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Original Article

Catalytic cofactors (Mg^{2+} and Zn^{2+} ions) influence the pattern of vanadate Inhibition of the monoesterase activity of calf intestinal alkaline phosphatase

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ABSTRACT: The mechanism of modulation of vanadate inhibition of alkaline phosphatase activity by catalytic cofactors has not been fully characterized. We investigated the effect of the interaction of catalytic cofactors (Mg^{2+} and Zn^{2+}) and vanadate (an active site inhibitor) on the rate of hydrolysis of para-nitrophenyl phosphate (pNPP) (monoesterase reaction) by calf intestinal alkaline phosphatase (CIAP). The results showed that vanadate significantly inhibited 'cofactor-free' CIAP, and the inhibition was relieved by the presence of the catalytic cofactors in the reaction. Our results show that the absence of the cofactors did not significantly alter the K_m of the reaction, but caused a decrease in the V_{max}. Kinetic analyses showed that vanadate inhibited CIAP-catalyzed hydrolysis of pNPP by decreasing the V_{max} and increasing the K_m of the reaction. The presence of cofactors in the reaction alleviated the effect of vanadate by increasing the V_{max} and decreasing the K_m. The activity of the dialyzed CIAP was increased by the addition of catalytic cofactors to vanadate-inhibited enzyme. This study provides preliminary data that reversible inhibition of CIAP is subject to the influence of catalytic cofactors. Further studies will reveal detailed mechanistic aspects of this observation and its significance in the biological system.

KEYWORDS: alkaline phosphatase, monoesterase reaction, vanadate inhibition, catalytic cofactors.

INTRODUCTION

Phosphoryl transfer reactions are a common theme in biological processes. The central roles of nucleic acids, high-energy nucleotides, and other phosphate containing molecules in cellular systems necessitate well-regulated mechanisms for controlling the transfer of phosphate groups from donor molecules to acceptor molecules. Rate enhancement and specificity are key issues in phosphoryl transfer reactions that require tight regulations. Frequently, metal ion cofactors and inhibitors are participants in regulating these key aspects of catalysis. Alkaline phosphatase (AP) is a metalloenzyme that catalyses phosphoryl transfer reaction and thus a good model for studying cofactor-inhibitor interactions in enzyme catalysis. The enzyme is comprised of two identical subunits, each of which contains two zinc and one magnesium ions (Kim and Wyckoff, 1991).

Alkaline phosphatase exists in organisms of all kinds and plays a vital role in survival under phosphate starvation (Prada *et al*., 1996; Zappa *et al*., 2001; Ayantika and Parames, 2007). There is only one *Escherichia coli* alkaline phosphatase (ECAP), but four alkaline phosphatase isoenzymes, that have been isolated in humans. In humans, three out of four AP isozymes are tissue-specific: one is placental (PLAP), the second appears in germ cells (GCAP), and the third in the intestine (IAP). They are 90–98%

homologous, and their genes are clustered on chromosome 2q37.1. The fourth, tissue-nonspecific (TNAP), 50% identical to the other three, is nonspecific and can be found in bone, liver, and kidney (Weiss *et al.,* 1986; Millan and Manes, 1988; Millan, 2006). Its gene is located on chromosome 1p34–36 (Greenberg *et al*., 1990), and mutations in the TNAP gene have been associated with hypophosphatasia, a rare inherited disorder, characterized by defective bone mineralization (Whyte, 1994). Moreover, at least seven isoenzymes of intestinal alkaline phosphatase have been revealed in bovine intestinal alkaline phosphatase (Manes *et al*., 1998). Alkaline phosphatase is present in both soluble and membrane forms, but more than 95% of the intestinal isoenzyme is normally associated with the brush border membrane (Seetharam *et al*., 1977; Steven *et al*., 1981).

