BIOKEMISTRI 17(2):129-136 (Dec 2005)
Available online at http://www.bioline.org.br/bk.
Abstracted online at www.ajol.info/journals/biokem

Printed in Nigeria

An international journal published by the

Nigerian Society for Experimental Biology

Regulatory effect of divalent cations on rat liver alkaline phosphatase activity: *How Mg^{2+} activates (and inhibits) the hydrolysis of p-nitrophenylphosphate*

Rotimi O. ARISE[1], Femi F. BOLAJI[1,4], Olalekan A. JIMOH[1,4], Joseph O. ADEBAYO[2], Femi J. OLORUNNIJI[1,3*] and Sylvia O. MALOMO[1]

1. Department of Biochemistry, Faculty of Science, University of Ilorin, P.M.B 1515, Ilorin, Nigeria.
2. Department of Biochemistry & Physiology, Faculty of Health Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Nigeria.
3. Division of Molecular Genetics, Institute of Biomedical and Life Science, Anderson College, University of Glasgow, Glasgow, G11 6NU, United Kingdom.
4. Deceased

Received 19 April 2005

MS/No BKM/2005/020,

Abstract

The concentration-dependent stimulation of rat liver alkaline phosphatase (ALP) catalyzed hydrolysis of para- nitrophenylphosphate (pNPP) was studied. ALP displayed some activity even in the absence of exogenous Mg^{2+}. Kinetic analyses show that activation by Mg^{2+} is exerted at the V_{max} level without necessarily enhancing the affinity of the enzyme for the ion. However, the hyperbolic activation operates only within the optimal level of 0 to 5mM concentrations of the metal ion. Higher concentrations were actually inhibitory in a pure non-competitive manner. Mg^{2+}, either as an activator (optimal concentrations) or inhibitor (supra-optimal levels) exerts its action via a V_{max} effect with only negligible effect on K_m for the substrate.

Key words: Magnesium ion, Alkaline phosphatase, Supra optimal regulation

*Author to whom all correspondence should be addressed.
E-mail: femijohn@gmail.com; F.Olorunniji@bio.gla.ac.uk **Tel**: +44 1413 571866

INTRODUCTION

Despite intensive efforts towards understanding the biochemical nature of alkaline phosphatase, the role of this enzyme in biological processes is still not completely understood[1,2]. Although, a variety of evidence points towards diverse mechanisms of action of this enzyme, it is well known that alkaline phosphatase is a zinc metalloenzyme that can be activated by magnesium ion[3]. On the other hand, excess zinc has been reported to inhibit several alkaline phosphatase by replacing magnesium ions on the enzyme molecule[4].

Various divalent metal ions have been shown to have both activating and inhibitory effects on mammalian alkaline phosphatases[5,6], but the question of how the mechanisms of activation and inhibition is accomplished, however, remains unanswered. This is in spite of the different effects that have been described in the presence of various divalent metal ion[7].

It has been shown that alkaline phosphatase have three classes of metal binding sites, which have been designated to perform catalytic, regulatory and structural roles. The alkaline phosphatase of *E. coli* is a dimer composed of two identical subunits with four atoms of zinc and two atoms of magnesium[8]. Removal of the zinc ions leads to loss of catalytic activity while replacement of the zinc ions by other divalent cations resulted in lower maximal activity. Three metal ions (two Zn^{2+} and one Mg^{2+}) in ALP active sites are essential for enzymatic activity[8]. The inhibition of alkaline phosphatase by excess Zn^{2+} has been proposed to be due to the replacement of Mg^{2+} by Zn^{2+} at one site of the enzyme[5]. In an earlier report we showed that vanadate and L-phenylalanine display positive synergistic interactions in their inhibition of rat liver alkaline phosphatase[9].

This report describes an attempt to examine the effect of low (optimal) and high (supra-optimal) magnesium ion concentrations on rat liver alkaline phosphatase, with a view to understanding how the metal ion performs the dual role.

MATERIALS AND METHODS

P-nitrophenylphosphate (pNPP) was obtained from sigma chemical company, St. Louis, US. Magnesium salt of chloride was a product of British Drug House, Poole, UK. All other chemicals used in this study were of high quality research grade.

