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ARTICLE

Mechanistic and *In silico* Characterization of Metal ion Requirements of *Escherichia coli* Zinc Phosphodiesterase Activity

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Abstract

Zinc phosphodiesterase (ZiPD) participates in the maturation of tRNA precursors. The roles of metal ions in promoting phosphoryl transfer reaction on zinc phosphodiesterase (ZiPD) activity have not been fully characterized. Therefore, this study investigated the effects of some metal ions on phosphodiesterase activity of *Escherichia coli* ZiPD as well as the binding site and binding affinity of the metal ions. ZiPD activity was measured by monitoring the rate of hydrolysis of bis-para-nitrophenyl phosphate (bis-pNPP) in the presence of some selected divalent metal ions (Mn^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+}). The results obtained revealed that Mn^{2+} at 1 mM activated ZiPD activity by 4-fold with binding affinity score of 1.795. Co^{2+} at 0.5 mM activated ZiPD activity by 2-fold with binding affinity score of 1.773. Mg^{2+} at 0.5 mM enhanced the binding affinity of ZiPD for bis-pNPP but did not increase the turnover rate of ZiPD. Zn^{2+} at 1.5 mM activated ZiPD activity by 2-fold via increased affinity of ZiPD for bis-pNPP. In conclusion, the findings from this study showed that Mn^{2+} and Zn^{2+} are the most effective stimulatory ions of ZiPD for bis-pNPP while Zn^{2+} exerted the highest binding affinity of ZiPD for bis-pNPP.

Keywords: Zinc phosphodiesterase; phosphodiesterase activity; binding affinity; metal ions

1.0 Introduction

Phosphoryl transfer reactions are important phenomena in biological systems that require well-controlled mechanisms for the transfer of phosphate groups from donor molecules to molecules accepting the phosphate group. Enzymes that catalyse phosphoryl transfer reactions (phosphomonoesterases, phosphodiesterases and phospho-triesterases) employ metal ion cofactors as key participants in regulating their catalytic actions (Igunnu *et al.,* 2014). Zinc phosphodiesterase (ZiPD) is a member of tRNase Z enzymes that belong to the family of metal dependent *β*-lactamases (Tavtigian *et al.,* 2001), a group of metalloproteins which perform a variety of functions (Daiyasu *et al.,* 2001). ZiPD is encoded by *ElaC* gene that is prevalent in eubacteria, archaebacteria, and mammals with a highly conserved sequence (Minagawa *et al.,* 2004).

Most single tRNA molecules end with the 3́ terminal sequence CCA (Minagawa, *et al*., 2006) which is necessary for aminoacylation of the tRNAs and for translation on the ribosome (Green and Noller, 1997). The tRNAs are transcribed as larger precursors, which subsequently undergo different processing steps such as removal of 5́ and 3́ extra nucleotides to produce mature tRNAs (Morl and Marchfelder, 2001). Specific Nucleases carry out a precise cleavage of precursor tRNA (pre-tRNA) after its transcription at both the 5́ and 3́ extensions which is a vital activity in the maturation of tRNA (Morl and Marchfelder, 2001). ZiPD participates in the maturation of tRNA precursors in both *E. coli* mutant strains without the 3́-tRNA maturation exoribonucleases and in wild type *E. coli* strains (Dutta *et al.,* 2012; Kelly and Deutscher, 1992). ZiPD exhibits both endoribonuclease and exoribonuclease activity. It functions as an exoribonuclease on tRNA precursors lacking a CCA sequence and acts as an endoribonuclease on tRNA precursors in which CCA sequence is present at the 3́ terminal end (Dutta *et al.,* 2012).

Zinc phosphodiesterase is a homodimeric enzyme containing two subunits with each monomer showing the typical metallo-β-lactamase fold (de la Sierra-Gallay *et al*., 2005), with a core of two-seven-strand β-sheets which are flanked on each side by α-helices. The two sheets have similar topologies, consisting of four antiparallel β-strands followed by three parallel β-strands. There is an exosite which extends from the body of the protein between β-strands β9 and β12. The protein core width is approximately 50Å, and its height and depth are about 32Å and 28Å, respectively; the exosite extends from the core of the protein at a slight angle and has a total length of approximately 40Å (Vogel *et al*., 2004; de la Sierra-Gallay *et al*., 2005). The ZiPD dimerization interface is in the region of helices α1, α2, and α3, and the dimer is arranged with the monomers in a head-to-head fashion with the exosite tails extending away from each other. A narrow cleft is formed jointly by the two monomers in which the active site and metal coordination residues are present (Dutta and Deutscher, 2009). Furthermore, another narrow cleft corresponding to the proposed 3′ trailer RNA binding channel of the *B. subtilis* tRNase Z enzyme (de la Sierra-Gallay *et al*., 2005) is positioned directly adjacent to this active site cleft and is formed by a single monomer using the loop between α3 and β6 and several residues from the α2 helix.

Zinc phosphodiesterase exhibits a binuclear metal binding site (Daiyasu *et al.,* 2001). The metal coordination sites of the structurally characterized members of this enzyme family are identified partly by residues from the conserved sequence motif H*X*H*X*DH (Vogel *et al.,* 2005). In general, metallo-*β*-lactamases bind one or two metal ions, preferably zinc, iron, or manganese with high selectivity (Vogel, 2002). These metal ions are essential for numerous biocatalytic processes and also to achieve catalytic functionality. Although the monomers of ZiPD have a binuclear metal binding site, the roles of metal ions in promoting phosphoryl transfer catalysed by ZiPD are yet to be fully understood. Therefore, this study investigated the effects of some metal ions on the *E. coli* ZiPD activity as well as the binding site and binding affinity of the metal ions with the view to gaining further insight into the metabolic function of metal ions in ZiPD catalysis.

