Volume 260, number 1, 109-112 FEB 08022 January 1990

A biomimetic model for vanadium-containing bromoperoxidase

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Received 1 November 1989; revised version received 22 November 1989

A biomimetic model of vanadium-containing bromoperoxidase (BPO) was proposed. The vanadium-containing BPO possesses vanadate ion (+5 oxidation state) as a prosteric group and catalyses the formation of a carbon-bromide bond in the presence of hydrogen peroxide, bromide ion and bromide acceptor. The reaction mechanism is as yet unknown. Several vanadyl (VO^{2+}, +4 oxidation state) complexes with oxalate, glutarate, succinate, malonate and acetate ligands showed similar bromination activity to that of BPO. Especially, the vanadyl-oxalato complex provided the highest BPO-like activity. Both vanadate ion and vanadyl complexes with nitrogen-containing ligands such as EDTA, glycine and serine showed essentially no BPO-like activity. Thus, the vanadyl-oxalato complex is proposed to be not only a possible chemical model of BPO, but a useful compound to elucidate the reaction mechanism of BPO.

Vanadium; Bromoperoxidase; Biomimetic model; Oxygen consumption; Electron spin resonance; Spin-trapping

1. INTRODUCTION

Bromoperoxidase (BPO) can use bromide ion in the presence of hydrogen peroxide (H_2O_2) and a bromide acceptor for the catalytic formation of carbon-bromide bonds. Recently, a novel BPO, that contains vanadium ion as a prosteric group, has been found in algae and lichen [1-7]. The BPO from *Ascophyllum nodosum* was the second vanadium-containing enzyme found in nature [1-3]. The first vanadium-containing enzyme was reported for nitrogen fixing nitrogenases from *Azotobacter vinelandiie* [8,9] and *A. chroococcum* [10,11].

Purified BPO from *A. nodosum* has the following characteristics [1-3,7]. (1) The enzyme loses its brominating activity upon dialysis against EDTA, and the activity is restored specifically by vanadate ion (+5 oxidation state). (2) The ratio of vanadium:protein is rather low, being 0.3-2.0 mol vanadium per mol enzyme. (3) In the resting enzyme vanadium ion is present in the vanadate state. But little is known about the enzyme in vivo. (4) ESR analysis of the enzyme reduced with sodium dithionite suggests that the ligand environment largely consists of oxygen donors [12]. The mechanism of bromination is as yet unknown.

During investigation of the chemistry and biochemistry of vanadium [13-15], we found that several simple vanadyl (VO^{2+}) (+4 oxidation state) complexes possess similar BPO activity to that of the enzyme. This paper reports chemical models of vanadium-containing BPO, and a possible mechanism of the BPO-like activity based on the results of studies by ESR (electron spin resonance) and with an oxygen electrode.

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2. MATERIALS AND METHODS

Monochlorodimedone (MCD, 2-chloro-5,5-dimethyl-1,3-cyclohexanedione) was obtained from Sigma Co. 5.5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec Co. Vanadyl sulfate ($VOSO_4 \cdot 6H_2O$) was standardized complexometrically with ethylenediamminetetraacetic acid (EDTA). All other reagents were of analytically pure grade. BPO activity was assayed by measuring bromination of MCD using $\epsilon = 20.2\ mM^{-1} \cdot cm^{-1}$ at 290 nm for MCD and $\epsilon = 0.2\ mM^{-1} \cdot cm^{-1}$ at 290 nm for monochloromonobromodimedone by a reported method [2,3]. The standard assay mixture was prepared in an optical cuvette under air and contained 0.1 M potassium phosphate (pH 6.0) containing 0.2 M sodium sulfate (Na_2SO_4), 50 mM potassium bromide (KBr), 50 μM MCD, 0.1 mM vanadium ion ($VOSO_4$ or $NaVO_3$) and various types of ligand, and 2 mM hydrogen peroxide (H_2O_2). Each compound was added in the following order: 0.1 M potassium phosphate buffer (pH 6.0), 50 μM MCD, 50 mM KBr, ligands and 0.1 mM vanadium compounds, and the reaction was initiated by adding of 2 mM H_2O_2. Oxygen concentration in solution was measured as reported previously [14]. ESR spectra were recorded at 22°C or 77K using a JEOL FE1XG X-band spectrometer operated at 100 kHz field modulation. The microwave frequency was monitored by a Takeda Riken frequency counter, TR5212. The magnetic field strength was calibrated by the hyperfine coupling constant of the Mn(II) ion doped in MgO powder (86.9 G). The *g*-values were estimated based on the *g*-values of the TCNQ-Li (tetracyanoquinodimethane-lithium) salt ($g = 2.00252$) as a standard.