The skeletal isoenzyme of AP has been implicated in matrix vesicle-mediated calcification during bone formation and phosphate transport (Cyboron *et al*., 1982). Inorganic pyrophosphate (PPi) is a potent inhibitor of calcification and AP has been identified as one of the central regulators of mineralization via its ability to control the pool of extracellular PPi, (i.e., PPi that is generated, or transported to the outside of the cells). Previous studies have reported that the major role for TNAP in bone tissue is hydrolysis of PPi to maintain proper levels of this inhibitor of mineralization, thus ensuring normal bone mineralization (Hessle *et al*., 2002; Johnson *et al*., 2003). Another recognized function of AP is its role as a lipopolysaccharide (LPS)-detoxifying enzyme. This function of AP was first established by Poelstra *et al.* (1997). LPS plays a major role in the development and/or aggravation of several multifactoral diseases, including sepsis, atherosclerosis, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and multiple sclerosis (MS) (Vincent *et al*., 1992; Patel *et al*., 1995). The toxic moiety of lipopolysaccharide is the well-preserved Lipid A part. Lipid A contains two phosphate groups attached to two glucosamine which determine the toxicity level and are crucial for the biological activities of LPS. AP catalyses the enzymatic removal of phosphate groups from LPS, thereby detoxifying it (Poelstra *et al*., 1997; Bentala *et al*., 2002).

Quite a number of substrate and transition state analogue inhibitors of AP have been studied. AP is inhibited by several compounds on the basis of different mechanisms. L-phenylalanine, imidazole, histamine and theophylline are known to inhibit alkaline phosphatase uncompetitively while phosphate, phosphoethanolamine and phenylphosphonate are competitive inhibitors (Ghosh and Fishman, 1966; Farley *et al*., 1987; Hoylaerts *et al.,* 1992). Vanadate is known to markedly inhibit intestinal, placental, and renal isoenzyme (Chen and Zhou, 1999; Cuncic *et al*., 1999) by competitive inhibition mechanism (Tromp *et al*., 1991) or mixed inhibition mechanism (Shirazi *et al*., 1981; Malomo *et al*., 2003). Vanadate and L-phenylalanine have been shown to display positive synergistic interactions in their inhibition of rat liver alkaline phosphatase (Malomo *et al*., 2003).

In earlier reports, we showed that optimal concentrations of Mg^{2+} and Zn^{2+} activated CIAP catalysed hydrolysis of para-nitrophenyl phosphate (pNPP) (monoesterase reaction) (Olorunniji *et al*., 2007; Igunnu *et al.,* 2011). In view of the biological functions of alkaline phosphatase, the mechanistic details of the interaction between the metal cofactors and inhibitors are important to our understanding of how AP inhibition is modulated in the body. Thus, the effects of Mg^{2+} and Zn^{2+} on vanadate-inhibited monoesterase activity of calf intestinal AP (CIAP) were investigated in this study with a view to gaining understanding of the interaction between the metal ions and vanadate in AP catalysis. The mechanistic details and the implication of how the metal cofactors modulated the extent of inhibition of CIAP by vanadate are herein discussed.

MATERIALS AND METHODS

Materials

Alkaline phosphatase substrate, the sodium salt of para-nitrophenyl phosphate (pNPP), was obtained from Sigma-Aldrich, UK. Magnesium chloride ($MgCl_2$), zinc chloride ($ZnCl_2$) and sodium orthovanadate were obtained from Fisher Scientific, UK. All other chemicals used in this study were of analytical grade. Purified homogenous CIAP was obtained from New England Biolabs, UK.

Preparation of Dialyzed CIAP

Loosely bound metal ions were removed from CIAP according to the method of Koutsoulis *et al.* (2010). A 500 μl sample of 28 μM CIAP was dialysed in 20 mM Tris-HCl (pH 7.6) at 4 ^{o}C for 24 hours.

CIAP-Catalyzed Hydrolysis of pNPP

Monoesterase activity of CIAP was measured by the rate of hydrolysis of pNPP in the presence of catalytic cofactors (Zn^{2+} and Mg^{2+}) and vanadate at 37 ºC in 50 mM Tris-HCl (pH 8.5). Details of each experiment are described in the Figure Legends. Reaction mixtures containing 10 μM CIAP and 50 mM Tris-HCl (pH 8.5) was pre-incubated at 37 ^{o}C with vanadate for 10 minutes and reactions were initiated by the addition of appropriate concentration of pNPP in the presence of catalytic cofactors. Reactions were carried out for 10 minutes at 37 ^{o}C, and stopped by the addition of 500 mM NaOH and 10 mM EDTA. The absorbance was read at 405 nm against a blank of the buffered substrate. Spectrophotometric readings were taking in a Perkin Elmer Lambda 45 UV/VIS instrument. Activities are expressed as change in A_{405} per minute reflecting the release of p-nitrophenol from the substrate p-nitrophenylphosphate (pNPP). Curve fitting was carried out using GraphPad Prism 4.0 (2005).

