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Modulatory Effects of Mg^{2+} and Zn^{2+} Ions on Monoesterase Activity of Wild-type and Mutant *E. coli* Alkaline Phosphatases

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Abstract

Metal cofactors and arginine-166 residue are active site participants in alkaline phosphatase catalysis. However, the mechanism by which the metal cofactors coordinate with arginine-166 residue during alkaline phosphatase catalysis is elusive. This study investigated the effects of Mg^{2+} and Zn^{2+} on monoesterase activity of wild-type and mutant *E. coli* alkaline phosphatases (ECAPs). The intact arginine-166 residue of wild-type ECAP was replaced by alanine and serine in the mutant ECAPs, R166A and R166S, respectively. Monoesterase activity of ECAP was measured by monitoring the rate of hydrolysis of para-nitrophenyl phosphate (pNPP). The monoesterase activity of wild-type ECAP was approximately 2-fold higher than the mutant ECAPs. Mg^{2+} (0.1-10mM) increased the activities of wild-type and mutant enzymes in a concentration-dependent manner. Zn^{2+} (0.05-0.1mM) slightly increased the activities of wild-type and mutants ECAPs. In the absence and in the presence of 10mM Mg^{2+} or 0.1mM Zn^{2+}, the maximum reaction rate of wild-type ECAP was higher than those of the mutant ECAPs while its Michaelis constant was lower than those of the mutant ECAPs. Findings in this study revealed that monoesterase activity of ECAP was greatly reduced by the loss of arginine-166 residue but its modulation by Mg^{2+} and Zn^{2+} ions was independent of arginine-166 residue.

Keywords: Alkaline phosphatase, arginine-166 residue, monoesterase activity, metal cofactors.

1. Introduction

Alkaline phosphatase (AP; EC 3.1.3.1) is a nonspecific dimeric metalloenzyme that catalyses the hydrolytic transfer of phosphate to water or its transphosphorylation to amino alcohols (Hoylaerts *et al.*, 1998). The active site of each monomer of AP contains some residues including serine and arginine, two zinc atoms, and one magnesium atom crucial to its catalytic function, and it is optimally active at alkaline pH environments (Millan, 2006).

Alkaline phosphatase is one of the best-studied enzymes that serve as a prototype for a wide

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variety of enzymes that use two metal ions to catalyse phosphoryl transfer reactions (Coleman, 1992). Previous studies on *E. coli* alkaline phosphatase (ECAP) revealed that the two zinc ions are directly involved in catalysis (Sowadski *et al.*, 1985; Kim and Wyckoff, 1991). The two active-site Zn^{2+} ions coordinate the nucleophile and the leaving group, respectively, and a non-bridging oxygen atom of the transferred phosphoryl group is coordinated between the two Zn^{2+} ions (Figure 1). A third metal ion site near the bimetallo site contains a Mg^{2+} ion, and it has been suggested that a Mg^{2+} bound hydroxide ion acts as a general base to deprotonate the serine nucleophile (Kim and Wyckoff, 1991; Zalatan *et al.*, 2008). Divalent ions such as Mg^{2+}, Co^{2+} and Mn^{2+} are activators of the AP while Zn^{2+} is a constituent metal ion (Ray *et al.*, 2017).

Alkaline phosphatases exhibit a high degree of sequence homology among species from bacteria to man with strong sequence conservation seen in regions where the catalytic residues are located (Le Du *et al*., 2001). Studies of the active site-ligand interaction are always important in investigations of enzymes.The function of arginine 166 (Arg-166) residue in the active site of ECAP has been investigated and kinetic data analysis suggests that loss of Arg-166 residue leads to decrease in monoesterase activity of ECAP (O'Brien *et al*., 2008). Previous studies have also shown that optimal concentrations of both Mg^{2+} and Zn^{2+}ions are required for AP catalysis (Olorunniji *et al*., 2007; Igunnu *et al*., 2011). Since metal cofactors (Mg^{2+} and Zn^{2+}) and Arg-166 residue are active site participants in alkaline phosphatase catalysis, it is not clear how they coordinate together to promote alkaline phosphatase catalysis. This study therefore investigated the effects of Mg^{2+} and Zn^{2+} on monoesterase activities of wild-type and mutant ECAPs.