Preparation of rat liver alkaline phosphatase

Crude homogenates of rat liver was concentrated for ALP activity using ammonium sulphate according to a modified procedure of Hung and Melnykovych[10]. Rat liver was homogenized in 0.25M sucrose solution at 4^0C and the crude homogenate was centrifuged at 5000 rpm for 20 minutes at the same temperature. To the supernatant fraction was added a 0.55g/ml-(4.17M) solution of $(NH4)_2SO_4$ gradually with stirring until 30% saturation was achieved. The precipitate was collected by centrifugation at 5000 rpm for 20 minutes and re-dissolved in 0.1M carbonate-bicarbonate buffers, pH10.1. The crude preparation was further fractionated on a Sephadex G-100 column to obtain a rich and highly active alkaline phosphatase fraction. The activities of the ALP prepared this way and used in this study were highly reproducible and gave linear results with a correlation level sufficient for kinetic work[9].

Determination of alkaline phosphatase hydrolysis of pNPP

Alkaline phosphatase activity was measured by the hydrolysis of p-nitrophenylphosphate (pNPP) at 25^0C in the presence of a previously added magnesium chloride in 0.1M Na_2CO_3/ $NaHCO_3$ buffers, pH 10.1 as previously described[11]. Enzyme activity is expressed as the μM of p-nitrophenol released per minute. The concentrations of Mg2+ investigated were 0, 0.2 and 1.0mM at *p*NPP concentrations ranging from 0.63-5.1mM. Protein concentration was determined using the Folin-phenol method of Lowry *et. Al.*[12] with Bovine Serum Albumin (BSA) as standard. Spectrophotometric readings were taken on spectronic-21 UV-VIS spectrophotometer.

Investigation of the effect of Mg^{2+} concentration on alkaline phosphatase activity

In order to determine the concentration dependent effect of Mg^{2+} within optimal levels, the reaction medium was set up essentially as described by Wright and Plummer[13] but Mg^{2+} concentration was varied in the range of 0.1-3.8mM. Investigations on the effect of supra-optimal levels of Mg^{2+} on ALP activity were carried out at concentrations ranging from 12.5-25.4mM. The concentration range of pNPP was kept constant (0.63-5.1mM) for all Mg^{2+} concentrations studied and incubation was allowed for 10 minutes before stopping the reaction by the addition of 0.1M NaOH. The absorbance was monitored at 400nm against a blank of the buffered substrate and the corresponding activities were recorded.

Analysis of inhibition data

Cornish-Bowden[14] and Cortes *et. al*[15] proposed a relationship between inhibition constants, inhibitor concentrations for 50% inhibition ($i_{0.5}$) and types of inhibition. This 'higher resolution' kinetic procedure allows for detailed and non-confounding analysis of inhibition mechanism. The quantitative expressions of $i_{0.5}$ were derived by Cheng and Prusoff[16] for competitive, uncompetitive and mixed inhibition. When a straight line is obtained by plotting a function of the rate 'v' against the inhibitor concentration '$i_{0.5}$' whether this is the reciprocal[17] rate '$\frac{i}{v}$' or the reciprocal rate multiplied by the substrate[18] concentration 'a' that is '$\frac{a}{v}$'; the intercept of the extrapolated line on the 'i' axis is $-i_{0.5}$.This provides a simple and accurate way of estimating $i_{0.5}$. This is however easily demonstrated by reference to the expression[15]

$$\frac{a}{v} = \frac{k_m}{v}\left[\frac{1+i}{K_{ic}}\right] + \frac{a}{v}\left[\frac{1+i}{K_{iu}}\right] \quad \text{.....(1)}$$

Where 'v' is the limiting rate, 'K_m' is Michaelis constant, 'K_{ic}' is the competitive inhibition constant and 'K_{iu}' is the uncompetitive inhibition constant. A plot of $\frac{1}{i_{0.5}}$ against $\frac{v}{V}$ is a secondary plot, which gives a straight line according to the following expression[15]

$$\frac{1}{i_{0.5}} = \frac{1}{k_{ic}} + \frac{v}{V}\left[\frac{1}{k_{iu}} - \frac{1}{k_{ic}}\right] \quad \text{..... (2)}$$

The combination of the two plots discriminates clearly between all the different types of linear inhibition, and supplies the values of both inhibition constants; furthermore, it provides a direct link between the biochemical characteristics of the inhibition. The plots of [pNPP]/v versus [Mg^{2+}] for the Cornish-Bowden analysis and the secondary plot were made accordingly.