2.0 Experimental

Materials

Bis-para-nitrophenyl phosphate (bis-pNPP) was a product of Sigma-Aldrich, UK. Purified homogenous *Escherichia coli* zinc phosphodiesterase (ZiPD) was obtained from the Institute of Molecular Cell and Systems Biology, University of Glasgow, UK. All other chemicals used were of analytical grade.

Determination of E. coli Zinc Phosphodiesterase Catalyzed Hydrolysis of Bis-para-nitrophenyl Phosphate

Phosphodiesterase activities of ZiPD were measured by the rate of hydrolysis of bis-pNPP by the modification of the method of Dutta *et al.* (2012) in the presence of divalent metal ions (Mn^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+}) at 25^{o}C in 0.1 M Tris buffer, pH 7.5. The rate of appearance of para-nitrophenol was measured spectrophotometrically and enzyme activity was expressed as the change in absorbance per minute. Reaction mixture containing ZiPD and buffers in the presence of the appropriate ligand at specific concentrations was pre-incubated at 25^{o}C for 10 minutes. Reaction was initiated by the addition of the appropriate concentrations of the substrate for 15 minutes, and stopped by the addition of 0.5 M KOH. The absorbance was read at 405nm against a blank of the buffered substrate for bis-pNPP hydrolysis. Spectrophotometric readings were taken on a visible spectrophotometer. All measurements of reaction rate were carried out in triplicates and average was taken. Activities are expressed as change in A_{405} per minute reflecting the release of para-nitrophenol from the substrate bis-para-nitrophenyl phosphate (bis-pNPP).

Effects of Ligands on E. coli Zinc Phosphodiesterase Activity

In order to investigate the concentration dependent effects of the metal ions (Mn^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}) on the phosphodiesterase activities of ZiPD, aliquots of 20 µL of 1.25 µM ZiPD were pre- incubated with varying concentrations (0-3000 µM) of the individual ligands in 0.1 M Tris buffer at 25^{o}C for 10 minutes to ensure binding to the metal ion sites of the enzyme. Reaction was initiated by the addition of (50 µL of 5 mM) bis-pNPP to the reaction mixture for 15 minutes and stopped by the addition of 500 µL 0.5 M KOH as previously described (Dutta *et al.,* (2012).

Effect of Ligands on Kinetic Parameters of E. coli Zinc Phosphodiesterase Activity

In order to investigate the effects of the metal ions (Mn^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}) on the substrate kinetics of ZiPD, aliquots of 20 µL of 1.25 µM ZiPD were pre-incubated with specific concentrations of the ligands (1000 µM Mn^{2+} or 500 µM Co^{2+} or 500 µM Mg^{2+} or 1500 µM Zn^{2+}) in 0.1 M Tris buffer at 25°C for 10 minutes to ensure binding to the metal ion sites of the enzyme. Reaction was initiated by the addition of varying concentrations of substrate (100-2000 µM) to the reaction mixture for 15 minutes and stopped by the addition of 500 µL 0.5 M KOH as previously described (Dutta *et al.,* (2012). The data were fitted to the Michaelis-Menten curve and Lineweaver-Burk plot using Microsoft Excel and kinetic constants (V_{max} and K_m) were obtained.

Accession and Preparation of 3D Structure of Zinc Phosphodiesterase

The protein, *Escherichia coli* zinc phosphodiesterase (ZiPD) was prepared by retrieving the three-dimension crystal structure of ZiPD, (PDB ID: 2CBN) from RCSB PDB (http://www.rcsb.org/pdb/home/home.do). The bound complex molecules with the proteins were removed. The non-essential water molecules and all heteroatoms were removed using Pymol tool and Discovery studio 2017R2 respectively.

Metal Ion Binding based on Fragment Transformation Method

Metal ion binding (MIB) is a binding sites prediction server for metal ions, and this server provides an accurate, integrated method to search the residues in metal ion-binding sites by using the fragment transformation method (Chih-Hao *et al.,* 2012). Mn^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} binding to ZiPD were predicted following the MIB workflow (Figure 1)

Visualization of Molecular Docked Complexes

After the binding sites prediction of the metal ions was carried out, docking of Mn^{2+}, Co^{2+}, Mg^{2+} and Zn^{2+} was performed using the MIB server and the docked complexes were visualized using the Pymol graphical interphase.

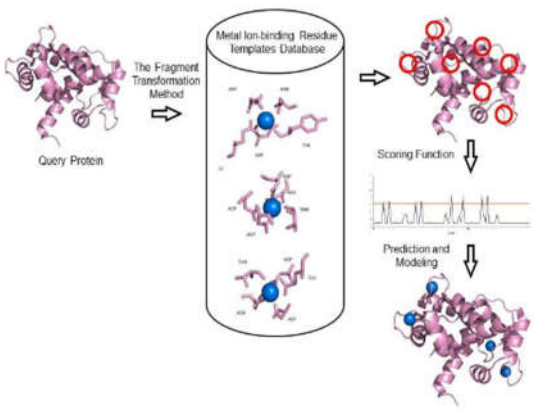


Figure 1: Metal-ion Binding workflow. This combines both structural and sequence information to identify the local structure of protein-metal interaction sites (Chih-Hao *et al.,* 2012)

3.0 Results and Discussion

Zinc phosphodiesterase has been established to be a binuclear zinc enzyme that hydrolyses phosphodiester bonds present in biomolecules; naturally occurring phosphodiesters includes nucleic acids and phospholipids. Assaying for this diesterase activity in the presence of zinc and other physiological divalent cations involves monitoring the rate at which it hydrolyzes bis-para-nitrophenyl phosphate (bis-pNPP) into para-nitrophenol.