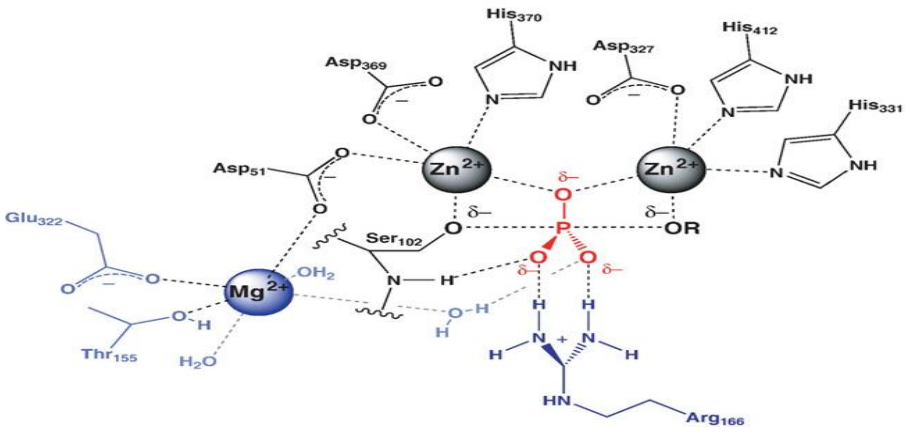

Figure 1: Active-site Schematics of *E. coli* Alkaline Phosphatase (Zalatan *et al.*, 2006).

2. Materials and Methods

Materials

Para-nitrophenyl phosphate (disodium salt) and orthovanadate (trisodium salt) were products of Sigma-Aldrich (Dorset, Poole, UK). Magnesium chloride ($MgCl_2$) and zinc chloride ($ZnCl_2$) were obtained from Fisher Scientific, UK. All other reagents used are of analytical grade. Homogenous wild-type, mutant R166A and mutant R166S *E. coli* alkaline phosphatases (ECAPs) were synthesized and purified by Dr. F. J. Olorunniji at Institute of Molecular Cell and Systems Biology, University of Glasgow, Bower Building, Glasgow G12 8QQ, Scotland, UK. The intact Arg-166 residue at the active side of wild-type ECAP was replaced by alanine and serine in the mutant ECAPs, R166A and R166S, respectively.

Procedure adopted for synthesis and purification of wild-type and mutant *E. coli* alkaline phosphatases.

The wild-type and mutant ECAP genes were chemically synthesized. Three custom-built plasmids from GeneArt each carrying different alkaline phosphatase genes namely wild-type gene (R166; with intact Arg-166 residue), mutant gene 1 (R166A; Arg-166 residue replaced with alanine) and mutant gene 2 (R166S; Arg-166 residue replaced with alanine) were made. The three plasmid DNAs were transformed into *E. coli* BL21 (DE3) pLysS and overexpressed by induction of T7 promoter system using IPTG. The expressed His-tag recombinant ECAPs were purified to homogeneity by Ni^{2+} affinity chromatography using AKTA HPLC purification machine.

Determination of time course of para-nitrophenyl phosphate hydrolysis catalysed by wild-type and mutant *E. coli* alkaline phosphatases.

The rate of hydrolysis of para-nitrophenyl phosphate (pNPP) by wild-type ECAP and mutant ECAPs (R166A and R166S) was compared using a simple time-course analysis. Reaction mixture containing 0.3 ml of 0.25 M sodium carbonate buffer buffer, pH 10.1, 1.1 ml of distilled water and 0.1 ml of enzyme (wild-type or mutant) was equilibrated in a water bath at 37°C for 10 minutes after which 1.0ml of 6mM pNPP solution was added to initiate the reaction. Thereafter, the mixture was incubated at 37°C for 2, 5, 10, 15 and 30 minutes before the reaction was stopped by the addition of 1.0 ml of 0.5 M NaOH. The absorbance was read against buffered substrate blank at 400 nm.

Effects of Mg^{2+} and Zn^{2+} on monoesterase activity of wild-type and mutant *E. coli* alkaline phosphatases.

Monoesterase activity of wild-type and mutant ECAPs was determined by measuring the rate of hydrolysis of pNPP at optimum conditions (temperature 37°C and pH 10.1). Reaction mixture containing 0.3 ml of 0.25 M $NaHCO_3/Na_2CO_3$ buffer, pH 10.1, 1.1 ml of distilled water and 0.1ml of enzyme was equilibrated in a water bath at 37°C for 10 minutes. The reaction was initiated by the simultaneous addition of varying concentrations of Mg^{2+}(0.1-10.0 mM) or Zn^{2+}(0.05-2.0 mM) solutions and 1.0 ml of 6 mM pNPP. Following incubation at 37°C for 10 minutes, 1.0 ml of 0.5 M NaOH was added to terminate the reaction. The absorbance was read against buffered substrate blank at 400 nm.