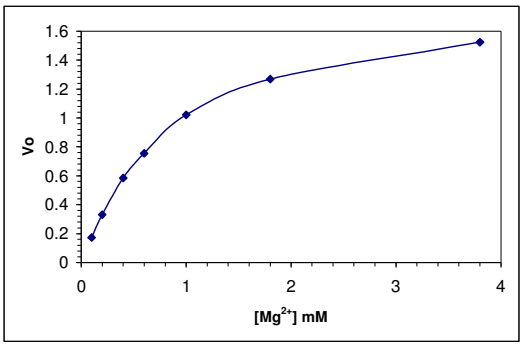
RESULTS

Activation of alkaline phosphatase with optimal concentrations of Mg^{2+}

Figure 1 shows the effect of Mg^{2+} concentration on the kinetics of ALP-catalyzed hydrolysis of pNPP. The stimulation was Mg^{2+} ion concentration dependent in the range of 0.1-3.8mM (panel A). Within this range, the activation was hyperbolic, an indication of a saturating process characteristic of Michaelis-Menten kinetics describing the saturating nature of the variation of reaction velocity with substrate concentration. This suggests that essentially all the enzyme molecules present were combined with the metal ion in the reaction solution. It is likely that the Mg^{2+} site in the protein is saturable in a way that influences catalysis. A double reciprocal transformation of the activation data is shown in panel B. This allows for estimations of Michaelis constant and maximum rates of 1.16mM and 2.19s^{-1} respectively. While the binding of Mg^{2+} to the enzyme is not a true enzyme-substrate phenomenon, the good fit of the data to the Michaelis-Menten equation affords a simple way of analyzing the data.

Figure 2A shows the hydrolysis of various concentrations of pNPP by alkaline phosphatase in the presence of 0, 0.2 and 1.0mM Mg^{2+}. Within the range of substrate concentration

A



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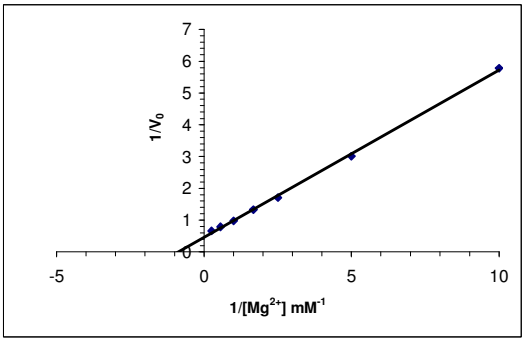


Figure 1: Activation of alkaline phosphatase by exogenous Mg^{2+}. Panel A shows the effect of increasing concentration of the cation on enzyme activity while the double reciprocal transformation of the data is shown in panel B.

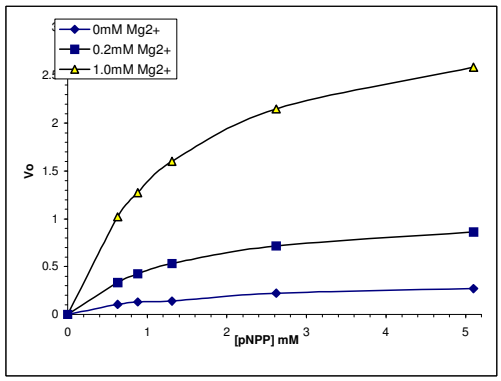
examined, the hydrolysis of pNPP by alkaline phosphatase obeyed Michaelis-Menten kinetics.

