Distinct Metal Ion Requirements for the Phosphomonoesterase and Phosphodiesterase Activities of Calf Intestinal Alkaline Phosphatase

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Abstract: The roles of Mg^{2+} and Zn^{2+} ions in promoting phosphoryl transfer catalysed by alkaline phosphatase are yet to be fully characterised. We investigated the divalent metal ion requirements for the monoesterase and diesterase activities of calf intestinal alkaline phosphatase. The synergistic effect of Mg^{2+} and Zn^{2+} in promoting the hydrolysis of para-nitrophenyl phosphate (monoesterase reaction) by alkaline phosphatase is not observed in the hydrolysis of the diesterase substrate, bis-para-nitrophenyl phosphate. Indeed, the diesterase reaction is inhibited by concentrations of Mg^{2+} that were optimal for the monoesterase reaction. This study reveals that the substrate specificities of alkaline phosphatases and related bimetalloenzymes are subject to regulation by changes in the nature and availability of cofactors, and the different cofactor requirements of the monoesterase and diesterase reactions of mammalian alkaline phosphatases could have significance for the biological functions of the enzymes.

Keywords: Alkaline phosphatase, phosphodiester hydrolysis, metal ion cofactors.

INTRODUCTION

Alkaline phosphatases (APs) are dimeric metalloenzymes (Fig. **1**) that catalyse the hydrolytic transfer of phosphate to water or its transphosphorylation to amino alcohols, but when separated the monomeric subunits fail to display enzyme activity [1]. Each monomeric subunit of AP contains three divalent cations (two Zn^{2+} and one Mg^{2+}) and a serine residue in the active site [2]. Previous studies on *E. coli* AP (ECAP) have shown that the two zinc ions are directly involved in catalysis (Fig. **2A**) [2-4]. The two zinc ions are well positioned to activate the serine and water for nucleophilic attacks and they are involved in holding the phosphate moiety of substrate [2]. The Zn^{2+} ion at the first metal ion binding site (M1 site) is required for catalysis and plays an important role in binding both the substrate and the phosphate released upon hydrolysis (Fig. **2A**). The second Zn^{2+} ion interacts with the hydroxyl group of the active site serine to stabilize the deprotonated form of the residue required for the nucleophilic attack on the phosphate [2]. Recent evidence suggests that the Mg^{2+} ion in the active site stabilizes the transferred phosphoryl group *via* a water molecule (Fig. **2A**), and functions *via* a mechanism different from the two Zn^{2+} ions at the bimetallocentre [5].

APs have been classified into a superfamily of phospho-/sulfo-coordinating enzymes catalyzing the hydrolysis of phosphate monoesters, diesters, triesters, and sulfate esters [6]. The phosphodiesterase activity of AP has been demonstrated in the enzyme from rat osseous plate [7] and *E. coli* [6]. Mutational analysis suggests that common active site features contribute to hydrolysis of both phosphate monoesters and phosphate diesters. However mutation of the active site arginine to serine, R166S, decreases the phosphomonoesterase activity but not the diesterase activity, suggesting that interaction of this arginine with the nonbridging oxygen(s) of the phosphate monoester substrate is responsible for the preferential hydrolysis of phosphate monoesters [6].

Two-metal ion catalysis is a common feature of enzymes that catalyze phosphoryl transfer reactions including the hydrolysis of phosphate monoesters, diesters and triesters [8, 9]. The active sites of members of the alkaline phosphatase superfamily share several structural features underscored by the two-metal catalytic centre. Comparative analyses of the monoesterase and diesterase activities of APs are providing new insights into their catalytic mechanism [5, 7, 10].

The structural similarity shared by the active sites of related mono- and diesterases raises the possibility that any bimetallo active site might be able to catalyze these different classes of reactions. However, the partial negative charge on a phosphoryl oxygen atom of the phosphate monoester dianion is lost upon conversion to a diester monoanion, and the steric bulk of the added esterifying group of the diester could result in steric clashes within the active site [6].

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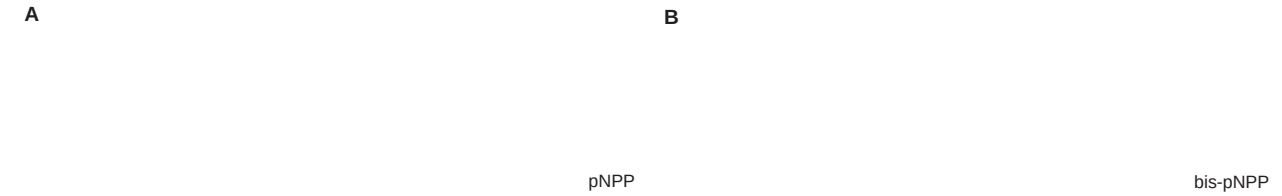

