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Effects of Oxodiperoxovanadate (V) Complexes on the Activity of Green Crab (*Scylla serrata*) Alkaline Phosphatase

X.-W. Zhou[1,2], Q.-X. Chen[1]*, Z. Chen[2], Z.-Q. He[1], and H.-M. Zhou[1,3]*

[1]Department of Biology, Xiamen University, Xiamen 361005, People's Republic of China
[2]Department of Chemistry, Xiamen University, Xiamen 361005, People's Republic of China
[3]Department of Biological Science and Biotechnology, Tsinghua University, Beijing 100084, People's Republic of China; fax: +8610 62785505; E-mail: zhm-dbs@mail.tsinghua.edu.cn

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Abstract—Green crab (*Scylla serrata*) alkaline phosphatase (EC 3.1.3.1) is a metalloenzyme that catalyzes the nonspecific hydrolysis of phosphate monoesters. The effects of some pollutants in seawater on the activity of the enzyme will result in the loss of the biological function of the enzyme, which will affect the exuviating crab shell and threaten the survival of the animal. In the present paper, the effects of four oxodiperoxovanadate (V) complexes on the activity of green crab alkaline phosphatase have been studied. The results show that these vanadate derivatives can lead to reversible inactivation. The equilibrium constants for binding of inhibitors with the enzyme and/or the enzyme–substrate complexes have been determined. The results show that sodium (2,2′-bipyridine)oxodiperoxovanadate, pV(bipy), and potassium oxodiperoxo-(1,10-phenanthroline)vanadate, pV(phen), are competitive inhibitors, while potassium picolinato-oxodiperoxo-vanadate, pV(pic), and oxalato-oxodiperoxovanadate, pV(ox), are mixed-type inhibitors. These results suggest that pV(bipy) is a considerably more potent competitive inhibitor than pV(phen) and that the competitive inhibition effect of pV(pic) is stronger than that of pV(ox), but the non-competitive inhibition effect of pV(ox) is stronger than that of pV(pic).

Key words: alkaline phosphatase, oxodiperoxovanadate, inhibition, inactivation

Alkaline phosphatases (EC 3.1.3.1), which exist widely in the animal and microorganism kingdoms, catalyze the transfer of phosphate groups to water (hydrolysis) or alcohol (transphosphorylation) using a wide variety of phosphomonoesters and are characterized by a high pH optima and a broad substrate specificity [1, 2]. Alkaline phosphatase from green crab is an important enzyme participating in cell phosphate metabolism, which is concerned with the absorption of phosphate and calcium from seawater and the change of the shell of the animal. Because of pollution of the breeding aquatic environment, such as a shift of acidity and alkalinity and heavy metal ions or organic solvents, the enzyme activity and its conformation can be affected, and the growth and survival of the animal is threatened.

Abbreviations: *p*NPP) *p*-nitrophenyl phosphate; pV(bipy)) sodium (2,2′-bipyridine)oxodiperoxovanadate, $Na[VO(O_2)_2(bipy)]\cdot 5H_2O$; pV(ox)) potassium oxalato-oxodiperoxovanadate, $K_3[VO(O_2)_2(ox)]\cdot 2H_2O$; pV(phen)) potassium oxodiperoxo-(1,10-phenanthroline)vanadate, $K[VO(O_2)_2(phen)]\cdot 3H_2O$; pV(pic)) potassium picolinato-oxodiperoxovanadate, $K_2[VO(O_2)_2(pic)]\cdot 2H_2O$.

* To whom correspondence should be addressed.

The enzymes from mammals and *E. coli* have been extensively studied [3-6]. Recently, the X-ray crystal structure of bacterial alkaline phosphatase was reported to 2.0 Å resolution in the presence of inorganic phosphate [7]. The active site is a tight cluster of two zinc ions (3.9 Å separation) and one magnesium ion (5 and 7 Å from the two zinc ions). The magnesium ion is octahedrally coordinated by three water molecules, the carboxylate oxygen of Asp51 and Glu322, and the hydroxyl group of Thr155. Asp153 interacts indirectly with the magnesium ion through two of the three water molecules and has been shown to be important for the binding of the magnesium ion [8-10]. Murphy et al. [11] reported mutations at positions 153 and 328 in *E. coli* alkaline phosphatase. They suggested that the X-ray structure of *E. coli* enzyme, particularly in the active site region, is similar to that of the mammalian and yeast enzymes. Alkaline phosphatase from green crab (*Scylla serrata*) is also a dimeric metalloenzyme containing zinc and magnesium ions, and the structure of its active site is probably similar to that of bacterial alkaline phosphatase. It is well known that green crab alkaline phosphatase can be inactivated by EDTA, and the complete kinetic course of EDTA inactivation has been reported