Values of V_{max} and Km for pNPP at the three levels of Mg^{2+} are derived from the double reciprocal transformations shown in Figure 2B. It is apparent that ALP displayed activity even in the absence of exogenous Mg^{2+}, though the activity was low. This finite activity is evident that the preparatory procedures employed in obtaining the enzyme did not lead to exhaustive loss of the bound Mg^{2+} in the enzyme. Stimulation of ALP hydrolysis of pNPP was also dependent on Mg^{2+} concentration as it was observed that ALP activity was higher at 1.0mM Mg^{2+} compared with 0.2mM.

Higher concentrations of Mg^{2+} inhibits ALP activity

The hyperbolic and concentration-dependent activation of ALP by Mg operates only at the

A



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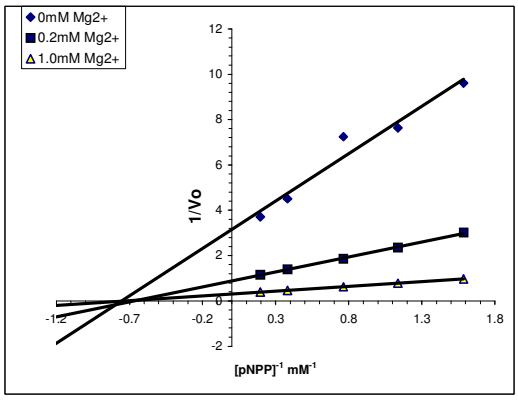

Figure 2: Effect of activating (optimal) concentrations of Mg^{2+} on Michaelis-Menten kinetics of pNPP hydrolysis by alkaline phosphatase.

optimal level of bout 0 to 5mM concentrations of the metal ion. Further experiments were carried out to study the kinetic pattern of this inhibition. The strategy used was to analyze how Mg^{2+} at 12.5, 16.7, 21.1 and 25.4mM affect the kinetics of pNPP hydrolysis within the substrate concentration range of 0.63 to 5.1mM. The basic pattern is shown in Figure 3 along with activity at an optimal (2mM) concentration of Mg. The presence of these higher concentrations of Mg^{2+} suppressed the hydrolysis of pNPP. It is noteworthy however that ALP-catalyzed hydrolysis of pNPP at these inhibitory levels of Mg^{2+} still obeys Michaelis-Menten kinetics. The double reciprocal transformation (Figure 3B) gave a series of linear fits characteristic of a pure non-competitive mechanism for the inhibition of pNPP hydrolysis by highest concentration of Mg^{2+}. This was further confirmed by subjecting

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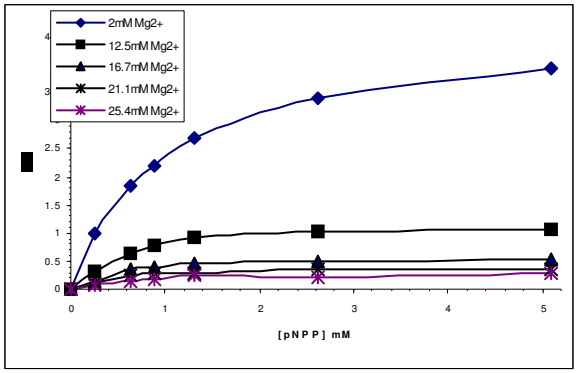

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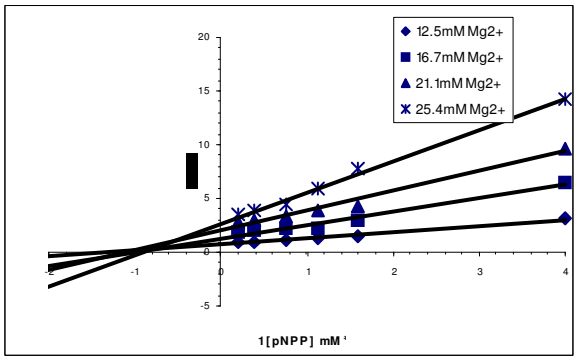
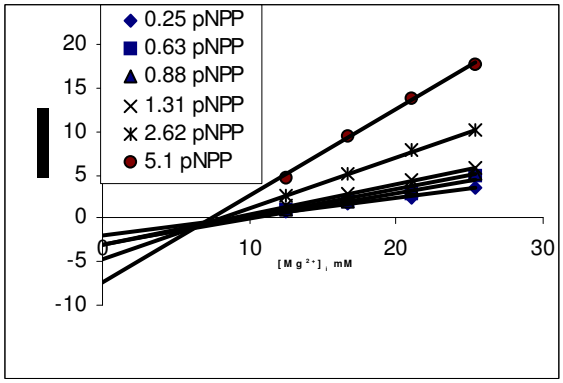


