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Metal Displacement Effects on Monoesterase Activity of Calf Intestinal Alkaline Phosphatase

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ABSTRACT: The mechanism of modulation of alkaline phosphatase activity by metal ions has not been fully elucidated. We investigated the time-dependent modulatory effects of Mg^{2+} and Zn^{2+} in promoting the hydrolysis of para-nitrophenyl phosphate (monoesterase reaction) by calf intestinal alkaline phosphatase (CIAP) and the effects of addition of the activating metal ions to the metal-inhibited enzyme. The CIAP was affected by changes in pre-incubation time in the presence of the two metal cofactors. Both Mg^{2+} (0.1 – 0.25 mM) and Zn^{2+} (0.1 – 5 mM) modulated Zn^{2+}- and Mg^{2+}- inhibited monoesterase activity of CIAP. The CIAP activity was inhibited when the enzyme was pre-incubated with 1 mM Ca^{2+}. Further addition of Mg^{2+} (0.1 – 2 mM) did not completely restore the activity though partly relieved the inhibition caused by Ca^{2+}- pre-incubated enzyme. Again, addition of 2mM Zn^{2+} to Ca^{2+}- pre-incubated alkaline phosphatase completely restored the activity of the enzyme. This study suggests that Mg^{2+} and Zn^{2+} regulate the catalytic property of each other and modulate the inhibitory effect of Ca^{2+} in alkaline phosphatase catalysis through a displacement effect. The modulation of Ca^{2+}-inhibited CIAP activity by Mg^{2+} and Zn^{2+} may be explored in the treatment of disorders of bone mineralization especially those arising from inhibited alkaline phosphatase activity.

KEYWORDS: Alkaline phosphatase, monoesterase reaction, metal ion cofactors, displacement reaction

1. Introduction

Metal cofactors are active sites features found in metalloenzymes which are critical to the catalytic process and essential to structure stabilization. Occupation of a site by a metal ion on the enzyme molecule could induce a conformational change that can either activate or inhibit the activity of the enzyme molecule (Bosron *et al*., 1977).

Alkaline phosphatases (APs; EC 3.1.3.1) are dimeric metalloenzymes that catalyse the hydrolytic transfer of phosphate to water or its transphosphorylation to amino alcohols. However, when separated the monomeric subunits fail to display enzyme activity (Hoylaerts *et al.,* 1997). Each monomeric subunit of AP contains three divalent cations (two Zn^{2+} and one Mg^{2+}) and a serine residue in the active site (Kim and Wyckoff, 1991). Previous studies on *E. coli* AP (ECAP) have shown that the two zinc ions are directly involved in catalysis (Sowadski *et al*., 1985; Kim and Wyckoff, 1991; Bortolato *et al*., 1999). Report by Zalatan *et al* (2008) suggests that the Mg^{2+} in the active site stabilizes the transferred phosphoryl group via a water molecule and functions via a mechanism different from the two Zn^{2+} at the bimetallocentre.

Four isoenzymes of AP exist; three of which are found exclusively in intestine, placenta and germ cells are termed tissue specific while the fourth isoenzyme is tissue non specific AP because of its wide distribution, being found in abundance in hepatic, skeletal and renal tissues (Moss, 1982; Hessle *et al*., 2002). Intestinal APs are distinguished by the highest catalytic activity and due to this; the isoenzyme has attracted special attention of researchers developing procedures for the determination of metal ions (Muginova *et al*., 2007). The enzyme is present in both soluble and membrane forms, but more than 95% of the intestinal AP is normally associated with the brush border membrane.

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Mammalian APs perform a variety of functions including bone mineralization and fat absorption in the intestine (Millan, 2006). They show structural similarity to ECAP, and strong sequence conservation in regions where the catalytic residues are located (Le Du *et al*., 2001).