For the effect of Mg^{2+} and Zn^{2+} on substrate kinetics of pNPP hydrolyses catalysed by wild-type, mutant (R166A) and mutant (R166S)*E. coli* alkaline phosphatases, the reaction was initiated by the simultaneous addition of 10 mM Mg^{2+} or 0.1 mMZn^{2+} and varying concentrations of pNPP (0.025-2.0 mM). The maximum reaction rate (V_{max}) and Michaelis constant (K_m) values were obtained from the Lineweaver-Burk Plot. All measurements of reaction rate were performed in triplicates.

3. Result and Discussion

Comparison of monoesterase activity of wild-type and mutant *E. coli* alkaline phosphatases.

The rate of hydrolysis of para-nitrophenyl phosphate (pNPP) by the wild-type, mutant (R166A) and (R166S) *E. coli* alkaline phosphatases (ECAPs) at varying time intervals (2, 5 10, 15 and 30 minutes) were compared using a simple time course analysis. The rate of pNPP hydrolysis by wild-type enzyme was about 2-fold faster than those of the mutant enzymes within the period of 30 minutes (Figure 2).

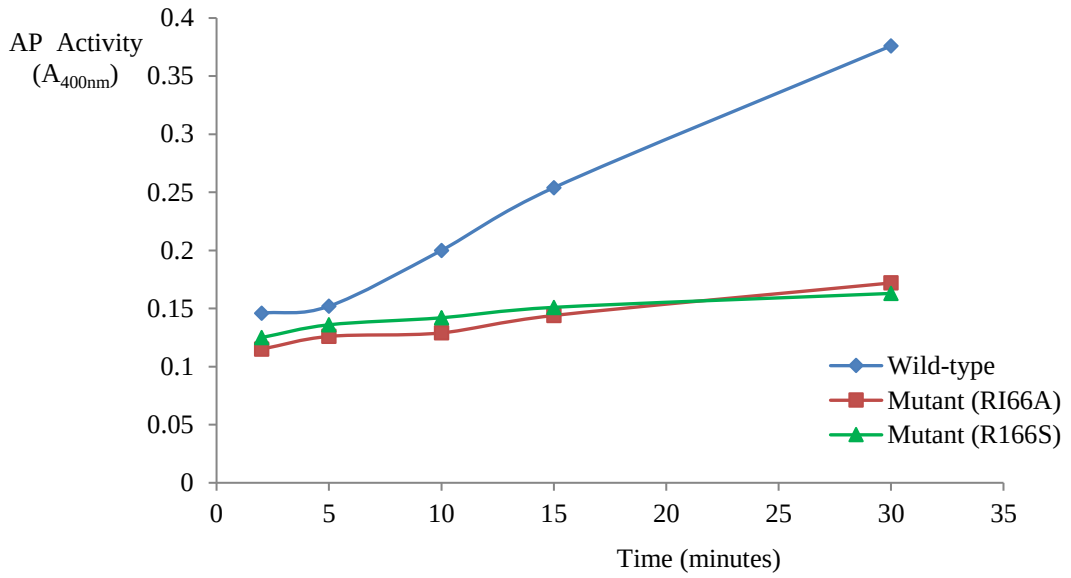

Figure 2: Time course of para-nitrophenyl phosphate hydrolysis catalysed by wild-type and mutant *E. coli* alkaline phosphatases.

It has been established that *E. coli* alkaline phosphatase (ECAP) contains three metal binding sites in which Zn^{2+} ions occupies the catalytic and structural sites, while Mg^{2+} ions are bound at the regulatory site (Borson *et al*., 1977). Occupation of a site on the enzyme molecule by a metal cofactor or modifier could induce a conformational change that can bring about activation or inhibition of the enzyme molecule (Bosron *et al.*, 1977). The catalytic role of Arg-166 in the active site of ECAP has been elucidated by site directed mutagenesis (O'Brien *et al*., 2008). In this study, the replacement of Arg-166 residue in ECAP with alanine or serine residue resulted in dramatic reduction in the rate of pNPP hydrolysis catalysed by the enzyme. This result suggests that Arg-166 residue is critical to monoesterase activity of ECAP and is in agreement with previous reports that loss of the arginine residue leads to decrease in monoesterase activity of APs (Serpersu *et al*., 1987; Zhang *et al*., 1994; O'Brien *et al*., 2008). Some other studies have also demonstrated that arginine-phosphoryl interactions are important for AP catalysis (Chaidaroglou *et al.* 1988; O'Brien *et al*., 2008).