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by monitoring the hydrolysis of *p*-nitrophenyl phosphate [12]. It has been reported that lysine [13], tryptophan [14, 15], and arginine residues [16] are essential for the enzyme activity and are situated at the active site of the enzyme. It has also been found that some inorganic salts, such as Na_2HPO_4, Na_2HAsO_4, and Na_3WO_4 [17], as well as some amino acids and N-thiophosphoryl amino acids, are inhibitors of green crab alkaline phosphatase [18]. The vanadium content of seawater amounts to 20-35 nM. Vanadium is thus the most abundant transition metal in the aquasphere [19]. Vanadate in the presence of hydrogen peroxide forms several peroxovanadate derivatives that display strong biological activity to phosphatase [20]. Therefore, oxodiperoxovanadate (V) complexes have increasingly attracted interest recently. However, the biological effects of peroxovanadate derivatives on alkaline phosphatase have not yet been fully investigated.

The present paper reports the effects of oxodiperoxovanadate (V) complexes on the activity of green crab alkaline phosphatase. The results show that pV(bipy) and pV(phen) are competitive inhibitors, while pV(pic) and pV(ox) are mixed-type inhibitors.

MATERIALS AND METHODS

The alkaline phosphatase was prepared from green crab (*Scylla serrata*) viscera as described by Chen et al. [15]. The crude preparation was further chromatographed by ion-exchange chromatography on DEAE-cellulose, then by gel filtration through Sephadex G-150 followed by DEAE-Sephadex A-50. The final preparation was homogeneous according to isoelectric focusing and HPLC. The specific activity of the purified enzyme was 3320 U per mg of enzyme protein. *p*-Nitrophenyl phosphate (*p*NPP) was from E. Merck (USA). Four oxodiperoxovanadate complexes—pV(ox), pV(bipy), pV(phen), and pV(pic)—were synthesized and described previously [21-23]. Their purity was confirmed by ^{51}V-NMR (D_2O). The ^{51}V-NMR chemical shifts of the four oxodiperoxovanadate complexes were at −732, −746, −742, and −743 ppm, respectively. All other reagents were local products of analytical grade.

The protein concentration was determined as described by Lowry [24], and the assay of green crab alkaline phosphatase activity was as described by Chen et al. [25, 26] with the use of 5 mM *p*-nitrophenyl phosphate as substrate in the standard assay. Inhibition studies were performed by dissolving the inhibitor in an assay system containing 2 mM *p*-nitrophenyl phosphate, 2 mM $MgCl_2$, and 50 mM $Na_2CO_3/NaHCO_3$ buffer (pH 10.0). The enzyme (in 5 μl) was added to 1 ml of the assay system at 37°C, and the rate of substrate hydrolysis was monitored for 10 min. The activity was calculated by the increased absorption at 405 nm using molar absorption coefficient $1.73\cdot10^4$ $M^{-1}\cdot cm^{-1}$ [15]. Absorption measurements were recorded using a Beckman DU-8B spectrophotometer.

RESULTS

Effects of four oxodiperoxovanadate complexes on the activity of green crab alkaline phosphatase. The effects of four oxodiperoxovanadate complexes on *p*NPP hydrolysis by the enzyme were studied. The inhibitions of green crab alkaline phosphatase by the oxodiperoxovanadate complexes were concentration dependent (Fig. 1). The presence of the inhibitors results in inactivation of the enzyme. With increasing concentrations of the inhibitors, the activity of green crab phosphatase marked decreased. When the concentration of inhibitor was 6 mM, pV(bipy), pV(phen), pV(pic), and pV(ox), caused the loss of the enzyme activity by 90, 75, 70, and 65%, respectively.