Information about the reaction mechanism of AP comes mostly from studies on the *E. coli* enzyme, which is believed to be generally the same for all APs. The reaction starts with a nucleophilic attack of a Serine 102 alkoxide on the phosphate group of the phosphomonoester substrate, forming the covalent phosphoenzyme intermediate. The second step is the hydrolysis of this phosphoseryl intermediate to the noncovalent enzyme-phosphate complex (Simopoulos and Jencks, 1994). In the presence of a phosphate acceptor, such as Tris or diethanolamine, the enzyme displays transphosphorylating activity, an organic nucleophile replacing water (Simopoulos and Jencks, 1994). The rate determining step of the *E. coli* enzyme reaction is pH- dependent. At acidic pH, the hydrolysis of the covalent phosphoseryl intermediate is the slowest step while at basic pH, the dissociation of the phosphate from the noncovalent enzyme-phosphate complex becomes the rate-determining step (Gettins and Coleman, 1983). The type of amino acid at position 328 in the *E. coli* AP has been reported to affect the affinity of the enzyme for inorganic phosphate (Murphy *et al*., 1995) and thus the rate-determining step. While the main features of the catalytic mechanism are conserved comparing mammalian and bacterial APs, mammalian APs have higher specific activity and K_m values; a more alkaline pH optimum; lower heat stability; membrane-bound and inhibited by L-amino acids and peptides through an uncompetitive mechanism. These properties, however, differ noticeably among the different mammalian AP isoenzymes and are likely to reflect very different *in vivo* functions (Millan, 2006).

The two metal cofactors (Mg^{2+} and Zn^{2+}) of AP have concentration dependent activating and inhibitory effects on mammalian AP (Petitclerc and Fecteau, 1977, Hoylaerts *et al.,* 1998, Arise *et al.*, 2005). Some divalent cations such as Ca^{2+} have been reported to be capable of replacing Zn^{2+} resulting in reduced maximal activity (Kim and Wyckoff, 1991). Studies have also shown that optimal concentrations of both Mg^{2+} and Zn^{2+} were required for the tissue non-specific AP (TNAP) to catalyse the hydrolysis of the monoester substrate, para-nitrophenyl phosphate (Olorunniji *et al*., 2007; Igunnu *et al*., 2011). While a detailed catalytic mechanism of the enzyme has been proposed (Kim and Wyckoff, 1991; Holtz *et al*., 1999), the mechanism of modulation of AP activity by metal ions has not been fully substantiated.

Thus, the time-dependent modulatory effects of Mg^{2+} and Zn^{2+} ions in promoting the hydrolysis of para-nitrophenyl phosphate (monoesterase reaction) by CIAP and the effects of addition of the activating metal ions to the metal- inhibited enzyme were investigated in this study with a view to gaining further insight into the interaction between the metal ions in AP catalysis.

2. Materials and methods

2.1 Chemicals

Sodium salt of para-nitrophenyl phosphate (pNPP) was a product of Sigma-Aldrich, UK while magnesium sulphate ($MgSO_4$), zinc sulphate ($ZnSO_4$) and calcium sulphate ($CaSO_4$) were from Fisher Scientific, UK. Purified homogenous CIAP was obtained from New England Biolabs, UK. All other chemicals used in this study were of analytical grade.

2.2 Determination of alkaline phosphatase-catalysed hydrolysis of pNPP

Phosphomonoesterase activity of CIAP was determined by the rate of hydrolysis of pNPP in the presence of the divalent metal ions (Mg^{2+}, Zn^{2+} and Ca^{2+}) in 0.1M Na_2CO_3/$NaHCO_3$ buffer, pH 10.1 according to the procedure described by Ahlers (1975) AP reacts with para-nitrophenyl phosphate to form para-nitrophenol, the rate of formation which is directly proportional to the activity of AP. The rate of appearance of para-nitrophenol was determined spectrophotometrically (Spectronic 21 UV-Vis) and enzyme activity expressed as mmol of para-nitrophenol released per minute. Reaction mixture containing CIAP and buffers in the presence of the appropriate ligand was incubated

at 37 ^oC for 10 minutes. Reaction was initiated by the addition of the appropriate concentration of the substrate for 10 minutes. The absorbance (A) was read at 400 nm against a blank of the buffered substrate for pNPP hydrolysis. All the determinations of the reaction rate were performed in triplicates.