Activation Effect and Binding Potential of Mn^{2+} on E. coli Zinc Phosphodiesterase Activity

In this study, the effect of varying concentrations of Mn^{2+} on *E. coli* ZiPD catalysed hydrolysis of bis-pNPP was investigated. Mn^{2+} increased ZiPD activity progressively from 200 µM to 1000 µM, being the optimal concentration for ZiPD activity (Figure 2). There was 3-fold increase in ZiPD activity at this optimal concentration. This optimal activity of ZiPD was sustained from 1000 µM to 2000 µM concentrations of Mn^{2+}. However, there was a decrease in the activation effect at 3000 µM Mn^{2+} (Figure 2). The Michealis-Menten curve and the Lineweaver-Burk plot for the substrate kinetics of ZiPD catalysed bis-pNPP hydrolysis both in the presence and absence of Mn^{2+} are shown in Figures 3a and 3b, respectively. Mn^{2+} at 1000 µM increased the V_{max} by 5-fold but slightly decreased the K_m (Table 1).

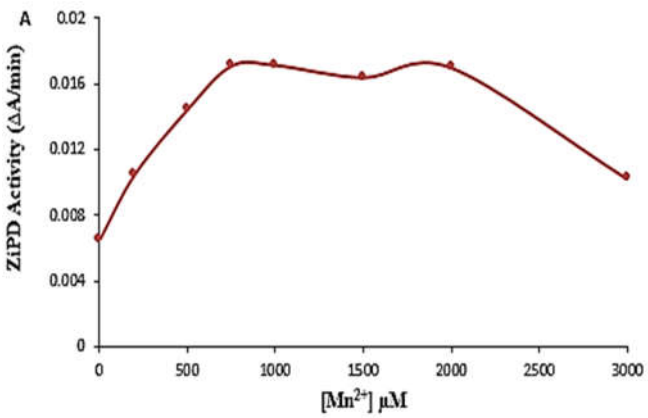


Figure 2: Activatory effect of Mn^{2+} on ZiPD catalysed hydrolysis of bis-para-nitrophenyl phosphate. Each value is a mean of three independent assays.

Furthermore, the binding affinity of each of the residues was calculated against Mn^{2+} and only residues with higher binding scores are predicted as Mn^{2+} binding residues as shown in the Figure 4a while the affinity binding of Mn^{2+} within the predicted Mn^{2+} binding sites reveals the interacting residues at optimum binding (Figure 4b).

Mn^{2+} enhances the hydrolytic activity of metalloenzymes and also participates in nucleic acid interaction (Shumilina *et al.,* 2014; Satya, 2018). The content of transition metals such as copper and manganese in ZiPD is negligible (Spath, *et al.,* 2007) but in this study, the activation effect of Mn^{2+} on ZiPD activity could be due to the increased turnover rate and enhanced binding affinity of the enzyme for bis-pNPP which is indicated by the increased V_{max} and decreased K_m, respectively. This might be due to the way Mn^{2+} binds to ZiPD by interacting with ARG

259 and GLU 260 which are present within the predicted binding sites as shown in Figure 4b. This Mn^{2+} coordinates additional water molecule that can be deprotonated by GLU-260 which in turn serves a nucleophile that attacks the phosphate group present on the substrate. Numerous amino acids have high binding potential for Mn^{2+} (Figure 4a) with Mn^{2+} having a binding affinity of 1.795, so it may be that the binding of Mn^{2+} to the enzyme at any of this amino acid residues alters its conformation that makes the enzyme to bind more tightly to the substrate which then leads to enhanced catalytic rate. This observation is in line with the report of Shumilina *et al.* (2014) that bis-pNPP hydrolysis is enhanced in the presence of Mn^{2+}. Mn^{2+} was the most effective cation stimulating ZiPD activity as observed in this study.

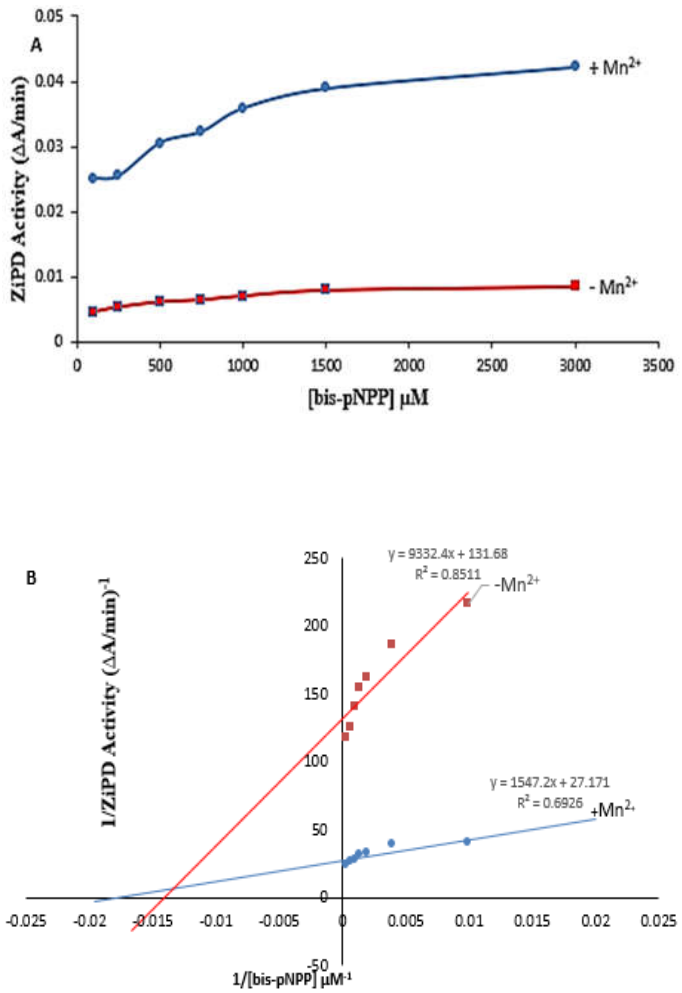


Figure 3: Substrate Kinetics of Zinc Phosphodiesterase catalysed Hydrolysis of bis-para-nitrophenyl phosphate (bis-pNPP) in the presence and absence of Manganese ion. (a) Michealis-Menten curve (b) Lineweaver-Burk plot. Each value is a mean of three independent assays.