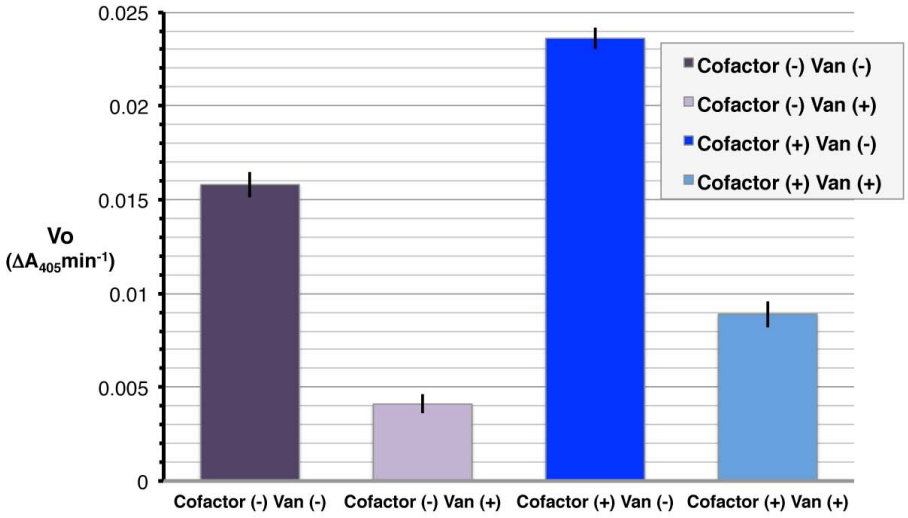


Figure 1: The effect of catalytic cofactors (Mg^{2+} and Zn^{2+}) on the inhibition of CIAP by vanadate. Reactions mixtures containing 50 mM Tris-HCl (pH 8.5) and 10 µM CIAP was pre-incubated at 37 °C with vanadate for 10 minutes and reactions were initiated by the addition of 400 µM pNPP. Reactions were carried out for 10 minutes at 37 °C, and stopped by the addition of 500 mM NaOH and 10 mM EDTA. Enzyme activity is expressed as the change in A_{405} per minute. Each bar represents the mean ± SD of five individual reactions.

RESULTS

In order to test the effect of cofactors on the activity of CIAP and its inhibition by vanadate, it was necessary to prepare enzyme samples that are significantly free of the two metal ion cofactors (Zn^{2+} and Mg^{2+}). Preparation of the 'cofactor-free' CIAP was done by extensively dialyzing the enzyme in a low ionic strength buffer (Koutsoulis *et al.* 2010). It has been shown that this procedure efficiently strips the enzyme of any loosely bound metal ions. These demetalated enzyme preparations were used for subsequent experiments designed to test the interaction of cofactors and vanadate during CIAP-catalysed hydrolysis of pNPP.

The effect of catalytic cofactors (Mg^{2+} and Zn^{2+}) on the inhibition of CIAP by vanadate is shown in Figure 1. Vanadate significantly inhibited dialyzed CIAP but its effect was modulated significantly by the addition of the catalytic cofactors. However, the activity of the enzyme in the presence of both vanadate and catalytic cofactors was significantly lower than in their absence.

Figure 2 shows the effect of catalytic cofactors (Mg^{2+} and Zn^{2+}) and vanadate on substrate kinetics of CIAP-catalyzed hydrolysis of pNPP.). The results showed that vanadate significantly inhibited 'cofactor-free' CIAP, and the inhibition was relieved by the presence of the catalytic cofactors in the reaction. Our results show that the absence of the cofactors did not significantly alter the Km of the reaction, but caused a decrease in the Vmax. Kinetic analyses showed that vanadate inhibited CIAP-catalyzed hydrolysis of pNPP by decreasing the V_{max} and increasing the K_m of the reaction.

The presence of cofactors in the reaction alleviated the effect of vanadate by increasing the V_{max} and decreasing the K_m. The activity of the dialyzed CIAP was increased by the addition of catalytic cofactors to vanadate-inhibited enzyme