Figure 3: Effect of inhibiting (supra-optimal) concentrations of Mg^{2+} on Michaelis-Menten kinetics of pNPP hydrolysis by alkaline phosphatase.

the data to more rigorous processing using the transformations of Cornish-Bowden[15].

The plots of [pNPP]/v versus [Mg^{2+}] for the Cornish-Bowden analysis and the secondary plot '$\frac{1}{i_{0.5}}$' against '$\frac{v}{V_{ma}}$' are shown in Figures 4A and 4B respectively. When these were compared with the classical patterns of Cornish-Bowden transformations, they gave a pure non-competitive mechanism, within the limits of experimental error. Figure 5A is a plot of '$\frac{1}{V}$' against Mg^{2+} concentration while 5B is a plot of Km/V_{max} against Mg^{2+} concentration. These plots evaluate the inhibition mechanism while showing the inhibitory effect of Mg^{2+} at high concentrations on the kinetic parameters of K_m and V_{max}. The plots reveal a systematic curvature, which is indicative of hyperbolic inhibition[14]. This provisionally happens when the 'EX' complex is less reactive than the free

A



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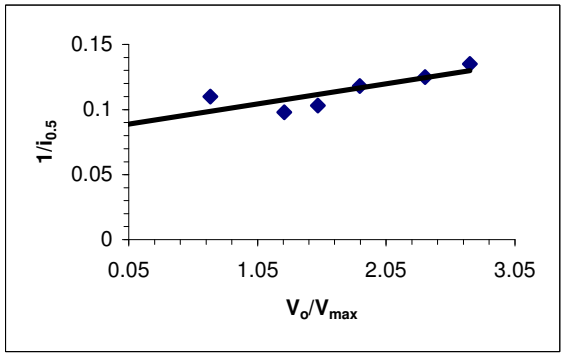
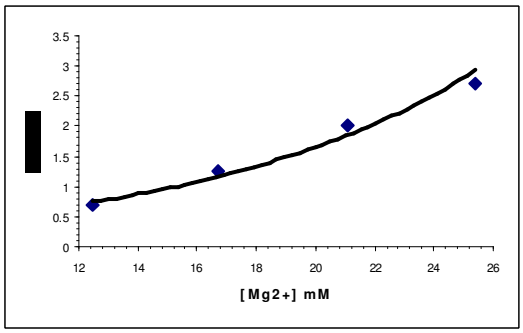


Figure 4: Cornish-Bowden plots for the analysis of alkaline inhibition by supra-optimal concentrations of Mg^{2+}



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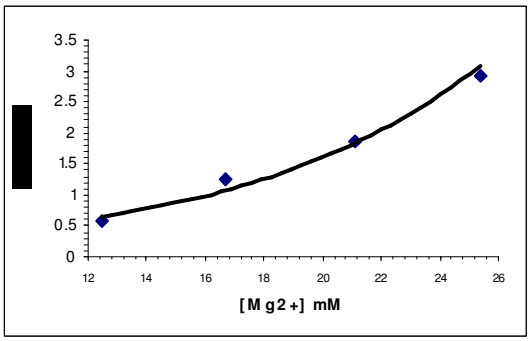

B

Figure 5: Effect of inhibiting (supra-optimal) concentrations of Mg^{2+} on some catalytic constants of pNPP hydrolysis by alkaline phosphatase.

enzyme, in this instance; 'X' is the normal substrate or cofactor. This is mechanistically different from the 'EI' complex of competitive inhibition since the 'EX' is still catalytically reactive, albeit at a slower rate[14]. It is interesting to note that Mg^{2+} as an activator (optimal concentration) and as an inhibitor (at higher concentration) exerted its action as a Vmax effect with only negligible effect on Km for the substrate.