Table 1: Kinetic parameters for *E. coli* zinc phosphodiesterase catalyzed hydrolysis of bis-para-nitrophenyl phosphate (bis-pNPP) in the absence and presence of 1 mM of Mn^{2+} ion

Reaction	V_{max} (ΔA/min)	K_m (µM)
ZiPD- bis-pNPP	0.007708	69.10
ZiPD- bis-pNPP + 1 mM Mn^{2+}	0.037730	53.65

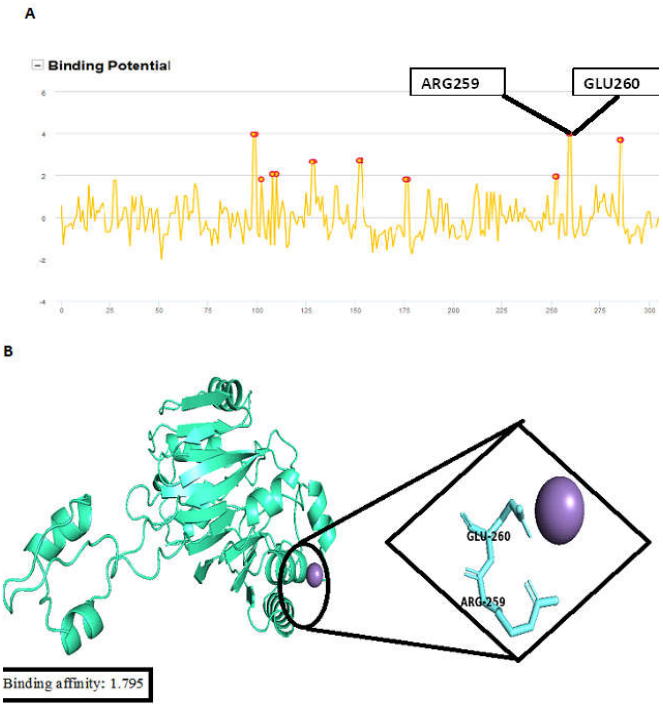


Figure 4: (a) Binding potential of each amino acid residues of ZiPD for $Mn^{2+}$and their corresponding binding affinities (b) Interacting residues within ZiPD Mn^{2+} binding site with the corresponding binding potential

Activation Effect and Binding Potential of Co^{2+} on E. coli Zinc Phosphodiesterase Activity

The effect of varying concentrations of Co^{2+} on *E. coli* zinc phosphodiesterase (ZiPD) catalysed hydrolysis of bis-(para-nitrophenyl) phosphate (bis-pNPP) was investigated. Co^{2+} increased ZiPD activity progressively from 200 µM to 500 µM, being the optimal concentration for ZiPD activity (Figure 5). There was 2-fold increase in ZiPD activity at this optimal concentration. This optimal activity of ZiPD was sustained from 500 µM to 1000 µM concentrations of Co^{2+}. However, there was a slight decrease in the activation effect from 1000 µM to 2000 µM Co^{2+} concentrations with a rise in activity at 3000 µM (Figure 5). The Michealis-Menten curve and the Lineweaver-Burk plot for the substrate kinetics of ZiPD catalysed bis-pNPP hydrolysis both in the presence and absence of Co^{2+} are shown in Figures 6a and 6b, respectively. Co^{2+} at 500 µM increased the V_{max} and K_m by 2-fold and 2.5-fold, respectively (Table 2).

As consequence of the increased activity of ZiPD in the presence of Co^{2+}, the binding affinity of each of the residues was calculated against Co^{2+} and only residues with higher binding scores are predicted as Co^{2+}

binding residues as shown in Figure 7a while the binding affinity of Co^{2+} within the predicted Co^{2+} binding sites reveals the interacting residues at optimum binding (Figure 7b).

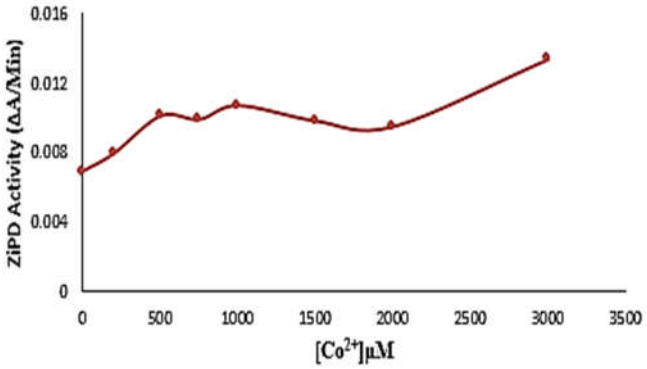
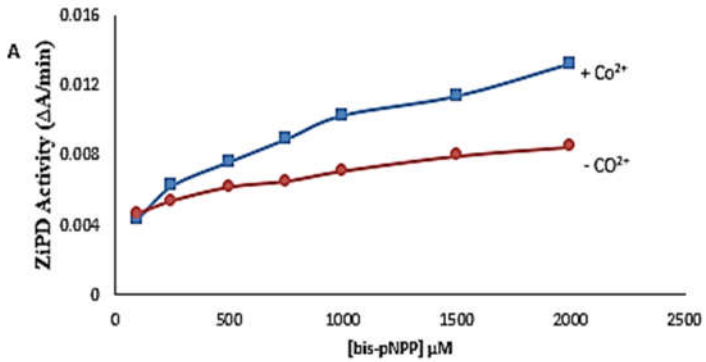


Figure 5: Activatory Effect of Co^{2+} on ZiPD catalysed hydrolysis of bis-para-nitrophenyl phosphate. Each value is a mean of three independent assays.