2.3 Determination of time dependent modulation of CIAP-catalysed pNPP hydrolysis by Mg^{2+} and Zn^{2+} ions

Reaction mixture containing 0.1M $Na_2CO_3/NaHCO_3$ buffer (pH 10.1) and 10 µM CIAP was pre-incubated at 37 ^oC with 0.5, 1 and 2 mM concentrations of Mg^{2+} or Zn^{2+} for 0, 5, 10, 15 and 30 minutes. The reaction was initiated by the addition of 2 mM pNPP to the reaction mixture, incubated at 37 ^oC for 10 minutes and stopped by the addition of 1.0 ml 0.5 M KOH.

2.4 Determination of cofactor displacement in CIAP-catalysed pNPP hydrolysis

The effects of addition of the activatory metal ions to the metal-inhibited CIAP catalysed hydrolysis of pNPP were investigated. Reaction mixture containing 0.1M $Na_2CO_3/NaHCO_3$ buffer (pH 10.1) and 10 µM CIAP was pre-incubated at 37 ^oC with an inhibitory metal ion concentration (15 mM Mg^{2+} or 5 mM Zn^{2+}) for 10 minutes followed by a challenge of the system with different activatory metal ion concentrations (0.1 – 2 mM Zn^{2+} or 0.1 - 5mM Mg^{2+}). Reaction was initiated by the addition of the activatory metal ion and 2 mM pNPP. Incubation was allowed for 10 minutes at 37 ^oC after which the reaction was stopped by the addition of 1.0 ml 0.5 M KOH.

2.5 Effects of Mg^{2+} and Zn^{2+} on CIAP pre-incubation with Ca^{2+}

Reaction mixture containing 0.1M $Na_2CO_3/NaHCO_3$ buffer (pH 10.1) and 10 µM CIAP was pre-incubated at 37 ^oC with Ca^{2+} followed by the addition of different concentrations of Mg^{2+} and Zn^{2+}. In the first experiment, 1mM Ca^{2+} ion was pre-incubated with CIAP at 37 ^oC for 15 minutes and the reaction was initiated by the addition of varying concentrations of Mg^{2+} in the range 0.1 - 2 mM and 2 mM pNPP. In the second experiment, AP was pre-incubated with 1 mM Ca^{2+} and the reaction was initiated by the addition of varying concentrations of Zn^{2+} in the range of 0.1 - 2 mM with 2 mM pNPP concentration. The control in each of the experiments was carried out without pre-incubation with Ca^{2+}; reaction was initiated by the addition of 1 mM Ca^{2+}, appropriate concentration of Mg^{2+} or Zn^{2+} and 2 mM pNPP. In all experiments, incubation was done at 37 ^oC for 10 minutes before stopping the reaction by addition of 1.0 ml 0.5 M KOH.

3. Results

The time dependent modulatory effects of Mg^{2+} and Zn^{2+} on CIAP-catalysed pNPP hydrolysis at 0, 0.5, 1 and 2 mM metal ion concentrations were investigated. It is apparent from Figures 1 and 2 that the monoesterase activity of CIAP was affected by changes in pre-incubation time in the presence of the two divalent cations. Mg^{2+} activated CIAP at the three concentrations investigated and within the period of incubation (0 – 30 minutes). On the other hand, Zn^{2+} only activated CIAP within 0 – 10 minutes of pre-incubation with the enzyme. Pre-incubation of CIAP with Zn^{2+} for 15 and 30 minutes before initiating the reaction with the addition of pNPP led to inhibition of the enzyme (Figure 2).