3. RESULTS AND DISCUSSION

Fig.1 shows the BPO-like activities of various types of vanadyl complex. The vanadyl-aquo complex showed BPO activity, but vanadate ion, which is in the same

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00145793/90/$3.50

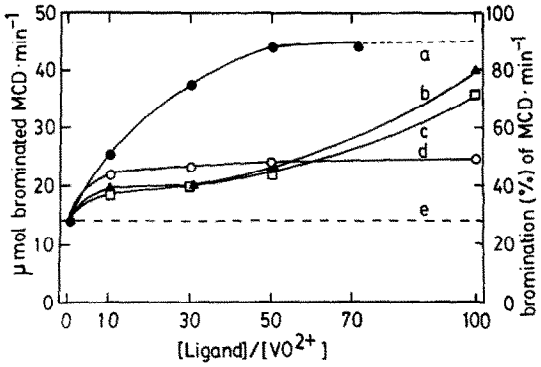


Fig.1. Bromoperoxidase-like activities of vanadyl complexes. The reaction mixture consisted of 50 mM KBr, 50 μM MCD, 0.1 mM $VOSO_4$, various concentrations of ligands and 2 mM H_2O_2 in 0.1 M potassium phosphate buffer, pH 6.0. The reactions were initiated by addition of H_2O_2. Results are for complexes with (a) oxalate, (b) glutarate, (c) succinate, (d) malonate and (e) aquo-vanadyl ion.

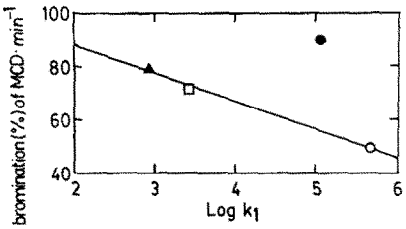


Fig.2. Correlation of bromoperoxidase-like activity and stability constant (log K_1) of vanadyl-oxygen containing ligand complexes. Results are for vanadyl-oxalato (●), -malonato (○), -succinato (□) and -glutarato (▲) complexes. Due to lack of stability constants for vanadyl-oxoligand complexes in the literature, we measured them as reported [12]. The log K_1 and log K_2 values for vanadyl-oxalato, -malonato, -succinato and -glutarato complexes were found to be 5.08 ± 0.14 and 2.56 ± 0.39, 5.67 ± 0.08 and 4.04 ± 0.43, 3.43 ± 0.08 and 3.11 ± 0.48, and 2.92 ± 0.15 and 2.34 ± 0.52, respectively, under the conditions of 25°C and μ = 0.1 (KNO_3). Thereby the proton dissociation constants, pK_1 and pK_2, of the oxoligand were determined as 1.86 ± 0.01 and 3.95 ± 0.01 for oxalic acid, 2.79 ± 0.01 and 5.36 ± 0.01 for malonic acid, 4.08 ± 0.03 and 5.37 ± 0.05 for succinic acid, and 4.23 ± 0.01 and 5.13 ± 0.02 for glutaric acid, under the same conditions.

