The Role of the Metal Ion Coordination Sites in Catalyzing the *Escherichia coli* Alkaline Phosphatase Reaction

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Abstract

Metallic ion cofactors are commonly used by enzymes to carry out reactions. Amino acid residues in enzymes create a metal ion binding site for the metallic cofactors to help the enzyme catalyze its specific reaction. This review examines the role of metallic ion binding sites and the function of the metal ions in Escherichia coli alkaline phosphatase, an enzyme that recruits three metal ions to hydrolyze a non-specific monophosphate ester. Two of the metal ion sites bind zinc, and are crucial for the catalysis of this reaction, as they are directly involved in the mechanism of this reaction. The third metal site binds magnesium in E. coli, however, this ion is not directly involved in the mechanism and thus, participates in the reaction by activating a nucleophile and stabilizing the monophosphate ester in the active site. Comparison of E. coli alkaline phosphatase to other members of the alkaline phosphatase family of different species reveals that the two zinc ion metal binding sites are highly conserved, but the magnesium ion binding site varies with the adaptation of the enzyme to different environments.

Introduction

Metal ion cofactors have been seen to be conserved between enzymes that catalyze similar reactions. Despite these similarities, minute differences in the metal ion binding sites contribute to variation in the overall structure, specificity, and structure in related enzymes.[1] In this review, we examine the roles of the metal ion binding sites of *Escherichia coli* alkaline phosphatase and other members of the alkaline phosphatase family. Understanding the role of each metal ion binding site within the active site of alkaline phosphatase can inform us about how *E. coli* has optimized the structure of alkaline phosphatase to perform its function. On a larger scale, this can inform us about the role of metal ion binding in enzymatic activity, as well as the evolutionary adaptations of alkaline phosphatase in a variety of species living in various environments.

E. coli alkaline phosphatase is one of the most widely studied members of the alkaline phosphatase family, which catalyzes the hydrolysis of a phosphate monoester.[1,2] It is a homodimer containing two active sites that catalyze a non-specific hydrolysis of phosphoryl groups from a variety of monophosphate esters in basic (pH=8.0) conditions.[2,3] Within the active site of each monomer, a phosphate substrate is bound with coordination from metal ions (Figure 1a). There are three metal ion binding sites in the active site: M1, M2, and M3. M1 and M2 are occupied by zinc ions, while M3 is occupied by a magnesium ion.[2] These sites help to position the phosphate substrate before hydrolysis occurs.[4] Catalysis of the reaction requires M1 site occupancy, although M1 site occupancy also greatly enhances M2 site occupancy.[5] While the magnesium ion in the M3 binding site is not directly involved in catalysis, it is important for the structural stability of the transition state.[4] The proposed and generally accepted mechanism for catalyzing hydrolysis of the

phosphate ester shows that both zinc ions at the M1 and M2 site are directly involved in stabilizing the substrate, as well as in catalysis (Figure 1b).[6] The magnesium ion in the M3 site has been found to be important for the enzyme, as it stabilizes the active form of the enzyme. The high activity and high affinity form of alkaline phosphatase does not exist without the bound magnesium ion in that site.[4]