The modulatory effect of Zn^{2+} on Mg^{2+}-inhibited CIAP catalysed hydrolysis of pNPP is shown in Figure 3. The result revealed that the addition of Zn^{2+} up to 0.25 mM led increased the activity of the enzyme, thereby modulating the inhibitory effect imposed by Mg^{2+}. However, further increase in Zn^{2+} concentration beyond 0.25 mM decreased the activity of CIAP. Similarly, the effect of Mg^{2+} on Zn^{2+}-inhibited CIAP catalysed hydrolysis of pNPP was carried out. As shown in Figure 4, addition of Mg^{2+} from 0.1 – 5 mM concentration steadily increased the activity of CIAP, thereby canceling out the inhibitory effect of the Zn^{2+}.

As shown in Figures 5 and 6, the activity of CIAP was inhibited when the enzyme was pre-incubated with Ca^{2+}. Increasing concentrations of Mg^{2+} relieved the inhibition caused by Ca^{2+}-pre-incubation with CIAP but did not completely restore the activity of the enzyme

(Figure 5). On the other hand, further addition of Zn^{2+} to Ca^{2+} - pre-incubated CIAP completely restored the activity at 2 mM concentration (Figure 6).

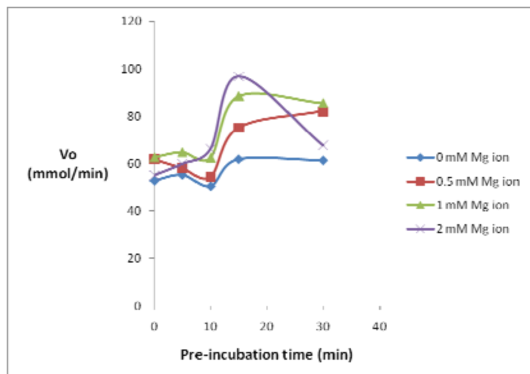

Figure 1: Time-dependent activation of CIAP-catalysed pNPP hydrolysis by Mg^{2+}

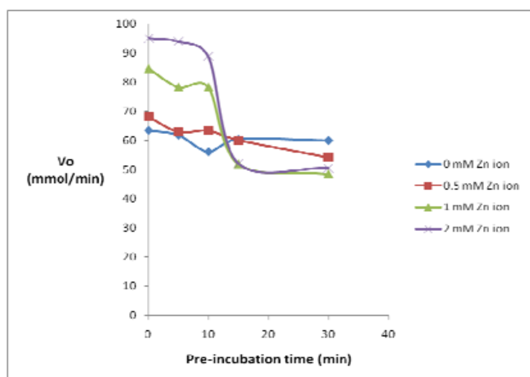

Figure 2: Time-dependent modulation of CIAP-catalysed pNPP hydrolysis by Zn^{2+}

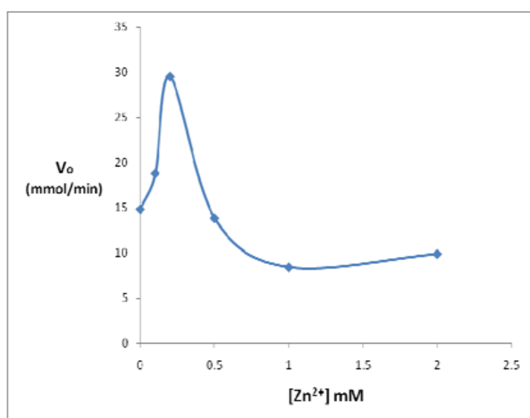

Figure 3: Modulatory effect of Zn^{2+} on Mg^{2+}-inhibited CIAP-catalysed hydrolysis of pNPP

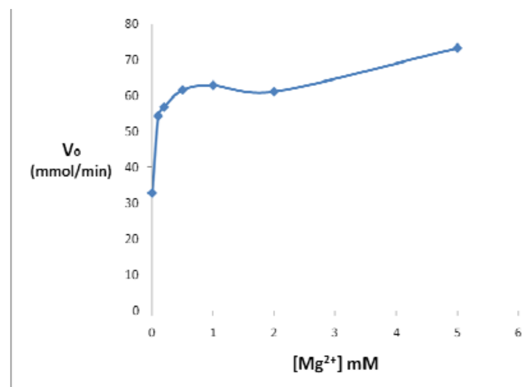

Figure 4: Modulatory effect of Mg^{2+} on Zn^{2+}-inhibited CIAP-catalysed hydrolysis of pNPP