Modulatory effects of Mg^{2+} and Zn^{2+}ions on monoesterase activity of wild-type and mutant *E. coli* alkaline phosphatases.

The effect of Mg^{2+} and Zn^{2+} on the rate of hydrolysis of pNPP catalysed by wild-type and mutant ECAPs was investigated in this study.Mg^{2+} (0.1-10 mM) increased the rate of pNPP hydrolysis catalysed by the wild-type and mutant enzymes in a concentration dependent

manner (Figure 3). Zn^{2+} slightly increased the activities of wild-type and mutants (R166A and R166S) ECAPs between 0.05mM to 0.1mM concentrations (Figure 4).

Alkaline phosphatase has been shown to be activated by Mg^{2+} (Brunel and Cathala, 1973; Arise *et al.*, 2005; Igunnu *et al.*, 2011; Ray *et al.*, 2017). Extensive evidence obtained by various enzymatic studies also indicated that while Zn^{2+} is essential for the catalytic activity of alkaline phosphatase (Kim and Wyckoff, 1991; Ray *et al.*, 2017), it can also serve as an activator for the enzyme (Igunnu *et al*., 2011). In this study, Mg^{2+} (0.1-10 mM) and Zn^{2+} (0.05-0.1 mM) activated monoesterase activity of wild-type and mutant (R166A and R166S) ECAPs. This suggests that the modulatory effects of ECAP by Mg^{2+} and Zn^{2+}ions were not influenced by the replacement of arginine residue at position 166 with alanine or serine.

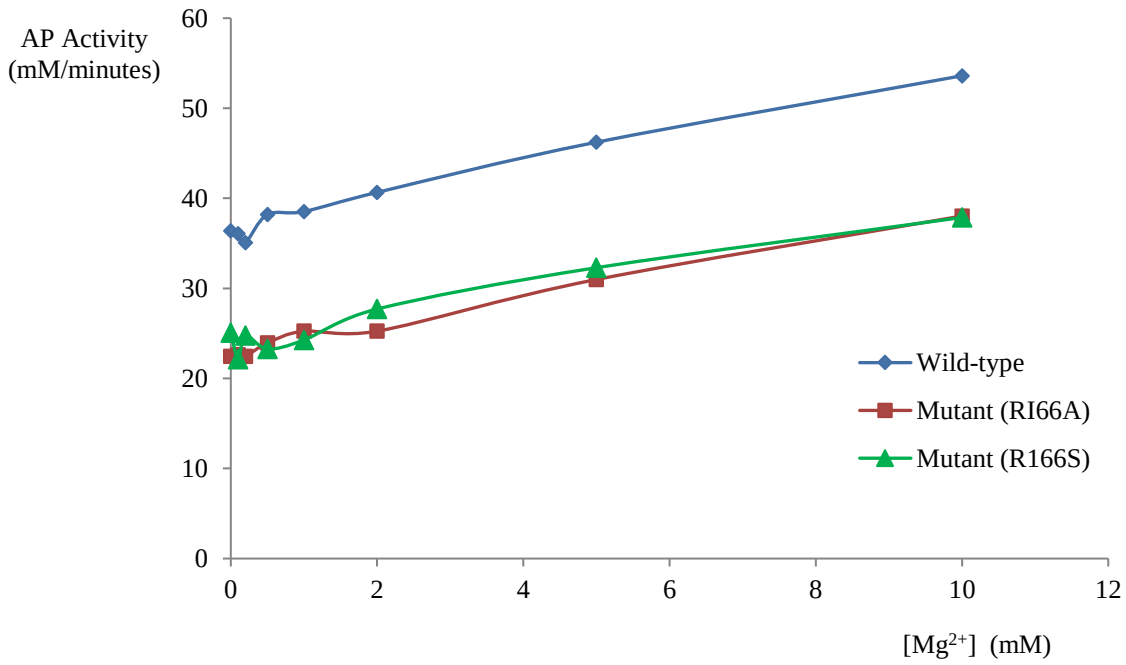

Figure 3: Hydrolysis of para-nitrophenyl phosphate catalysed by wild-type and mutant *E. coli* alkaline phosphatases in the presence of Mg^{2+} ion.

