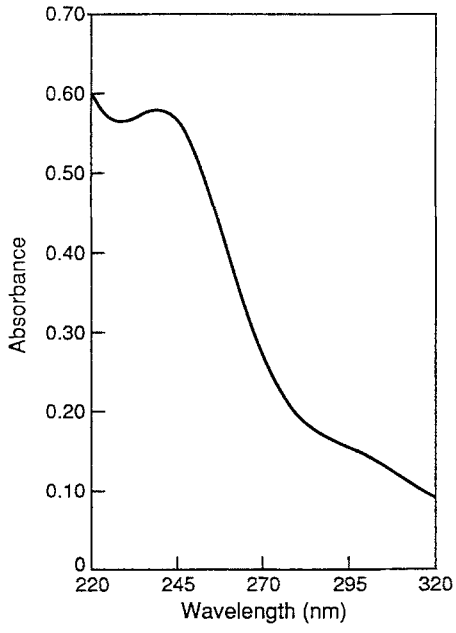


Fig. 1. UV spectrum of monochloroammonia formed enzymatically. 10 mM ammonia, 0.1 M Cl^-, 4 mM H_2O_2 and 0.5 μM V-BrPO. The reaction was carried out in 5 mM acetate, pH 5. The spectrum was run after 2.5 h and was referenced to equimolar ammonia in 5 mM acetate, pH 5, with 0.1 M Cl^-.

V-BrPO can catalyze the formation of transient monobromo derivatives of secondary (Mes (1-(*N*-morpholino)-ethane sulfonic acid)) and tertiary (Hepes and Mops (3-*N*-morpholino)-2-hydroxypropane sulfonic acid) amines [26].

In summary, V-BrPO is a promising enzyme for commercial applications, with substantial chemical and thermal stabilities. V-BrPO is not inactivated by an assortment of strong oxidants and it maintains its integrity in a variety of organic solvents. Phosphate is not a suitable buffer in which to store V-BrPO, because phosphate can displace the protein-bound vanadium(V), although, the activity of the phosphate-inactivated enzyme can be fully restored by subsequent addition of vanadium(V). Moreover hydrogen peroxide prevents V-BrPO inactivation by phosphate.

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