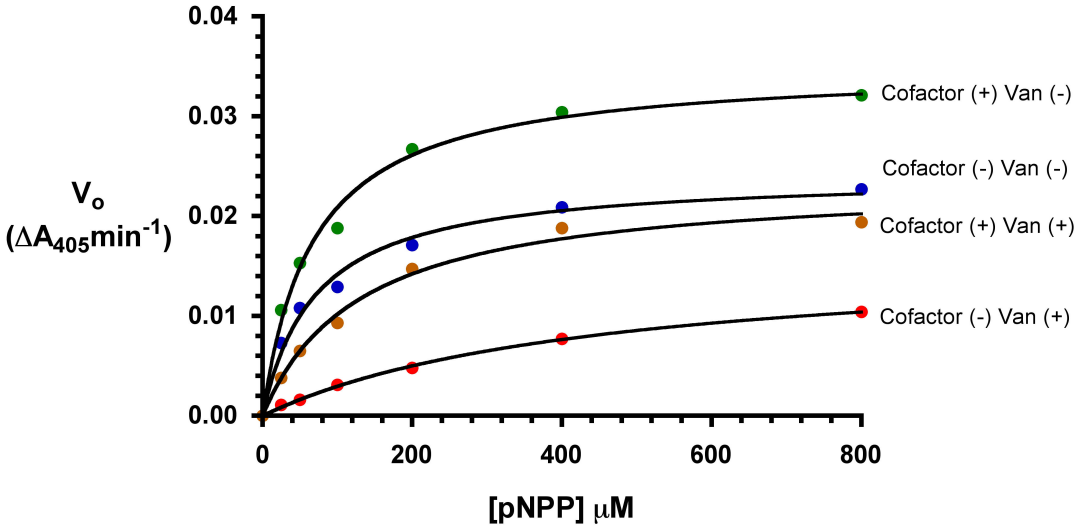
The effect of increasing concentration of vanadate on CIAP activity in the presence and absence of catalytic cofactors (Mg^{2+} and Zn^{2+}) is shown in Figure 3. The activity of the dialyzed CIAP was increased by the addition of catalytic cofactors to vanadate-inhibited enzyme.

DISCUSSION

Metal ions are essential to life and a major determinant of their functional relevance in living systems is that a substantial fraction of enzymes require metals for their catalytic activity (Andreini *et al.*, 2008). Magnesium is the most widespread metal present in enzymes. The main reason behind this observation is that magnesium plays a central role as the essential partner of phosphate-containing substrates which in cells is largely present as Mg^{2+} complex (Luthi *et al.*, 1999). The association between Mg^{2+} and phosphate is in fact a constant feature of key biological molecules (e.g. DNA, RNA), suggesting that biological systems have selected the abundant and highly soluble Mg^{2+} since the early stages of life (Andreini *et al.*, 2008). Recent evidence suggests that the Mg^{2+} in the active site of alkaline phosphatase stabilizes the transferred phosphoryl group via a water molecule and functions via a mechanism different

from the two Zn^{2+} ions at the bimetallocentre (Zalatan *et al*., 2008). Zinc is the second-most abundant metal ion in enzymes. The reason for the high occurrence of zinc in enzyme catalysis lies in the distinctive combination of properties displayed by this metal. From one point of view, zinc resembles the group II elements in that it is also stable in the +2 oxidation state, and is redox inert. In particular, the Zn^{2+} has a radius (0.74 Å) similar to that of Mg^{2+} and the electrostatic binding to negatively charged species, accounts for part of zinc functionality in the active site of enzymes (Andreini *et al*., 2008). Zn^{2+} is known to perform this function in a variety of enzymes such as alkaline phosphatase, containing dinuclear zinc sites, in which one of the ions contribute to activate water, which bridges the two metals, and the other ion is involved in substrate binding and activation (Aubert *et al*., 2004; Andreini *et al*., 2008).

The inhibition by vanadate has been hypothesized to be more potent in enzymes involving a stable covalent phosphoryl enzyme intermediate than those in which the product is formed without covalent involvement of the enzyme. It is particularly active against Na^+/K^+ ATPase, human AP and *E. coli* AP (Cyboron *et al.,* 1982). The results obtained in this study suggest that vanadate is an active site inhibitor which exerted its inhibitory effect on the dialyzed CIAP via a mechanism that decrease the binding affinity of the enzyme for pNPP. This observation corroborated the report of Stankiewicz *et al*. (1995) that ascribed the inhibitory effect of vanadate on phosphate-metabolizing enzymes to its ability to mimic the anionic character of inorganic phosphate suggesting a competitive mechanism of inhibition.