Fig. (5). Effects of Mg^{2+} on Zn^{2+}-activated hydrolysis of pNPP and bis-pNPP by CIAP. Reaction mixtures (200 µl) containing 25 mM Tris-HCl (pH 8.5), 2.5 mM p-nitrophenyl phosphate (**A**), or bis-p-nitrophenyl phosphate (**B**), 2 mM or 4 mM $ZnCl_2$ plus the indicated concentration of $MgCl_2$ (0-10 mM), and 10 µM CIAP were incubated at 37 ^oC for 10 minutes. Reactions were initiated by the addition of the substrate to the reaction mixture and stopped by the addition of 1.0 ml 0.5 M NaOH. Activities are expressed as change in A_{410} per minute for phosphomonoesterase activity and change in A_{410} per hour for the phosphodiesterase activity.

mechanistic differences have been reported between the monoesterase and diesterase functionalities of ECAP. O'Brien *et al.*, [17] reported that the R166S mutation in ECAP specifically inhibited monoesterase activity without affecting the hydrolysis of diester substrates, suggesting that the residue is important for binding and positioning of monoester substrates. The lack of effect of the R166S mutation on diester hydrolysis shows that this interaction does not contribute to the reaction of phosphate diesters, presumably because the introduction of steric bulk caused by esterification of a non-bridging oxygen of the transferred phosphoryl group prevents this interaction with the phosphate diester substrate [6].

Zalatan *et al.*, [5] showed that removal of the Mg^{2+} site in ECAP had no significant effect on the diesterase reaction, while causing a significant decrease in monoesterase activity. Structural analysis and comparative studies with sulphate monoester substrates show that the R' group of diester substrates is oriented away from the Mg^{2+} site in AP (Fig. **2B**). Hence, the active site Mg^{2+} has little or no contribution to rate acceleration of diester hydrolysis, in contrast to the monoesterase reaction that depends on Mg^{2+} in transition state stabilisation (Fig. **2A**). It follows that catalysis of the diesterase reaction relies more on interactions with the bimetallocentre occupied by the two Zn^{2+} ions. This unique property of diesterase catalysis by AP might explain the inhibitory effect of Mg^{2+} if it displaces Zn^{2+} from the bimetallocentre [15]. In a comparative analysis of the hydrolysis of several diester substrates by ECAP-R166S, Nikolic-Hughes *et al.*, [18] showed that rate enhancements correlated with the amount of negative charge localised between the two Zn^{2+} ions in the active site. Hence, the electrostatic features of the active site can be tuned to favour monoesterase or diesterase reactions when the coordinating properties of the bimetallocentres are altered. Such electrostatic contributions to the catalysis of diester hydrolysis by CIAP would be perturbed by the introduction of Mg^{2+} into one or both of the Zn^{2+} catalytic sites.

The synergistic effect of Mg^{2+} and Zn^{2+} in the activation of phosphomonoesterase activity of AP observed in this study confirmed our earlier report of the synergistic effects of Zn^{2+} and Mg^{2+} ions in the activation of TNAP [14, 15]. These and related findings [19, 20] suggest that the synergistic effect of the two cofactors is a recurrent theme of the two-metal ion mechanism of the alkaline phosphatases. However, the effect of interaction of Mg^{2+} and Zn^{2+} in the hydrolysis of bis-pNPP by CIAP as observed here did not show any synergistic effect though each metal ion in the absence of the other activates the enzyme. This would not be surprising if Mg^{2+} makes little contribution to rate enhancement of diester hydrolysis [5], in contrast to its significant impact in hydrolysis of phosphomonoester by alkaline phosphatase [15]. Activation of phosphodiesterase activity of CIAP by Mg^{2+} was limited, Zn^{2+} being the preferred metal ion for the hydrolysis of bis-pNPP. Interestingly, Mg^{2+} seems to displace Zn^{2+} from the enzyme and significantly inhibits the diesterase reaction. Keppetipola and Shuman [21] described mutants of a polynucleotide kinase/phosphatase from *Clostridium temocellum* with different cofactor requirements for its monoesterase and diesterase activities.

The alkaline phosphatase superfamily of hydrolase enzymes exhibits a high degree of substrate flexibility. This broad specificity means that the enzymes could be engineered to act with different affinities on a variety of phosphate esters. This can be achieved *via* random or directed mutagenesis of active site residues. The findings from this study offer the prospect of altering the substrate specificities of alkaline phosphatases and related bimetalloenzymes through changes in reaction cofactors in addition to efforts resulting from active site engineering.

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CONFLICT OF INTEREST

None declared.

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