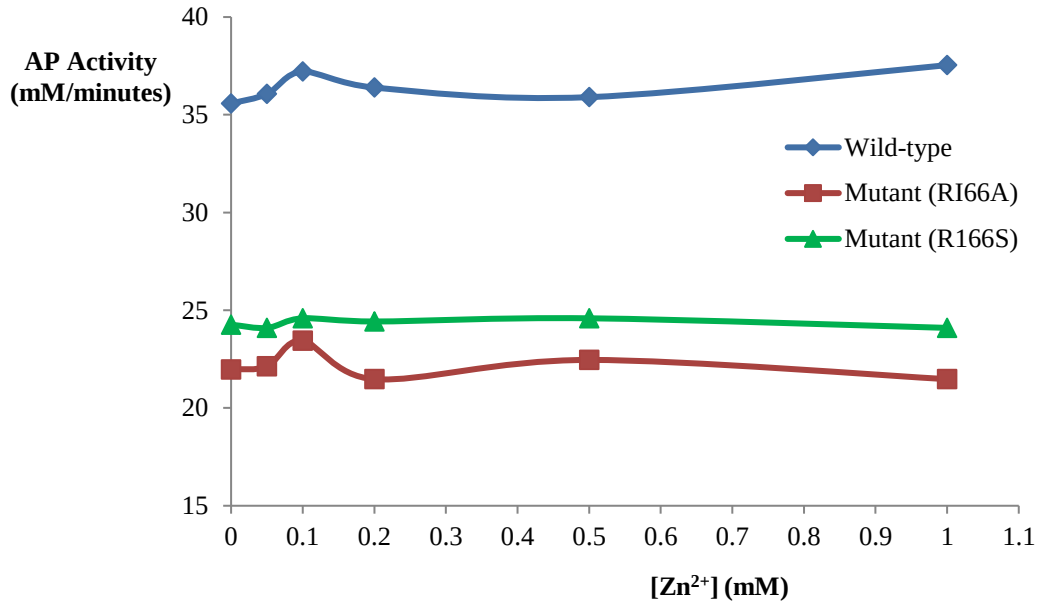

Figure 4: Hydrolysis of para-nitrophenyl phosphate catalyzed by wild-type and mutant *E. coli* alkaline phosphatases in the presence of Zn^{2+}.

Kinetic effects of Mg^{2+} and Zn^{2+} ions on monoesterase activity of wild-type and mutant *E. coli* alkaline phosphatases.

Substrate kinetics for the rate of pNPP hydrolysis catalysed by wild-type and mutants (R166A and R166S) ECAPs were investigated. Analysis of the Lineweaver-Burk plot (Figure 5) showed that maximum reaction rate (V_{max}) of the wild-type ECAP catalysed hydrolysis of pNPP was approximately 1.8-fold and 1.5-fold higher than those of R166A and R166S ECAPs, respectively (Table 1).On the other hand, the Michaelis constant (K_m) of the mutant ECAPs was approximately 3.8-fold higher than that of the wild-type ECAP (Table 1).

The effects of Mg^{2+} and Zn^{2+} ions on Substrate kinetics for the rate of pNPP hydrolysis catalysed by wild-type and mutants (R166 A and R166S) ECAPs were also investigated. Analysis of the Lineweaver-Burk plots (Figures 6 and 7) showed that in the presence of 10mM Mg^{2+}, the $V_{max}$of the wild-type ECAP catalysed hydrolysis of pNPP was approximately 1.5-fold and 1.4-fold higher than that of R166 A and R166S ECAPs, respectively while the K_m of R166A and R166S ECAPs were approximately 5.5-fold and 2.9-fold, respectively, higher than that of the wild-type ECAP (Table 1). In the presence of 0.1mM Zn^{2+}, the $V_{max}$of the wild-type ECAP catalysed hydrolysis of pNPP was approximately 1.7-fold higher than that of R166 A and R166S ECAPs while the K_m of R166A and R166S ECAPs were approximately 1.3-fold and 1.4-fold, respectively, higher than that of the wild-type ECAP (Table 1).