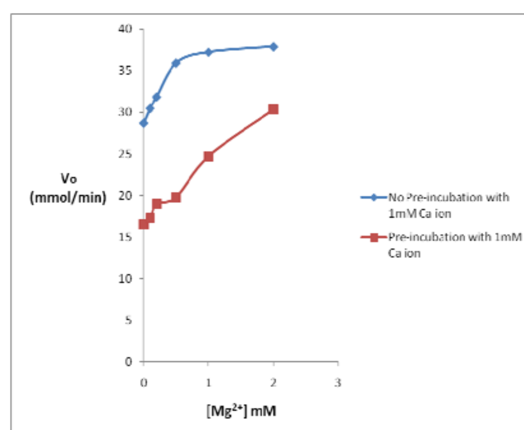

Figure 5: Effect of pre-incubation of CIAP with Ca^{2+} at different concentrations of Mg^{2+}

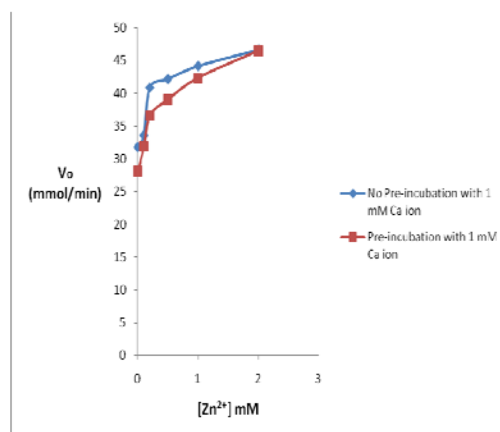

Figure 6: Effect of pre-incubation of CIAP with Ca^{2+} at different concentrations of Zn^{2+}

4. Discussion

The changes in CIAP activity resulting from variation in the time of pre-incubation of the

metal ions (Mg^{2+} and Zn^{2+}) with the enzyme in this study suggest that the modulatory effects of the two divalent cations mediated a conformational effect. Arise *et al.* (2005) reported that Mg^{2+} performs more of structural role by inducing conformational change in AP and that the metal ion hypothetically activates structural features needed for catalysis independent of substrate binding. The results here also corroborates previous reports that Mg^{2+} and Zn^{2+} activate AP activity at optimal concentration and inhibit the activity of the enzyme at supra-optimal concentrations (Ciancaglini *et al.*, 1989; Arise *et al.*, 2005). Excess Zn^{2+} have been reported to inhibit several APs by replacing Mg^{2+} on the enzyme molecule (Ciancaglini *et al.*, 1989). Similarly, in accounting for the inhibitory effect of excess Mg^{2+} on AP activity, it was proposed by Arise *et al* (2005) that since both Mg^{2+} and Zn^{2+} can bind to the same site on the enzyme molecule (Hung and Chang, 2001), excess Mg^{2+} could displace some structural and catalytic Zn^{2+} with a consequent down-regulation of activity.