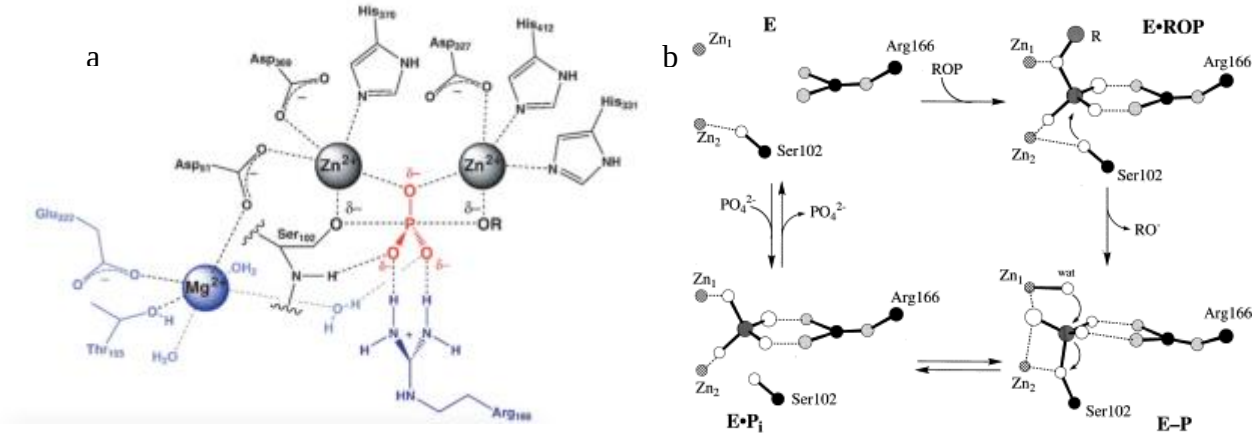


Figure 1. (a) Schematic diagram of the one of the active sites of *E. coli* alkaline phosphatase. Each metal ion is coordinated to multiple amino acid residues that create a binding site. The two zinc ions are directly involved in coordinating the phosphate in the active site.[5] (b) Proposed mechanism for the mechanism of alkaline phosphatase. After the substrate (ROP) binds, the zinc ions stabilize it within the active site. As the hydrolysis occurs through a ping-pong mechanism, the zinc ions, especially Zn_1, help the reaction proceed. At alkaline pH, the rate limiting step of the reaction is the release of the hydrolyzed substrate, while the rate limiting step at acidic pH is the binding of the substrate.[6]

Other members of the alkaline phosphatase family also catalyze similar reactions with monophosphate esters, however, some alkaline phosphatases have different metal binding characteristics than *E. coli* alkaline phosphatase. For example, mammalian and yeast alkaline phosphatases can be activated by high magnesium concentration, while E. coli alkaline phosphatase cannot.[7] Some require cobalt ions in place of magnesium (*Thermotoga maritima* alkaline phosphatase). Antarctic bacterium strain TAB5 alkaline phosphatase recruits the same three metal ions as E. coli alkaline phosphatase, yet the TAB5 alkaline phosphatase is more adapted to cold temperatures.[8] In some alkaline phosphatases, such as *Sphignomonas sp.* strain BSAR-1 alkaline phosphatase, the M3 metal binding site is removed altogether.[9] Despite the differences between members of the alkaline phosphatase family, all alkaline phosphatases still have the crucial zinc ion binding sites for the substrate. [1,7,8,9]

We will examine the role for each metal ion binding site within the active site of *E. coli* alkaline phosphatase (ECAP). The amino acids involved in binding each metal ion and how they affect the metal binding site will be discussed, as well as how each metal ion contributes to the function of alkaline phosphatase. Understanding the role of metal ion binding sites in ECAP will inform us about how metallic cofactors contribute to the overall structure, function, and specificity

of the enzyme. We will also understand better how differences between enzymes of the same family can lead to evolutionary adaptations and changes of the enzyme.

Discussion

Zn^{2+} in the M1 Metal Ion Binding Site is Required for Catalysis

The M1 metal ion binding site binds a Zn^{2+} ion under physiological conditions at pH=8.0; this Zn ion is known as Zn_1. In *E. coli*, Zn_1 is penta-coordinated by both carboxyl oxygens of Asp327, the imidazole nitrogens of His412 and His331, and an oxygen on the phosphate ligand (Figure 2).[3] The position of Zn_1 allows it to coordinate the negatively charged alkoxide leaving group before the first step of the mechanism, and allows it to activate a water molecule (hydroxide at high pH) in the second step of the reaction.[6]