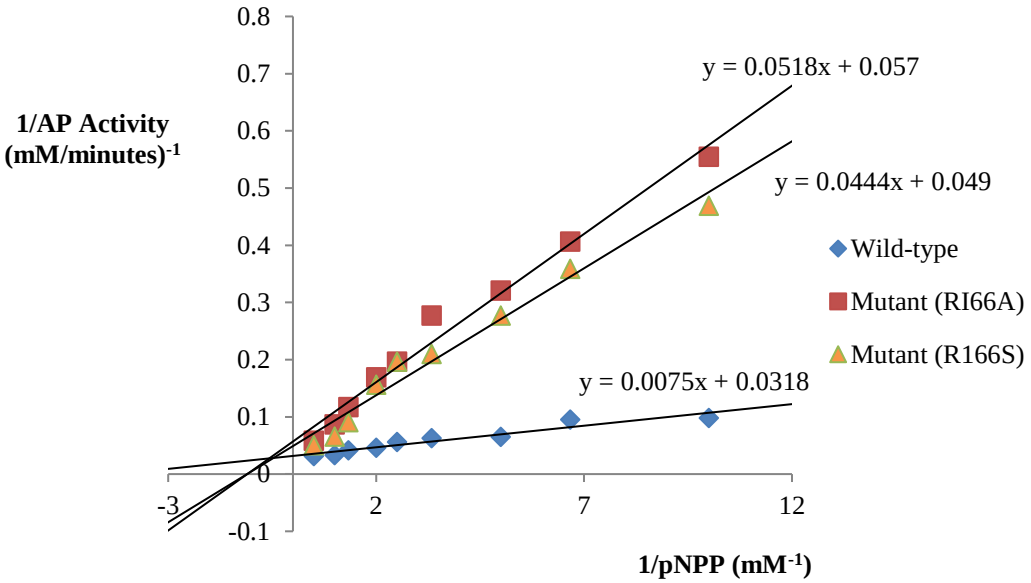

Figure 5: Lineweaver-Burk plot of para-nitrophenyl phosphate hydrolysis catalysed by wild-type and mutant *E. coli* alkaline phosphatases.

Table 1: Kinetic Parameters of para-nitrophenyl phosphate hydrolysis catalysed by wild-type and mutant *E. coli* alkaline phosphatases in the presence of $Mg^{2+}$and Zn^{2+} ions.

	Kinetic parameters	
***E. coli* alkaline phosphatase + Metal ion**	**V_{max} (mM/min)**	**K_m (mM)**
Wild-type	31.447	0.236
Mutant (R166A)	17.544	0.909
Mutant (R166S)	20.408	0.906
Wild-type + 10 mM Mg^{2+}	33.333	0.123
Mutant (R166A) + 10 mM Mg^{2+}	22.371	0.655
Mutant (R166S) + 10 mM Mg^{2+}	23.041	0.355
Wild-type + 0.1 mM Zn^{2+}	42.017	0.382
Mutant (R166A) + 0.1 mM Zn^{2+}	25.316	0.486
Mutant (R166S) + 0.1 mM Zn^{2+}	25.316	0.537

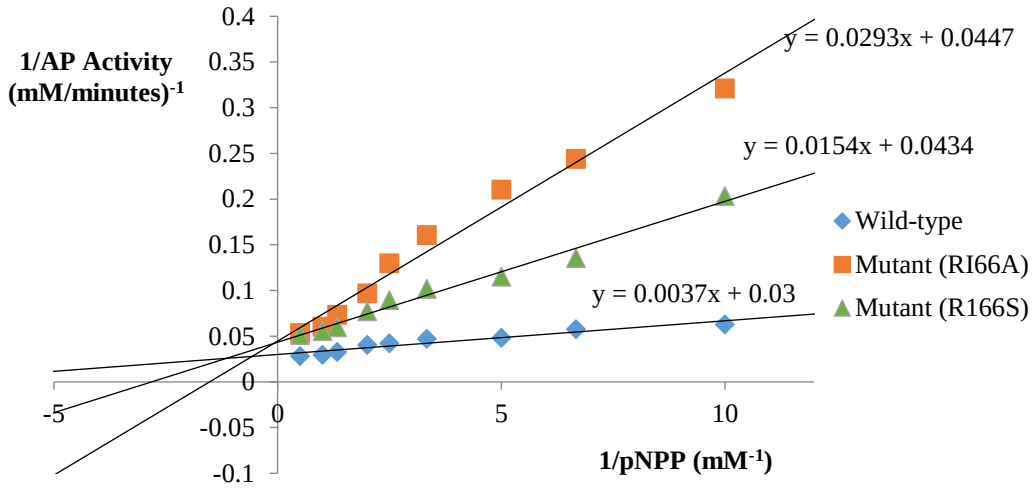

Figure 6: Lineweaver-Burk plot of para-nitrophenyl phosphate hydrolysis catalysed by wild-type and mutant *E. coli* alkaline phosphatases in the presence of 10 mM Mg^{2+} ion.