oxidation state as that proposed for vanadium in the resting enzyme [2,3], had no activity. The vanadyl-oxalate complex in the presence of excess ligand (50–100-fold) caused almost complete bromination and had the highest activity (3.1) of all the complexes tested, when the bromination activity of vanadyl-aquo complex (16.1 $\mu M \cdot min^{-1}$) was 1, followed in order by the glutarato (2.6), succinato (2.3), malonate (2.0) and acetato (1.2) complexes. It is noteworthy that vanadyl complexes ligated with oxygen donors, such as the oxalato and glutarato, had the BPO activity, while complexes with nitrogen-containing ligands, such as EDTA, glycine and serine, showed essentially no BPO activity. The BPO activity provided by oxo-vanadyl complexes was found to be correlated inversely with the stability constants of the complexes, except for the vanadyl-oxalato complex (fig.2). These results suggest that the lack of BPO activity of the complexes could be due to tight binding of vanadyl ion with ligand. Indeed, a high stability constant (like K_1 = 18.77) of vanadyl-EDTA complex was reported in the literature [17]. The reason why the parameter for the vanadyl-oxalato complex falls far from the straight-line figured by other vanadyl complexes, is not clear at present, and requires further investigation.

The ESR spectrum of the vanadyl-oxalate complex in fig.3A and B clearly shows a typical vanadyl form and the vanadium ion is in the +4 oxidation state. The feature of the ESR spectrum (g_o = 1.970, $g_\parallel$ = 1.942 and $g_\perp$ = 1.984; A_o = 104 G, $A_\parallel$ = 194 G and $A_\perp$ = 59 G) agree with a $VO(O_4)$ coordination mode [15] and are similar to those of the BPOs from *A. nodosum* and *L. saccharina* g_o = 1.968, $g_\parallel$ = 1.948 and $g_\perp$ = 1.978; A_o = 95 G, $A_\parallel$ = 176 G and $A_\perp$ = 55 G for *A. nodosum*) [3].

For mechanistic investigation of the BPO-like activity of the vanadyl species, a spin-trapping method was applied using DMPO (5.5-dimethyl-1-pyrroline-*N*-oxide). On addition of DMPO to a solution containing the same concentrations of vanadyl-aquo complex, KBr, MCD and H_2O_2 as in the standard assay mixture at pH 6.0, formation of a DMPO-OH radical adduct was clearly observed (hyperfine coupling constants a^N = 14.9 G and a^H = 14.9 G), consisting of 4 signals with intensities of 1:2:2:1 (fig.3C). These values are essentially identical to those found for DMPO-OH radical adduct, prepared from ultrasound aqueous

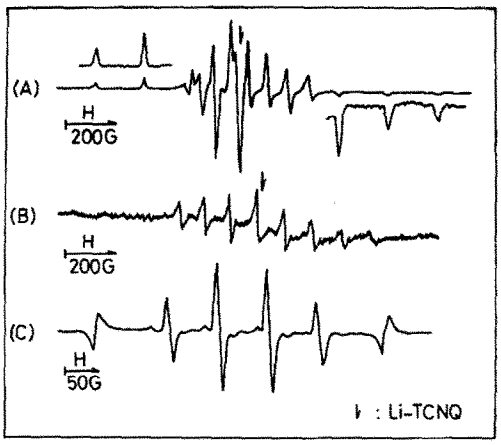
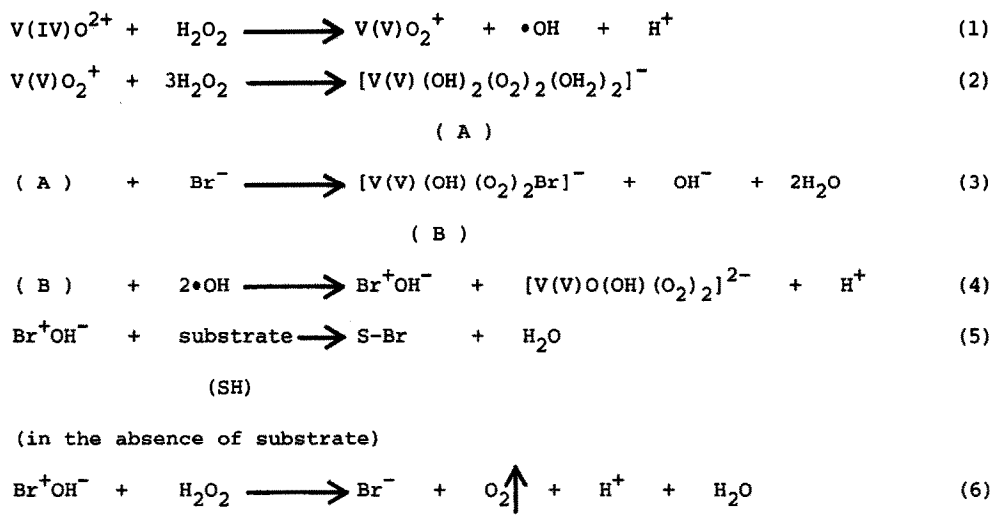