Inhibition of pV(bipy) and pV(phen) following the competitive mechanism. In the present study, the concentration of pV(bipy) was held at different constant values, while the substrate concentrations were varied, permitting measurement of the effect of increasing substrate concentration [S] on the initial reaction rate (v). Plots of $1/v$ versus $1/[S]$ for different concentrations of inhibitor (Fig. 2) shows that pV(bipy) was a competitive

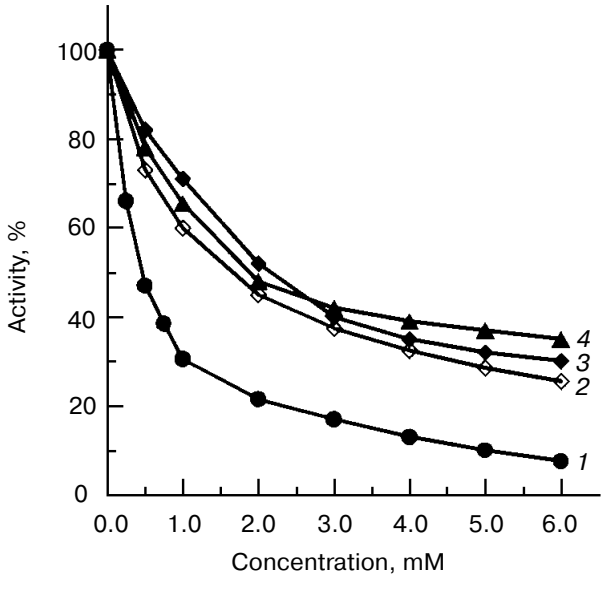


Fig. 1. Inactivation of green crab alkaline phosphatase by oxodiperoxovanadate complexes: *1*) pV(bipy); *2*) pV(phen); *3*) pV(ox); *4*) pV(pic). The enzyme was incubated for 10 min in 50 mM Na_2CO_3-$NaHCO_3$ buffer, pH 10.0, containing effectors at the different concentrations before determination of the remaining activity. The final concentration of the enzyme was 0.075 μM.

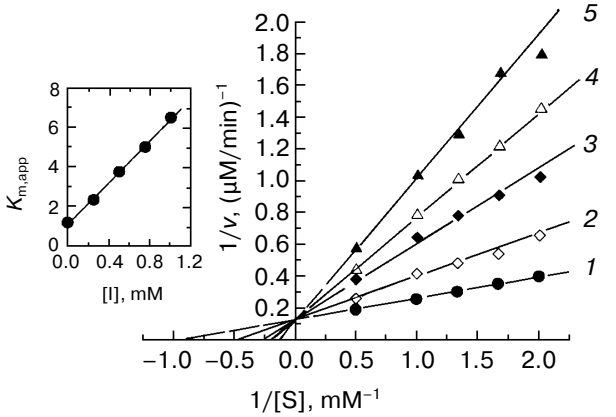


Fig. 2. Lineweaver–Burk plots for competitive inhibition of pV(bipy) on the green crab alkaline phosphatase for the hydrolysis of *p*-nitrophenyl phosphate. The experimental conditions were as for Fig. 1. pV(bipy) concentration (mM): *1*) 0; *2*) 0.25; *3*) 0.50; *4*) 0.75; *5*) 1.0. The inset represents the plot of apparent Michaelis constant ($K_{m,app}$) versus the concentration of pV(bipy) to determine the inhibition constant. The line is drawn using a linear least squares fit.

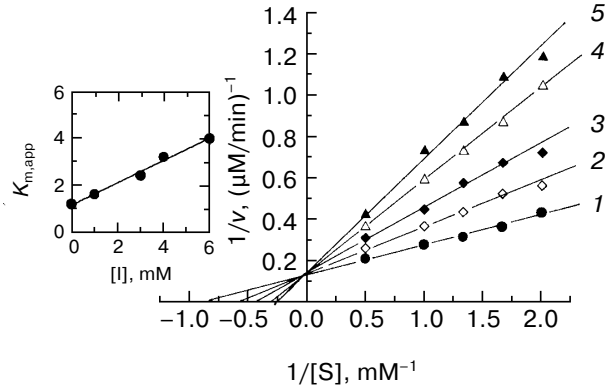


Fig. 3. Lineweaver–Burk plots for competitive inhibition of pV(phen) on the green crab alkaline phosphatase for the hydrolysis of *p*-nitrophenyl phosphate. The experimental conditions were as for Fig. 1. pV(phen) concentration (mM): *1*) 0; *2*) 1.0; *3*) 3.0; *4*) 4.0; *5*) 6.0. The inset represents the plot of apparent Michaelis constant ($K_{m,app}$) versus the concentration of pV(phen) to determine the inhibition constant. The line is drawn using a linear least squares fit.