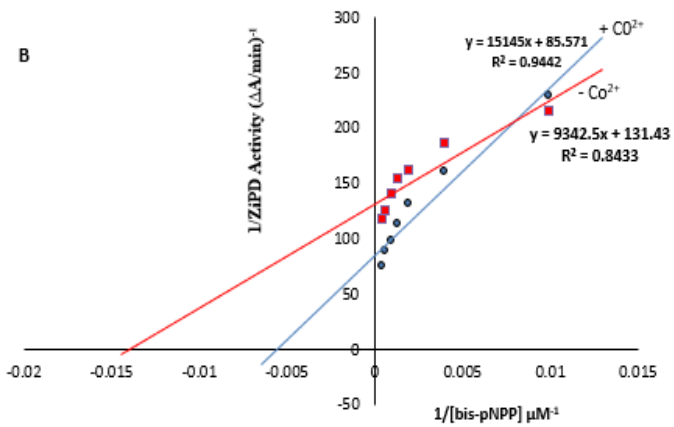


Figure 6: Substrate Kinetics of Zinc Phosphodiesterase catalysed Hydrolysis of bis-para-nitrophenyl phosphate (Bis-pNPP) in the presence and absence of Co^{2+} (a) Michealis-Menten curve (b) Lineweaver-Burk plot. Each value is a mean of three independent assays.

Table 2: Kinetic parameters for *E. coli* zinc phosphodiesterase catalyzed hydrolysis of bis-para-nitrophenyl phosphate (bis-pNPP) in the absence and presence of 0.5 mM of Co^{2+} ion

Reaction	V_{max} (ΔA/min)	K_m (µM)
ZiPD-bis-pNPP	0.007594	70.87
ZiPD-bis-pNPP + 0.5 mM Co^{2+}	0.011620	175.45

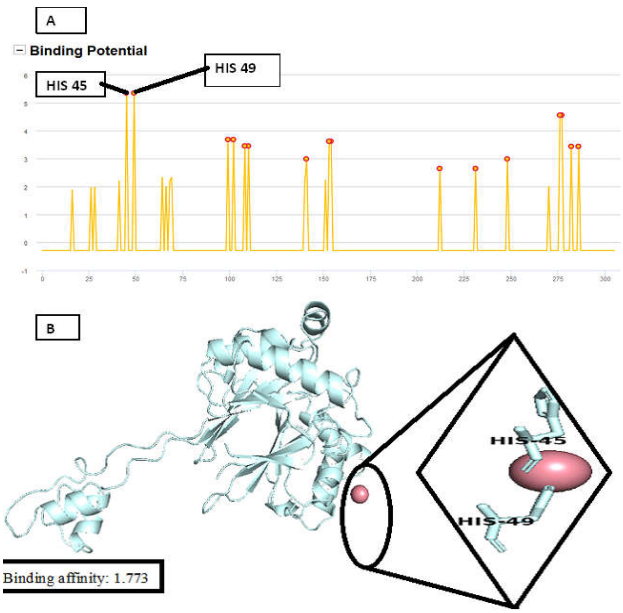


Figure 7: (a) Binding potential of each amino acid residues of ZiPD for $Co^{2+}$and their corresponding binding affinities (b) Interacting residues within ZiPD Co^{2+} binding site with the corresponding binding potential

Co^{2+} ion is found almost exclusively in B_{12}-dependent enzymes and is essential in maintaining the structure, function and stability of metalloenzymes (Jaishankar *et al.,* 2014; Singh and Verma, 2018). In this study, there was an increase in the phosphodiesterase activity of ZiPD in the presence of Co^{2+} as indicated by an increase in V_{max} (Figure 6). Less amino acid residues have potentials of binding to Co^{2+} ions but those that are capable have relatively higher binding potential (1.773); this increase might be as a result of the ability of the Co^{2+} ion to bind to sites other than the active sites which either leads to a conformational change or helps to coordinate more water molecules. This might be the reason why the presence of Co^{2+}, strongly favors the exoribonucleolytic activity of ZiPD as reported by (Dutta *et al.,* 2012). The increase in the K_m might be due to the displacement of the binuclear Zn^{2+} by Co^{2+} since its lower in the electrochemical series; this binuclear Zn^{2+} (at HIS-248 and HIS-270) is responsible for the coordination of the substrate and the cooperativity of ZiPD. Co^{2+} and Mn^{2+} ions were observed to be the most efficient in stimulating activity against bis-*p*-nitrophenyl phosphate. This corresponds to the findings of Arise *et al.* (2008) that Co^{2+} is the preferred cation, both for phosphodiesterase activity and for action on RNA.