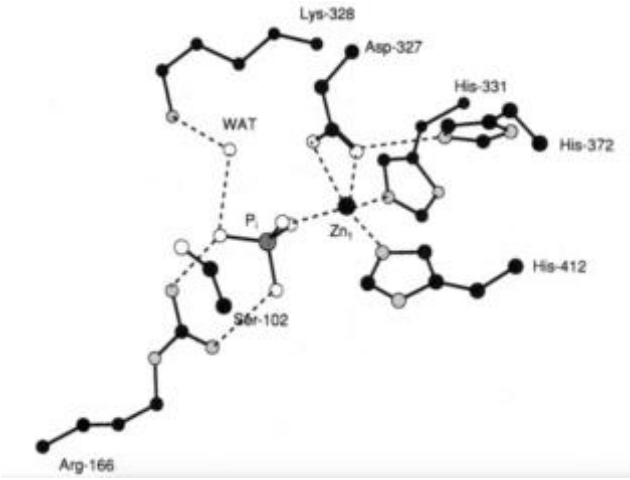


Figure 2. Schematic of the M1 binding site.

Binding of Zn^{+2} at the M1 site is required for the hydrolysis of monophosphate esters. The aspartate residue at position 327 is especially important for the coordination of the Zn_1 ion in M1, as both of the carboxyl oxygens contribute to zinc binding in this site. Site-specific mutagenesis of Asp327 to Ala327 (D327A) revealed that removal of the carboxyl oxygens resulted in extremely low hydrolysis activity compared to the wild type enzyme. Even at high zinc concentrations, catalytic activity is not enhanced, suggesting that zinc does not coordinate to Ala327, thus rendering catalytic activity nonexistent. Without the zinc ion to coordinate phosphate binding and the hydroxide attack, there is no catalytic activity. The D327N mutant (Figure 3a) exhibits more catalytic activity than the D327A mutant, however, the enzymatic activity is much still much lower than wild type levels, suggesting Zn_1 binding is greater in the D327N mutant than D327A. This implies that only one carboxyl in the wild type enzyme is essential for binding Zn_1, but the other carboxyl of Asp327 is also enhances Zn^{2+} binding at the M1 site. Therefore, Zn^{2+} binding at the M1 site is crucial for catalytic activity, and it plays a large role in phosphate binding.[10]

The requirement of Zn_1 binding for sufficient catalysis is also suggested by examination of the H412N mutant. Comparison of the x-ray crystal structures of H412N mutant to wild type alkaline phosphatase showed that Asn412 did not create a well-defined M1 binding site, and as a result, Zn_1 was not bound. The rate of catalysis of this mutant was almost nonexistent, as Zn_1 was absent and unable to coordinate the phosphate substrate, as well as activate a water molecule in the second step of the reaction. However, at a higher concentration of zinc, the rate of hydrolysis by the H412N mutant improved, showing that this mutant has a strong zinc dependence and implying that the higher activity of the H412N mutant is due to increased Zn_1 binding, which also increases substrate binding. The crystal structure of the mutant lacking Zn_1 also showed the phosphate ligand was being held loosely in its place by Arg166 and His412 and was no longer

coordinated to Zn_2, implying that phosphate binding is affected by the shift in Zn_1, which correlates with the low level of hydrolysis in the H412N mutant. Therefore, from the studies of the H412N mutant, we can conclude that Zn1 is required for catalysis, through its important role of coordinating the phosphate ligand in its active site.[11]

As the H412N mutant did not have a suitable binding site for conditions suitable for the wild type enzyme, further studies led to the examination of the H412Q mutant, which may coordinate Zn_1 better due to the longer side chain (Figure 3b). While zinc binding at M1 was improved in H412Q as compared to H412N, Gln412 was oriented in a way that did not allow for direct was also activated a lower concentration of zinc relative to H412N, showing that zinc binding is improved in Gln412 relative to Asn412. However, neither mutant was activated under the physiological levels of the wild type enzyme. These results imply that the imidazole ring of His412 in the M1 binding site is crucial for correct positioning of Zn_1 in the M1 binding site, and therefore indirectly, the positioning of the phosphate and binding of water.[12]