inhibitor. Increasing pV(bipy) concentration results in the production of a family of lines with a common intercept on the $1/v$ axis but with different slopes, indicating that V_{max} is unchanged by the presence of different concentrations of the inhibitor. The equilibrium constant for inhibitor binding, K_I, can be obtained from the plot of the apparent Michaelis–Menten constant ($K_{m,app}$) versus the concentration of pV(bipy), as shown in the inset. The inhibition constant (K_I) of pV(bipy) obtained from the experimental data is recorded in the table.

Inhibition types and inhibition constants of the activity of green crab alkaline phosphatase by oxodiperoxovanadate complexes

Inhibitor	Inhibition type	Inhibition constant	
		K_I, mM	K_{IS}, mM
pV(bipy)	competitive	0.20	
pV(phen)	competitive	1.95	
pV(pic)	mixed*	1.84	19.6
pV(ox)	mixed*	2.28	8.42

* Mixed type includes competitive and non-competitive inhibition.

The inhibition by pV(phen) also was competitive (Fig. 3). By the same methods, we could determine its inhibition constant. The K_I values obtained are also summarized in the table. It can be seen that pV(bipy) is a considerably more potent inhibitor than pV(phen).

Inhibition of pV(ox) and pV(pic) following the mixtyped mechanism. Inhibition by pV(ox) of green crab alkaline phosphatase was also studied. The results illustrated in Fig. 4 showed that the double-reciprocal plots yielded a family of straight lines with a point of intersection in the second quadrant. The inhibition of the enzyme by pV(ox) was of the mixed competitive and non-competitive type. The equilibrium constant for inhibitor binding with free enzyme (E), K_I, can be obtained from the plot of the slopes of the straight lines (with coordinates $\{1/v; 1/[S]\}$) versus the concentrations of pV(ox). The equilibrium constant for inhibitor binding with enzyme–substrate complex (ES), K_{IS}, can be obtained from the plot of the intercepts versus the pV(ox) concentrations. The inhibition constants (K_I) of pV(ox) obtained from the experimental data is recorded in the table. The inhibition of pV(pic) also showed mixed competitive and non-competitive type (Fig. 5). By the same methods, we determined its inhibition constants. The K_I and K_{IS} values so obtained are also summarized in the table.

DISCUSSION

It is well known that alkaline phosphatase can catalyze the hydrolysis of a wide variety of phosphomo-

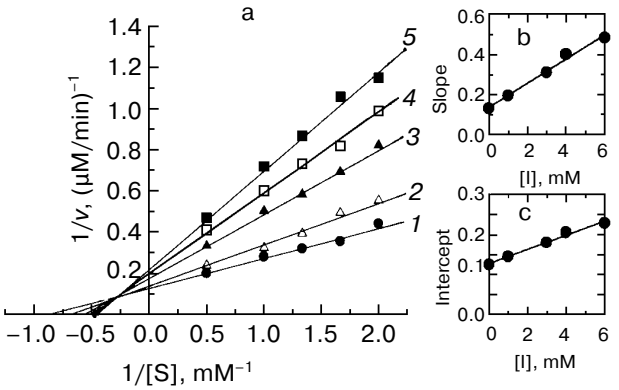


Fig. 4. Mixed-typed inhibition by pV(ox) of the green crab alkaline phosphatase for the hydrolysis of *p*-nitrophenyl phosphate. The experimental conditions were as for Fig. 1 except that the inhibitor was pV(ox). a) Lineweaver–Burk plots. pV(ox) concentration (mM): *1*) 0; *2*) 1.0; *3*) 3.0; *4*) 4.0; *5*) 6.0. b) Secondary plot of the slopes of the straight lines versus pV(ox) concentration to determine the free enzyme inhibition constant (K_I). c) Secondary plot of the intercept of the straight lines versus the concentration of pV(ox) to determine the ES complex inhibition constant (K_{IS}). The line is drawn using a linear least squares fit.