Activation Effect and Binding Potential of Mg^{2+} on E. coli Zinc Phosphodiesterase Activity

The effect of varying concentrations of Mg^{2+} on *E. coli* zinc phosphodiesterase (ZiPD) catalysed hydrolysis of bis-para-nitrophenyl phosphate (bis-pNPP) was investigated. Mg^{2+} increased ZiPD activity progressively from 200 µM to 500 µM, being the optimal concentration for ZiPD activity (Figure 8). There was a fold increase in ZiPD activity at this optimal concentration. Furthermore, there was a progressive decrease in the activation effect from 500 µM to 1500 µM Mg^{2+} concentrations. However, there was an increased activity at 2000 µM concentration (Figure 8). The Michealis-Menten curve and the Lineweaver-Burk plot for the substrate kinetics of ZiPD catalysed bis-pNPP hydrolysis both in the presence and absence of Mg^{2+} are shown in Figures 9a and 9b, respectively. Mg^{2+} at 500 µM slightly reduced the V_{max} and also decreased the K_m (Table 3).

The binding affinity of each of the residues was calculated against Mg^{2+} and only residues with higher binding scores are predicted as Mg^{2+} binding residues as shown in the Figure 10a while the affinity binding of Mg^{2+} within the predicted Mg^{2+} binding sites reveals the interacting residues at optimum binding (Figure 10b).

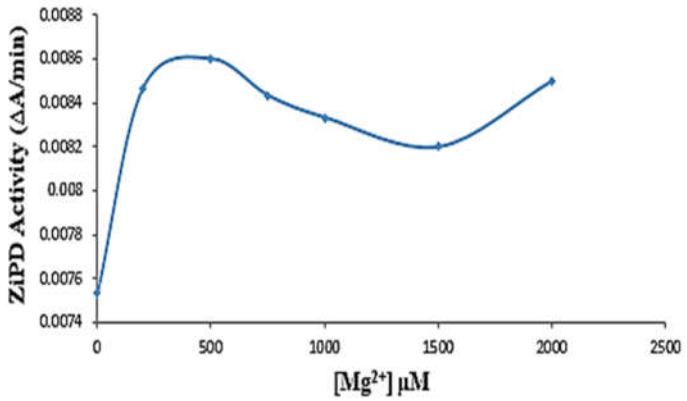
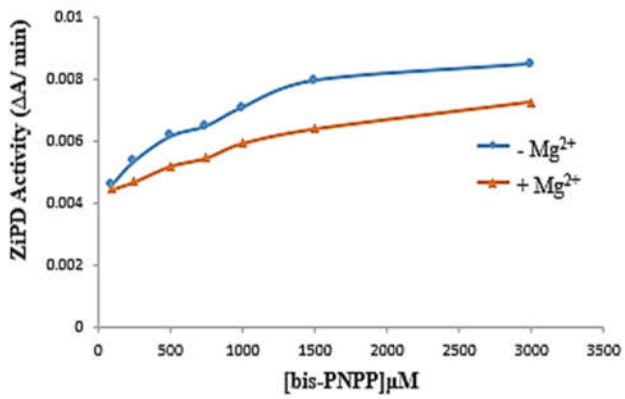


Figure 8: Activatory effect of Mg^{2+} on E. coli zinc phosphodiesterase catalysed hydrolysis of bis-para-nitrophenyl phosphate. Each value is a mean of three independent assays.

A



B

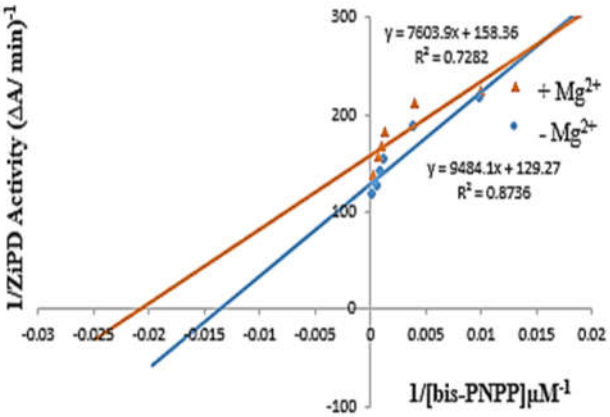


Figure 9: Substrate Kinetics of Zinc Phosphodiesterase catalysed Hydrolysis of bis-para-nitrophenyl phosphate (Bis-pNPP) in the presence and absence of Mg^{2+} (a) Michealis-Menten curve (b) Lineweaver-Burk plot. Each value is a mean of three independent assays.

Table 3: Kinetic parameters for *E. coli* zinc phosphodiesterase catalyzed hydrolysis of bis-para-nitrophenyl phosphate (bis-pNPP) in the absence and presence of 0.5 mM of Mg^{2+}

Reaction	V_{max} (ΔA/min)	K_m (µM)
ZiPD- bis-pNPP	0.007736	73.37
ZiPD- bis-pNPP + 0.5 mM Mg^{2+}	0.006315	48.02

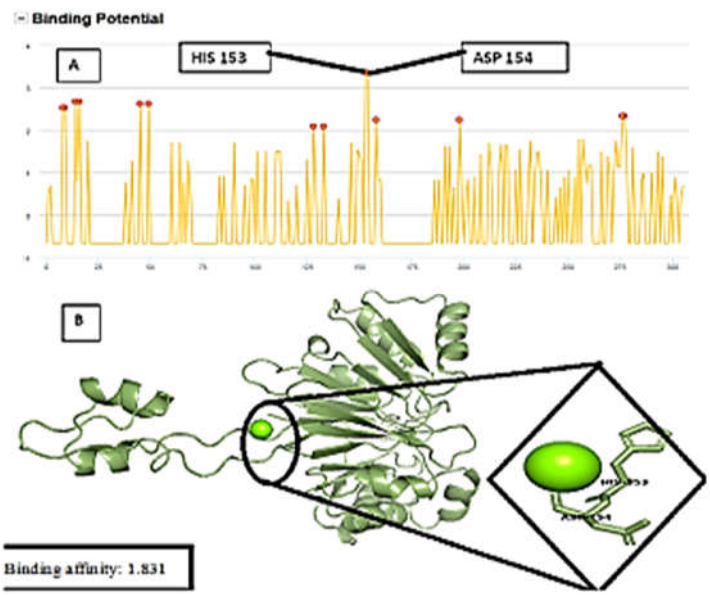


Figure 10: (a) Binding potential of each amino acid residues of ZiPD for $Mg^{2+}$and their corresponding binding affinities (b) Interacting residues within ZiPD Mg^{2+} binding site with the corresponding binding potential