Fig.3. ESR spectra of vanadyl-oxalato complex (A and B) and of the spin-trapping solution (C). The vanadyl-oxalato complex was prepared at pH 6.0 with vanadyl ion:oxalato = 1:100 (vanadyl ion = 0.1 mM). The ESR spectra were measured at 77K (A) and 21°C (B). The solution for spin-trapping contained 50 mM KBr, 50 μM MCD, 0.1 mM $VOSO_4$, 2 mM H_2O_2 and DMPO 5 μl, and the ESR spectrum (C) was measured within 30 s after addition of DMPO at 21°C, under the following conditions: power 8 mW, field 3370 ± 50 Gauss, modulation 100 kHz, 2.0 Gauss, sweep time 30 s and amplitude 1000. Small signals of both sides in (C) are the 3rd and 4th signals (ΔH_{3-4} = 86.9 Gauss) due to standard manganese (II) ion.



Scheme 1. Proposed mechanism of bromoperoxidase reaction of vanadyl complexes.

solution [18]. Our result indicates the generation of a hydroxyl radical ($OH^{\cdot}$) in the solution, similar to that in the Fenton reaction [19]. Formation of molecular oxygen (O_2) was monitored with an oxygen electrode. A solution containing the 0.1 mM vanadyl-aquo complexes, 50 mM KBr and 2 mM H_2O_2 in phosphate buffer (pH 6.0) evolved O_2 at a rate of 50.7 $\mu M \cdot min^{-1}$. On lacking of KBr in the solution, the formation of O_2 was 27.9 $\mu M \cdot min^{-1}$. However, in a similar solution containing vanadate in place of the vanadyl species no O_2 was formed. When MCD was added to the solution containing the vanadyl species, O_2 evolution decreased with an increase in the amount of MCD.

On the basis of these results, we propose that the mechanism for the BPO-like activities of vanadyl complexes is as shown in Scheme 1. Reaction (1) is supported by the detection of $OH^{\cdot}$ by the spin-trapping method as well as by the proposed scheme of Brooks and Sicilo [20]. An intermediate (A) formed at pH 6 in reaction (2) was determined by Harrison and Howarth [21]. On lacking of Br^- in the solution, the intermediate (A) will probably decompose to evolve O_2 [(A)$\rightarrow VO_2^+ + 2O_2 + 2H_2O + 2H^+$]. In the presence of Br^-, the formation of an intermediate (B) in the reaction (3) is proposed on the basis of results on the reaction of vanadate-H_2O_2 system by iodide [22]. When the intermediate (B) reacts with $OH^{\cdot}$ produced by reaction (1), hypobromous acid (Br^+OH^-), which may be considered as an active intermediate for bromination [7,23], will be generated by reaction (4), with subsequent bromination of the substrate, MCD (reaction (5)). The bromination, thus, proceeds by reaction (1–5). When no substrate is present in the system, the Br^+OH^- will react with H_2O_2 to form O_2 according to reaction (6), as demonstrated with the oxygen electrode.