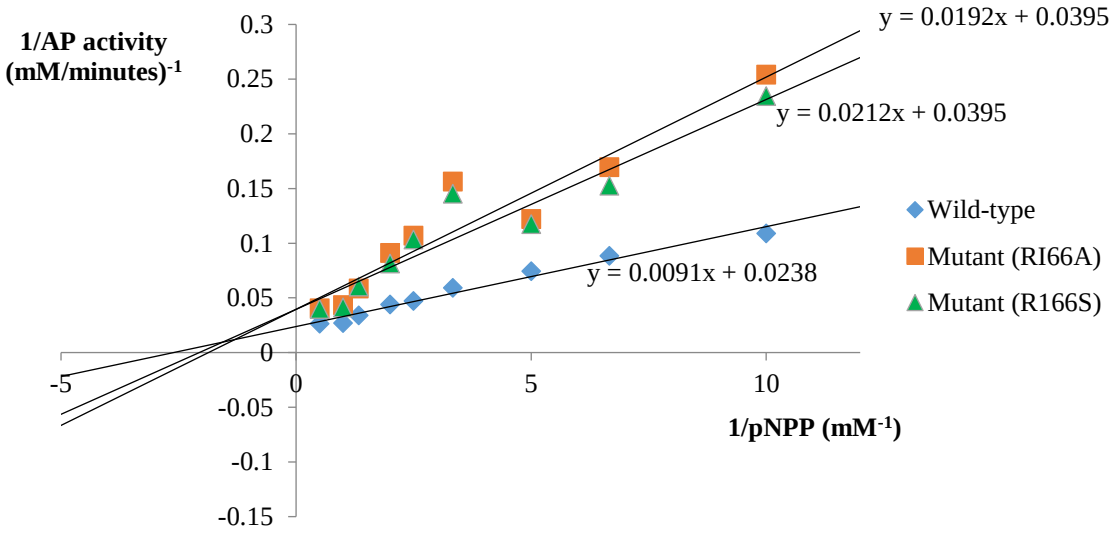

Figure 7: Lineweaver-Burk plot of para-nitrophenyl phosphate hydrolysis catalysed by wild-type and mutant *E. coli* alkaline phosphatases in the presence of 0.1 mM Zn^{2+} ion.

Kinetic analyses are useful in gaining insight into the mechanism of catalysis at the active site of the enzyme. In this study, kinetic analyses revealed that the maximum reaction rate of wild-type ECAP was higher than those of the mutant ECAPs in the absence and in the presence of optimal concentrations of metal cofactors (10mM Mg^{2+} or 0.1mM Zn^{2+}). Also, the Michaelis constant (K_m) of wild-type ECAP was lower than those of the mutant ECAPs in the absence and in the presence of 10mM Mg^{2+} or 0.1mM Zn^{2+}. The higher maximum reaction rate obtained

for wild-type ECAP catalysed hydrolysis of pNPP in the absence of exogenous Mg^{2+} or Zn^{2+} ion agrees with the earlier observations in this study and is in conformity with the report of Chaidaroglou *et al.* (1988) who showed that mutant AP enzymes with serine and alanine at position 166 have decreased turnover rate by 30-fold compared to wild-type enzyme. The decrease in the activities of mutant ECAPs may be as a result of diminished rate of hydrolysis of the covalent phosphoenzyme, the mechanism previously proposed by Chaidaroglou *et al.* (1988).The higher K_m values obtained in the mutant ECAPs suggests that the reduction in the activity of the enzymes was due to their decreased binding affinity with pNPP. This shows that the replacement of Arg-166 residue in ECAP with alanine or serine resulted in the decreased binding affinity of the enzyme with the substrate (pNPP). The results of this study further show that activatory effect of Mg^{2+} and Zn^{2+} did not alter the disparity between the kinetic properties of wild-type and mutant ECAPS.

4. Conclusion

Findings in this study revealed that monoesterase activity of ECAP was greatly reduced by the loss of arginine-166 residue but its modulation by Mg^{2+} and Zn^{2+} ions was independent of arginine-166 residue.

Acknowledgement

The authors wish to thank Dr. F. J. Olorunniji of the School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, UK for the expression and purification of wild-type and mutant (R166 A and R166S) *E. coli* alkaline phosphatases while he was at Institute of Molecular Cell and Systems Biology, University of Glasgow, Bower Building, Glasgow G12 8QQ, Scotland, UK.

References

Arise, R. O., Bolaji, F. F., Jimoh, O. A., Adebayo, J. O., Olorunniji, F. J. and Malomo, S. O. (2005): Regulatory effect of divalent cations on rat liver alkaline phosphatase activity: How Mg^{2+} activates (and inhibits) the hydrolysis of p-nitrophenylphosphate. *Biokemistri*. **17**, 129-136.