Mg^{2+} is the most prevalent metal ions present in enzymes (Isani and Carpenè, 2014). This is due to the fact that most phosphate-containing substrates have magnesium as essential cofactor (Igunnu *et al.,* 2014). It has been reported that ZiPD generates approximately equal amounts of exo- and endoribonucleolytic cleavage products in the presence of Mg^{2+} (Dutta *et al.,* 2012). In this study, Mg^{2+} enhanced the affinity of ZiPD for bis-pNPP as indicated by the reduced K_m but with a slightly reduced turnover rate indicating that the Mg^{2+} compete with the binuclear zinc for the substrate. The high binding potential of numerous amino acid residues also indicate that Mg^{2+} can bind at numerous sites other than the active site with very high binding affinity which can either positively or negatively modulate the enzyme activity. The increase in the substrate affinity of ZiPD for bis-pNPP can also be a mechanism by which the enzyme accommodates larger substrates since previous study demonstrated that Mg^{2+} ion stimulates the endoribonuclease activity of ZiPD to thymidine-5´-(para-nitrophenyl) phosphate (TpNPP) substrate (Spath *et al.,* 2007). It could be that Mg^{2+} favors the activity of ZiPD on a TpNPP substrate than on a bis-pNPP used in this study.

Activation Effect and Binding Potential of Zn^{2+} on E. coli Zinc Phosphodiesterase Activity

The effect of varying concentrations of Zn^{2+} on *E. coli* zinc phosphodiesterase (ZiPD) catalysed hydrolysis of bis-pNPP was investigated. Zn^{2+} increased ZiPD activity progressively from 200 µM to 1500 µM, being the optimal concentration for ZiPD activity (Figure 11). There was 2-fold increase in ZiPD activity at this optimal concentration. However, there was a decrease in the activation effect from 1500 µM to 3000 µM Zn^{2+} (Figure 11). The Michealis-Menten curve and the Lineweaver-Burk plot for the substrate kinetics of ZiPD catalysed bis-pNPP hydrolysis both in the presence and absence of Zn^{2+} are shown in Figures 12a and 12b, respectively. Zn^{2+} at 1500 µM increased the V_{max} by 2-fold but reduced the K_m by 4-fold (Table 4).
The binding affinity of each of the residues was calculated against Zn^{2+} and only residues with higher binding scores are predicted as Zn^{2+} binding residues as shown in the Figure 13a while the affinity binding of Zn^{2+} within the predicted Zn^{2+} binding sites reveals the interacting residues at optimum binding (Figure 13b).

Zn^{2+} is the second-most abundant metal ion in enzymes (Vogel *et al.,* 2002; Arora *et al.,* 2017). Zn^{2+} is very essential to life because of its inherent role in proteins which are involved in virtually all aspects of metabolisms, genetics and growth (Yan *et al.,* 2001; Jaishankar *et al.,* 2014). In this study, the activation effect of Zn^{2+} on ZiPD activity could be due to the increased maximum velocity and reduced K_m. Zn^{2+} has the highest binding affinity (2.636) for ZiPD which is not surprising since it is naturally found in this metalloenzyme. The consequence of higher binding of Zn^{2+} in comparison to other metal ions is revealed by its molecular interactions with more amino acids residues than other metal ions. Higher Zn^{2+} from 1500 µM to 3000 µM led to a decrease in the activity of the enzyme, which was accompanied with a precipitation of the protein. It is therefore in correlation with what was reported by Spath *et al.* (2007) that the inactivation is probably due to denaturation of ZiPD rather than to an enzymatic inhibition by zinc.

Structural stability is governed by hydrophilic and hydrophobic interactions between the backbone and side chains of individual amino acids in a protein. Hydrogen bonds and Van der Waals interactions are often the largest contributors to overall protein stability, but individually, salt bridges can contribute substantial energy to protein folding and stability (Vogt and Argos, 1997; Robinson *et a*l., 2006; Feller, 2010). Salt bridge is the strongest electrostatic interactions in proteins. The salt bridge most often arises from the anionic carboxylate ($RCOO^-$) of either aspartic acid or glutamic acid (Dougherty, 2006). Previous study has shown that six mutations at two catalytic residues, Glu-11 and Asp-20, within the active site of T4 lysozyme abolished or reduced enzymatic activity but increased thermal stability by 0.7-1.7 kcal.mol-1 (Shoichet *et al*., 1995). Thus, the highest binding affinity attributed to Zn^{2+} (2.636) in relation to Co^{2+} with the least binding affinity (1.773) may be due to its interaction with ASP68 and ASP212 and not necessarily the histidine residues since Co^{2+} interacted with two histidine residues (HIS45 and HIS49) as did Zn^{2+} (HIS69 and HIS270) (Table 5). Hence, the activation effect of Zn^{2+} on ZiPD activity might be due to the increased maximum velocity and reduced K_m. Furthermore, Mhaindarkar *et al.* (2018) demonstrated that loss of salt bridges due to mutation resulted in decrease in the substrate affinity and thus lead to an overall lower catalytic efficiency.