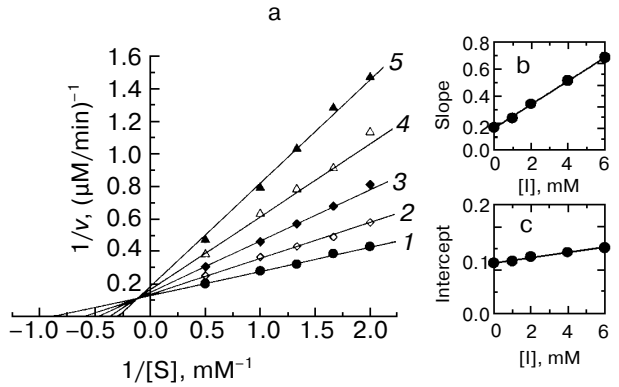
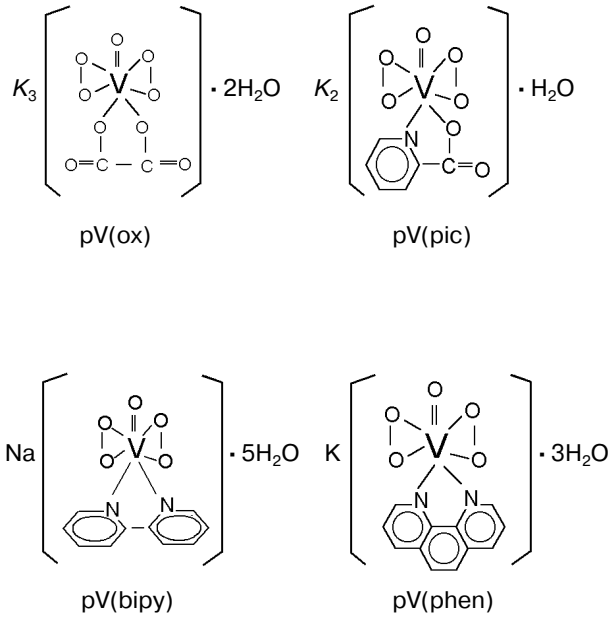


Fig. 5. Mixed-typed inhibition by pV(pic) of the green crab alkaline phosphatase for the hydrolysis of *p*-nitrophenyl phosphate. The experimental conditions were as for Fig. 1 except that inhibitor was pV(pic). a) Lineweaver–Burk plots. pV(pic) concentration (mM): *1*) 0; *2*) 1.0; *3*) 2.0; *4*) 4.0; *5*) 6.0. b) Secondary plot of the slopes of the straight lines versus pV(pic) concentration to determine the free enzyme inhibition constant (K_I). c) Secondary plot of the intercept of the straight lines versus the concentration of pV(pic) to determine the ES complex inhibition constant (K_{IS}). The line is drawn using a linear least squares fit.

noesters and produce inorganic phosphate salt and corresponding alcohol, phenol, or sugar. The enzyme is important in cell phosphorous metabolism, and the product HPO_4^{2-} is a competitive inhibitor. It has been reported that some amino acids and substrate analogs inhibit the activity of alkaline phosphatase [18, 27, 28]. However, the effects of vanadate (Na_3VO_4), which is a structural analog of the inorganic phosphate product, on alkaline phosphatase activity have been little explored. We investigated earlier the inhibition of green crab alkaline phosphatase activity by Na_3VO_4. The results showed that Na_3VO_4 is a strong competitive inhibitor (K_I = 0.135 mM) [29]. In this paper, inhibitions of the enzyme activity by four oxodiperoxovanadate derivatives were investigated. The results show that the four complexes also obviously inhibit the green crab alkaline phosphatase activity. Among the four oxodiperoxovanadates, both pV(bipy) and pV(phen) are competitive inhibitors, the other two pV(ox) and pV(pic) were shown to be mix-typed inhibitors.

The structures of the four oxodiperoxovanadate derivatives are well known [20]. They are shown below.



It can be seen from the table that of the two competitive inhibitors, pV(bipy), whose inhibition constant (K_I) is 0.20 mM, is considerable more potent than pV(phen) (K_I = 1.95 mM). The ligand of pV(phen), 1,10-phenanthroline, is larger than that of pV(bipy), 2,2′-bipyridine; this may explain the difference in inhibition as being due to spatial hindrance. However, the other two inhibitors showed a mixed competitive and non-competitive type. The results given in the table suggest that the competitive inhibition effect of pV(pic) is stronger than that of pV(ox), but the non-competitive inhibition effect of pV(ox) is stronger than that of pV(pic).

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