Bosron, W. F., Anderson, R. A., Falk, M. C., Kennedy, F. S. and Vallee, B. L. (1977): Effect of Magnesium on the properties of Zinc alkaline phosphatase. *Biochemistry.* **16**, 610-614.

Brunel, C. and Cathala, G. (1973): Activation and inhibition processes of alkaline phosphatase from bovine brain by metal ions (Mg^{2+} and Zn^{2+}). *Biochimica et Biophysica Acta (BBA)-Enzymology*. **309**, 104-115.

Chaidaroglou, A., Brezinski, D. J., Middleton, S. A. and Kantrowitz, E. R. (1988): Function of arginine 166 in the active site of *Escherichia coli* alkaline phosphatase. *Biochemistry.* **27**, 8338–8343.

Coleman, J. E. (1992): Structure and mechanism of alkaline phosphatase. *Annual Review of Biophysics and Biomolecular Structure*. **21**, 441–483.

Hoylaerts, M. F., Manes, T. and Millan, J. L. (1998): Molecular mechanism of uncompetitive inhibition of human placental and germ cell alkaline phosphatase. *Biochemical* Journal. **286**, 23-30.

Igunnu, A, Osalaye, D. S., Olorunsogo, O. O., Malomo, S. O. and Olorunniji F. J. (2011): Distinct metal ion requirements for the phosphomonoesterase and phosphodiesterase activities of calf intestinal alkaline phosphatase. *The Open Biochemistry Journal*. **5**, 45-50.

Kim, E. E. and Wyckoff, H. W. (1991): Reaction mechanism of alkaline phosphatases based on crystal structures: Two-metal ion catalysis. *Journal of Molecular Biology.* **218**, 449-464.

Le Du, M. H., Stigbrand, T., Taussig, M. J. and Stura, E. A. (2001): Crystal structure of alkaline phosphatase from human placenta at 1.8A resolution. Implication for a substratespecificity. *Journal of Biological Chemistry*. **276**, 9158-9165.

Millan, J. L. (2006): Alkaline Phosphatases, structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. *Purinergic Signalling.* **2**, 335-341.

O'Brien, P. J., Lassila, K. J., Fenn, T. D., Zalatan, J. G. and Herschlag, D. (2008): Arginine coordination in enzymatic phosphoryl transfer: Evaluation of the effect of Arg166 mutations in *Escherichia coli* alkaline phosphatase. *Biochemistry*. **47**, 7663-7672.

Olorunniji, F. J., Igunnu, A., Adebayo, J. O., Arise, R. O. and Malomo, S. O. (2007): Cofactor interaction in the activation of tissue non-specific Alkaline phosphatase: Synergistic effects of Zn^{2+} and Mg^{2+} ions. *Biokemistri*. **19**, 43-48.

Ray, C. S., Singh, B., Jena, I., Behera, S. and Ray, S. (2017). Low Alkaline Phosphatase (ALP) In Adult Population an Indicator of Zinc (Zn) and Magnesium (Mg) Deficiency. *Current Research in Nutrition and Food Science*. **5**(3), 347-352.

Serpersu, E. H., Shortle, D. and Mildvan, A. S. (1987): Kinetic and magnetic resonance studies of active-site mutants of staphylococcal nuclease: Factors contributing to catalysis. *Biochemistry*. **26**, 1289–1300.

Sowadski, J. M., Handschumacher, M. D., Murthy, H. M. K., Foster, B. A. and Wyckoff, H. W. (1985): Refined structure of alkaline phosphatase from *Escherichia coli* at 2.8Å resolution. *Journal of Molecular Biology*. **186**, 417-433.

Zhang, Z. Y., Wang, Y., Wu, L., Fauman, E. B., Stuckey, J. A., Schubert, H. L., Saper, M. A. and Dixon, J. E. (1994): The Cys (X) 5 Arg catalytic motif in phosphoester hydrolysis. *Biochemistry*. **33**, 15266–15270.

Zalatan, J. G., Fenn, T. D. and Hersclag, D. (2008): Comparative enzymology in the alkaline phosphatase superfamily to determine the catalytic role of an active site metal ion. *Journal of Molecular Biology*. **384**, 1174-1189.

Zalatan, J. G., Fenn, T. D., Brunger, A. T. and Herschlag, D. (2006): Structural and functional comparisons of nucleotide pyrophosphatase/phosphodiesterase and alkaline phosphatase: implications for mechanism and evolution. *Biochemistry*. **45**, 9788–9803.