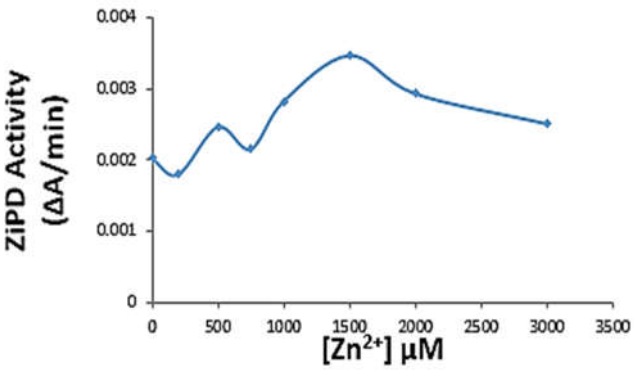
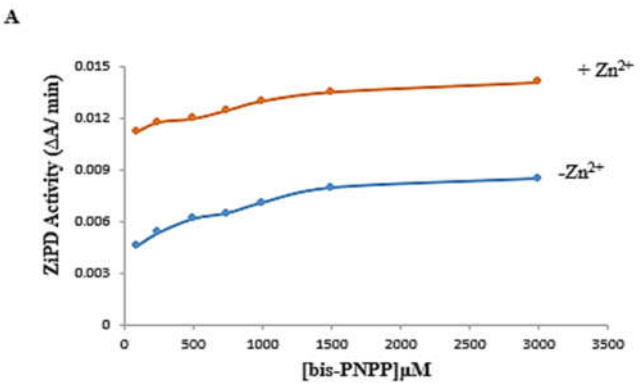


Figure 11: (a) Activation effect of Zn^{2+}on E. coli zinc phosphodiesterase catalysed hydrolysis of bis-para-nitrophenyl phosphate (Bis-pNPP). Each value is a mean of three independent assays.



B

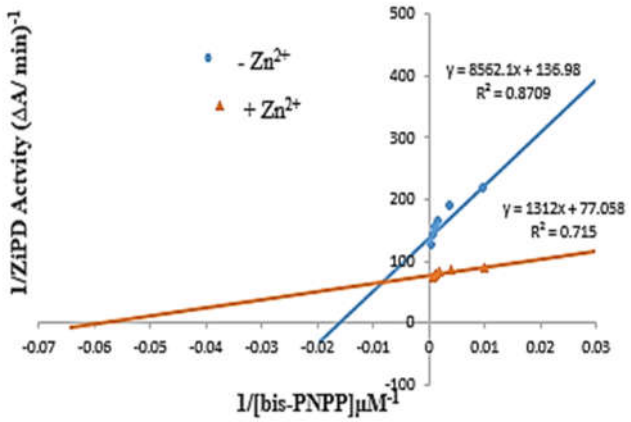


Figure 12: Substrate Kinetics of Zinc Phosphodiesterase catalysed Hydrolysis of bis-para-nitrophenyl phosphate in the presence and absence of Zn^{2+} (b) Michealis-Menten curve (c) Lineweaver-Burk plot. Each value is a mean of three independent assays.

Table 4: Kinetic parameters for *E. coli* zinc phosphodiesterase activities in the absence and presence of 1.5 mM of Zn^{2+}

Reaction	V_{max}(ΔA/min)	K_m(μM)
ZiPD- bis-pNPP	0.007700	62.50
ZiPD- bis-pNPP + 1.5 mM of Zn^{2+}	0.01298	16.13

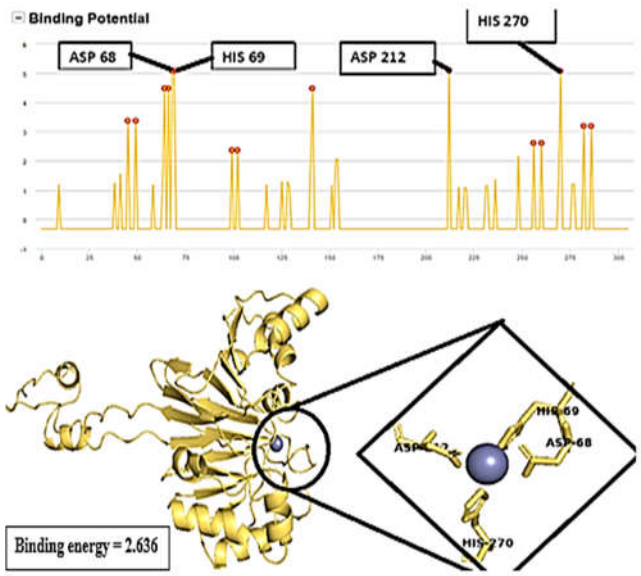


Figure 13: (A) Binding potential of each amino acid residues of ZiPD for $Zn^{2+}$and their corresponding binding affinities (B) Interacting residues within ZiPD Zn^{2+} binding site with the corresponding binding potential

Table 5: Binding affinities of the studied metal ions in relation to zinc phosphodiesterase activity

Ligand-ZiPD complex	Residues with the Highest Affinity with Ligands	Binding energy (kcal/mol)
Mn^{2+}-ZiPD	ARG259, GLU260	1.795
Co^{2+}-ZiPD	HIS45, HIS49	1.773
Mg^{2+}-ZiPD	HIS153, ASP154	1.831
Zn^{2+}-ZiPD	HIS69, HIS270, ASP68, ASP212	2.636

4. Conclusion

Our findings from this study showed that Mn^{2+} and Zn^{2+} ions are the most effective stimulatory ions of zinc phosphodiesterase with Zn^{2+} exerting the highest binding affinity for the enzyme. These findings may offer an insight into the metabolic roles of these ligands in zinc phosphodiesterase catalysis.

Conflicts of interest

The authors declare no conflict of interest.

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