In the present study, we found that vanadyl complexes containing oxygen ligands showed similar activities to vanadium-containing BPO, but that vanadate ion and its complexes showed no BPO activity. The vanadyl-oxalato complex with a $VO(O_4)$ coordination mode is a possible chemical model of vanadium-containing BPO. The present results will provide useful information to elucidate the reaction mechanism of vanadium-containing BPO.

REFERENCES

[1] Vilter, H. (1984) Phytochemistry 23, 1387–1390.
[2] Wever, R., Plat, H. and De Boer, E. (1985) Biochim. Biophys. Acta 830, 181–186.
[3] De Boer, E., Van Kooyk, Y., Tromp, M.G.M., Plat, H. and Wever, R. (1986) Biochim. Biophys. Acta 869, 48–53.
[4] De Boer, E., Tromp, M.G.M., Plat, H., Krenn, G.E., Wever, R. (1986) Biochim. Biophys. Acta 872, 104–115.
[5] Krenn, B.E., Plat, H. and Wever, R. (1987) Biochim. Biophys. Acta 912, 287–291.
[6] Wever, R., De Boer, E., Plat, H. and Krenn, B.E. (1987) FEBS Lett. 216, 1–3.
[7] Platt, H., Krenn, B.E. and Wever, R. (1987) Biochem. J. 248, 277–279.
[8] McKenna, C.E., Benemann, J.R. and Traylor, T.G. (1970) Biochem. Biophys. Res. Commun. 41, 1501–1508.
[9] Robson, R.L., Eady, R.R., Richardson, T.H., Miller, R.W., Hawkins, M. and Postgate, J.R. (1986) Nature 322, 388–390.
[10] Burns, R.C., Fucksman, W.H. and Hardy, R.W.F. (1971) Biochem. Biophys. Res. Commun. 42, 353–358.
[11] Hales, B.J., Case, E.E., Moringstar, J.E., Dzeda, M.F. and Mautner, A. (1986) Biochemistry 25, 7251–7255.
[12] De Boer, E., Keijzers, C.D., Klaassen, A.A.K., Reijerse, E.J., Collison, D., Garner, C.D. and Wever, R. (1988) FEBS Lett. 93–97.
[13] Sakurai, H.., Shimomura, S., Fukuzawa, K. and Ishizu, K. (1980) Biochem. Biophys. Res. Commun. 96, 293–298.
[14] Sakurai, H., Goda, T. and Shimomura, S. (1982) Biochem. Biophys. Res. Commun. 107, 1349–1354.
[15] Sakurai, H., Hirata, J. and Michibata, H. (1988) Inorg. Chim. Acta 152, 177–180.
[16] Takeshima, S. and Sakurai, H. (1982) Inorg. Chim. Acta 66, 119–124.

[17] Schwarzenbach, G. and Sandera, J. (1953) Helv. Chim. Acta 36, 1089–1101.
[18] Makino, K., Magdi, M.M. and Riesz, P. (1982) J. Am. Chem. Soc. 104, 3537–3539.
[19] Janzen, E.G., Nutter, jr D.E. and Davis, E.R. (1978) Can. J. Chem. 56, 2237–2242.
[20] Brooks, H.B. and Sicilo, F. (1971) Inorg. Chem. 10, 2530–2534.
[21] Harrison, A.T. and Howarth, O.W. (1985) J. Chem. Soc. Dalton Trans. 1173–1177.
[22] Secco, F. (1980) Inorg. Chem. 19, 2722–2725.
[23] Itoh, N., Izumi, Y. and Yamada, H. (1987) J. Biol. Chem. 262, 11982–11987.