The last residue involved in the M1 site, His331, also binds to Zn_1 via a nitrogen in the imidazole ring. The H331Q mutant showed about 50% activity compared to the wild type enzyme, suggesting that only the nitrogen atom is essential for Zn^{2+} binding (Figure 3c). Like His412, His331 coordinates Zn^{2+} in the correct position to allow for phosphate and substrate binding. The rate determining step of the H331Q mutant was also found to be the hydrolysis of the enzyme-phosphate complex, rather than the release of the phosphate, indicating that H331Q affects the ability of Zn1 to coordinate the water molecule to allow for hydrolysis.[13]

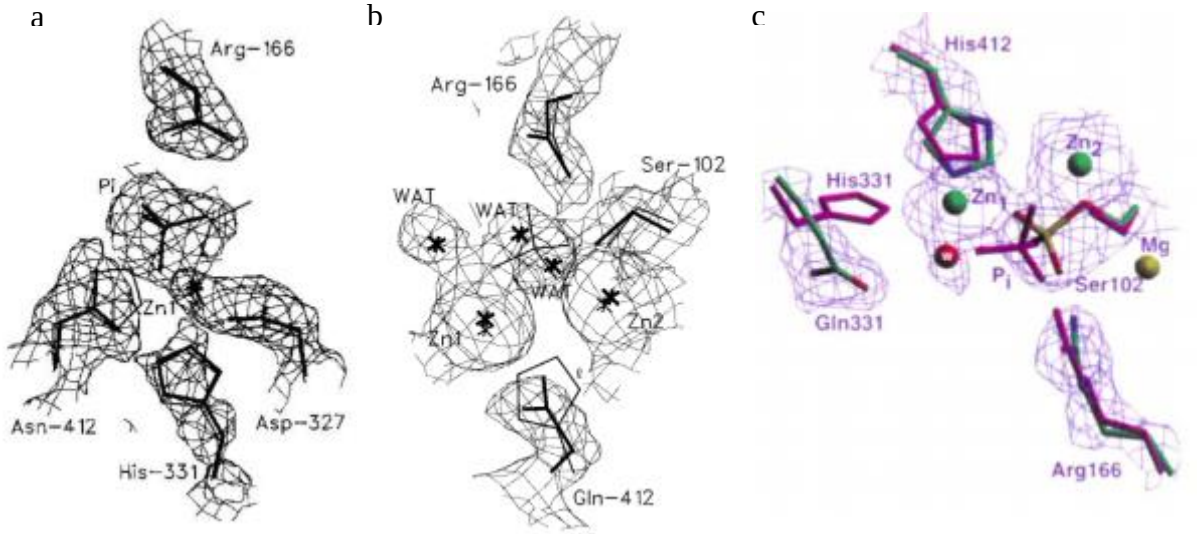


Figure 3. (a) The M1 site in the H412N mutant does not create a well-defined Zn2+ binding site. (b) The H412Q mutant coordinates Zn2+ in the M1 site. (c) In the H331Q mutant, Gln331 is no longer within bonding distance to Zn1. In a and b, thick lines represent the residues in the corresponding mutant; thin lines represent wild type ECAP. In c, magenta corresponds to wild type ECAP and green corresponds to H331Q.

Zn^{2+} in the M2 Metal Ion Binding Site Stabilizes Phosphate and Transition State

The M2 metal ion binding site binds a Zn^{2+} ion, known as Zn_2 to differentiate it from the Zn^{2+} ion in M1. It is coordinated tetrahedrally by the imidazole nitrogen of His370, one carboxyl oxygen of Asp51, one carboxyl oxygen of Asp369, and one oxygen of the phosphate ligand. In the absence of ligand, Ser102 serves as a ligand to Zn_2 (Figure 4).[3] The role of the Zn^{2+} the M2 binding site in the proposed mechanism is to stabilize the phosphate in the active site and to stabilize the formation of the serine oxyanion that is able to attack the phosphate ligand.[6] This has been shown through several mutations in the ECAP enzyme that affects the catalysis of the phosphatase reaction.