DISCUSSION

McCracken and Meighen[19] established the existence of three classes of metal binding sites in ALP. These are designated "catalytic" "structural" and "regulatory". Zn^{2+} occupies the catalytic and structural sites, while Mg^{2+} ions are bound at the regulatory site[20,21]. Brunel and Cathala[7] in explaining the role of metal ions in metal-activated enzymes systems proposed that the metal ion can act as a bridge between the enzyme and it's substrate or it can induce conformational changes and thereby convert an inactive or partially activated form of an enzyme into a catalytically active or more active form.

These considerations show the intimate role of Mg^{2+} in the general structure and function of ALP. Accordingly, it is expected on the basis of simple chemical considerations that changes in the level of the metal ion will sensitively affect the performance of the enzyme at structural and functional levels. It is conceivable that Mg^{2+} performs more of a structural role by inducing a conformational change in ALP as observed here. This may serve as a basis for its activating role in ALP assay. Occupation of a site on the enzyme molecule could induce a conformational change that brings about activation of the enzyme molecule. It is also possible that a Mg^{2+}-pNPP complex is the true substrate of the enzyme; hence, explaining the hyperbolic behavior. The effect of Mg^{2+} on pNPP hydrolysis by ALP as observed in this study is via the V_{max} effect and did not essentially affect k_m. This is in conformity with reports from other workers such as Brunnel and Cathala[7]. The fact that Mg^{2+} concentration may not affect K_m indicate that Mg^{2+} induces its activation effect on rat ALP through its binding to the enzyme-substrate complex rather than by an action on the free enzyme[14]. Mechanistically, the action of Mg^{2+} on the V_{max} may be to enhance the breakdown of the ES complex to form the free enzyme and product. The variable effect of Mg^{2+} on V_{max} values points to a substrate binding – independent activation pattern in which the cofactor, Mg^{2+} binds only to the ES complex to form the free enzyme and product.

The inhibition observed at higher Mg^{2+} concentration may be as a result of unproductive binding between ALP and excess Mg^{2+} as the same "agent" transits from an activator to an inhibitor - an interesting mechanistic puzzle. A positive explanation is that the excess Mg^{2+}, via a simple mass action effect, displaced Zn^{2+} from its site in the enzyme with a corresponding decrease in activity. The intact ALP requires four zinc and two magnesium atoms for catalytic activity and substrate binding[8]. Since both metal ions can bind to the same site, it is therefore possible for excess Mg^{2+} to displace some structural and catalytic Zn^{2+} with a consequent down – regulation of activity. In the reconstitution experiment of Zhang *et al.*[22], while low concentrations of Mg^{2+} stimulated the refolding of ALP, high concentration actually inhibited the reconstitution of active ALP. This offers another perspective on the role of Mg^{2+} as found in this study: Stabilization and destabilization of the catalytically active structure at low and high concentrations respectively[23].

The 'V_{max} effects' of Mg^{2+} as an activator at optimal concentration and, inhibitor at supra optimal concentration as observed in this study is consistent with the work of Park *et al.*[24] where it was shown that activation of ALP is time dependent and not a very rapid process. Generally, at optimal Mg^{2+} concentrations, V_{max} increased steadily while at inhibitory levels, the V_{max} was found to degenerate at a gradual rate. Non-competitive inhibitors bind to both free enzymes and the enzyme-substrate complex with equal affinity i.e. $K_{ic}=K_{iu}$. This, however may tend to conflict with earlier explanations about the binding of Mg^{2+} to the enzyme but this can be substantiated by the allostery ascribed to mammalian alkaline phosphatases[25], where they

concluded that mammalian ALP are allosteric enzymes in which both monomers act independently, especially when both ALP subunits are completely metalated.

In conclusion, if the primary mechanistic role of Mg^{2+} in ALP is structural as assumed from earlier studies, the V_{max} effect of the ion as observed in this study prescribes that the Mg^{2+} dependent structural role does not control substrate binding. Rather, Mg^{2+} hypothetically activates structural features needed for catalysis independent of substrate binding.

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