	K_m (μM)	V_{max}
Cofactor (-) Van (-)	70.6	0.0242
Cofactor (-) Van (+)	446.5	0.0162
Cofactor (+) Van (-)	67.6	0.0350
Cofactor (+) Van (+)	131.3	0.0236

Figure 2: The effect of catalytic cofactors (Mg^{2+} and Zn^{2+}) and vanadate on substrate kinetics of CIAP catalyzed hydrolysis of pNPP. Reactions were carried out as described in Figure 1. The concentrations of pNPP used were 20, 50, 100, 200, 400, and 800 µM. The kinetic parameters Km and Vmax estimated from the curves are shown in the lower panel. The curves represent the mean of three separate sets of experiments.

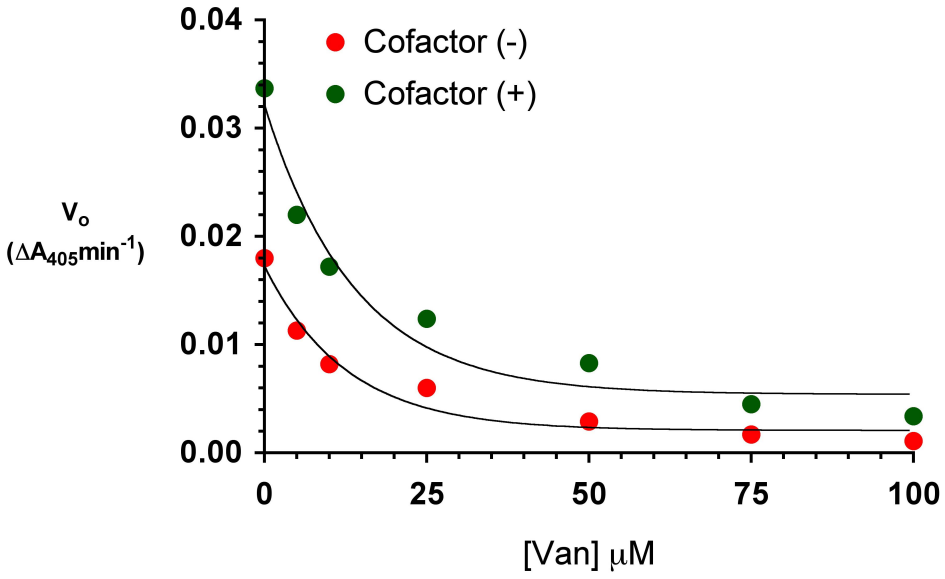


Figure 3: The effect of increasing concentration of vanadate on CIAP activity in the presence and absence of catalytic cofactors (Mg^{2+} and Zn^{2+}). All reactions were carried out as described in Figure 1, and initiated by the addition of 400 µM pNPP. The curves represent the mean of three separate sets of experiments.

It is apparent from this study that the addition of catalytic cofactors (Mg^{2+} and Zn^{2+}) modulated vanadate inhibition of CIAP-catalysed hydrolysis of pNPP by increasing the enzyme's turnover rate and binding affinity for its substrate. Subramaniam *et al.* (1995) described that Mg^{2+} plays an important role as a site-specific effector since it binds to the non-native structure, probably to the active site region of the enzyme, stabilizing transient conformations and guiding conformational reconstitution into productive pathways. Also, previous studies have shown that Zn^{2+} binding to TNAP are required for both catalysis and thermodynamic stability while Mg^{2+} enhances both characteristics (Coleman, 1992). One of the two Zn^{2+} ions in each monomer of the enzyme is involved in binding the substrate in AP catalysis (Kim and Wyckoff, 1991; Bortolato *et al.*, 1999). The relieve of vanadate inhibition of CIAP-catalyzed hydrolysis of pNPP by the catalytic cofactors as observed in this study also corroborates the report of Dirnbach *et al.* (2001) that the binding of one Mg^{2+} per enzyme dimer appears to cause a slight slowing down of the guanidine hydrochloride denaturation reaction; however, two bound Mg^{2+} ions have a dramatic protection effect. They also noted that just as Zn^{2+} provides AP with substantial thermodynamic stability, Mg^{2+} provides kinetic stability, slowing down the unfolding of AP under hostile, denaturing conditions. The results from this study reveal that vanadate inhibition of CIAP-catalyzed hydrolysis of pNPP can be relieved by the addition of catalytic cofactors. Further mechanistic studies could provide more insight into how inhibited AP can be reactivated which may be of significance in the biological system.

These findings also hint that the pattern of reversible inhibition of cofactor-requiring enzymes could be subject to the accessibility of the apoenzymes to the catalytic cofactors. Further studies will reveal detailed mechanistic aspects of this observation and its significance in natural biological systems.

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