Substitution of the metal ions of a metalloenzyme is one of the mildest and most specific procedures presently available to chemically modify an enzyme. Since both Mg^{2+} and Zn^{2+} can bind to the same site in the AP active site as earlier reported by Kim and Wyckoff (1991), Hung and Chang (2001), a possible explanation for the modulatory effect of Zn^{2+} on Mg^{2+} inhibition of CIAP activity could be due to the displacement of bound-Mg^{2+} from the Zn^{2+} binding-site, thereby promoting the binding of Zn^{2+} to its site thus enhancing catalysis. However, the decline in monoesterase activity of CIAP as concentrations of Zn^{2+} increased beyond 0.2 mM corroborate the report of Deen (2002) who showed that excess Zn^{2+} competes with Mg^{2+} and caused a progressive inhibition of the enzyme by replacing Mg^{2+} on the enzyme molecule. Similarly, the modulatory effect of Mg^{2+} on Zn^{2+} inhibition of CIAP could be due to the displacement of bound-Zn^{2+} from the Mg^{2+} binding site thereby causing reactivation of CIAP. These observations seem to establish the fact that metal ion displacement effects occur in AP catalysis and further suggest that both Mg^{2+} and Zn^{2+} regulate the catalytic property of each other in monoesterase activity of AP.

AP activity is frequently used as a marker for sites of mineral (e.g. calcium) deposition (Genge *et al.*, 1988; Orimo, 2010). Some divalent cations such as Ca^{2+} have been shown to be capable of replacing Zn^{2+} resulting in reduced maximal activity (Kim and Wyckoff, 1991). Thus, the effect of pre-incubation of CIAP with inhibitory concentration of Ca^{2+} followed by the addition of varying concentrations of Mg^{2+} and Zn^{2+} were carried out in this study to demonstrate the potential metal ion displacement effect in AP catalysis and to probe into the possibility of modulating Ca^{2+} inhibition of AP activity by the metal cofactors. In a previous attempt made to determine the correlation between alkaline phosphatase activity and accumulation of calcium during matrix vesicle mediated mineralization, a marked loss (up to 65-70%) in AP activity was found to accompany Ca^{2+} ion accumulation by matrix vesicle (Genge *et al.*, 1988). This implies that Ca^{2+} accumulation and consequent decline in AP activity are tightly associated. Investigation into the possible causes revealed that the decline in AP activity during Ca^{2+} uptake was not due to action of proteases but rather resulted from interaction with the developing mineral phase, loss of metal ions (Zn^{2+} and Mg^{2+}) from the active site of the enzyme, and concomitant irreversible denaturation of the enzyme (Genge *et al.*, 1988). Thus, the results obtained in this study as depicted in Figures 5 and 6 suggest that Ca^{2+} inhibits AP activity by displacing some of the metal ions from the active site of the enzyme. Increasing concentration of Zn^{2+} reversed the inhibitory action of Ca^{2+} possibly by displacing Ca^{2+} from the Zn^{2+} binding site. Addition of Mg^{2+} on the other hand, though up-regulated the enzyme activity, did not bring about complete reversal of loss of activity caused by Ca^{2+}. This seems to suggest that Ca^{2+} exerts more of its inhibitory effect on AP activity by binding to the Zn^{2+} binding site. It can also be deduced from this study that Ca^{2+} binding may not lead to irreversible denaturation of the enzyme as earlier proposed by Genge *et al* (1988).

The mechanistic devices that regulate tissue calcification are of major importance, as they ensure that calcification of the skeleton proceeds normally while mineralization is prevented elsewhere in the body. Alterations in these

regulatory mechanisms, either due to genetic defects or as a result of ageing could lead to diseases/malfunctioning such as osteoarthritis and arterial calcification. Tissue calcification is an active process that is under the control of factors that regulate normal bone formation. 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, thereby ensuring normal bone mineralization (Hessle *et al*., 2002). Thus, the modulatory effects of Mg^{2+} and Zn^{2+} on Ca^{2+}-inhibited CIAP activity as observed in this study may provide help in the treatment of resultant disorders due to alterations in the regulatory mechanisms of tissue calcification.

In conclusion, findings in this study suggest that Mg^{2+} and Zn^{2+} regulate the catalytic property of each other and modulate the inhibitory effect of Ca^{2+} in alkaline phosphatase catalysis through a displacement effect. The modulation of Ca^{2+}-inhibited CIAP activity by Mg^{2+} and Zn^{2+} may be explored in the treatment of disorders of bone mineralization resulting from inhibited alkaline phosphatase activity.

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