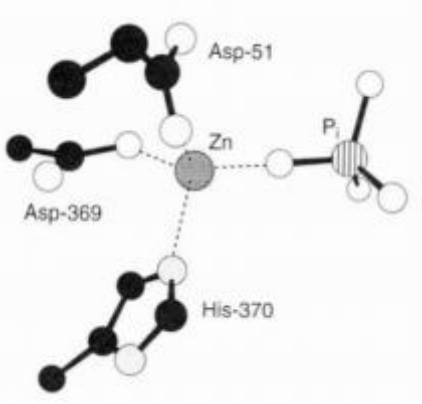
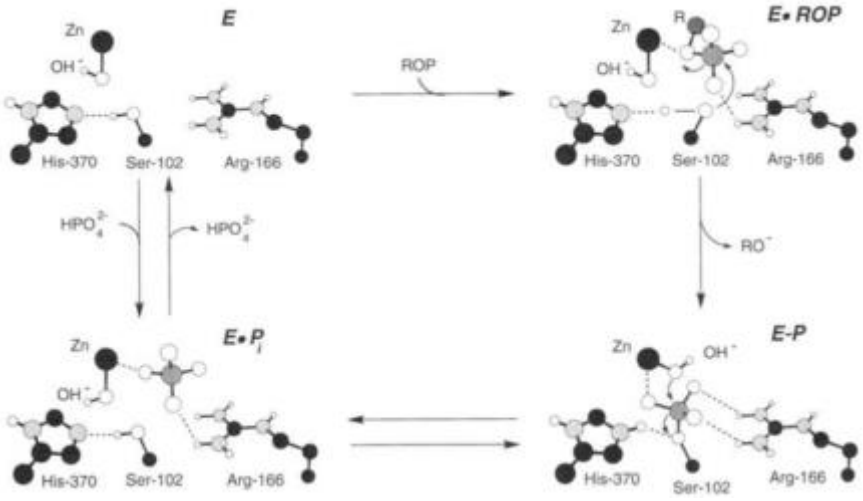


Figure 4. M2 binding site. Asp51is also bound to Mg2+ in the M3 site. (not shown)

The role of Zn_2 in stabilizing the phosphate ligand can be seen in the D369N mutation. The aspartic acid at position 369 normally coordinates the zinc ion in the M2 binding site. When it is replaced with an asparagine, a shift in the all the amino acid side chains within the M2 binding site is observed. Most noticeably, new hydrogen bonds form within the side chains of amino acids in the M2 and M3 binding sites, so that cations are unable to bind in the M2 and M3 sites. Given the shifts within the active site and the loss of two metal ions, the mutant enzyme is unable to catalyze the reaction in the same mechanism as the wild type enzyme, leading to a mechanism involving only one zinc atom. A proposed single-zinc mechanism (Figure 5) shows the phosphate ligand held loosely in the active site by Zn_1 and Arg166 only, as Zn_2 is not present. His370 is able to stabilize the serine oxyanion in place of Zn_2, but is unable to coordinate and stabilize the phosphate in the active site. This is consistent with the low catalytic activity of the D369N enzyme, implying that binding Zn_2 in the M2 site is important for drastically increasing the rate of catalysis.[14]



Another residue that plays an important role in binding Zn^{2+} in the M2 site is Asp51. This residue not only coordinates Zn^{2+} in the M2 binding site, but also coordinates Mg^{2+} binding in the M3 binding site. It acts as a carboxylate bridge between the M2 and M3 sites. The D51A mutant showed catalytic activity 400-fold lower than that of the wild type enzyme, implying that both carboxyl oxygens are needed for metal ion binding in M2 and for catalysis. The D51N mutant, which only has one site open to metal ion binding, is able to bind Zn^{2+} or Mg^{2+} in the M2 site; no metal ions were found in the M3 site (Figure 6). Catalytic activity was observed in both Zn^{2+} and Mg^{2+} bound mutants, suggesting that metal ions need to be bound in M2 in order for hydrolysis to occur, while binding in M3 is not necessarily for catalysis.[15] According to Kim and Wyckoff [6], these metal ions would stabilize the negatively charged Ser102 oxygen when no substrate is bound and would coordinate the substrate in the active site of the enzyme.

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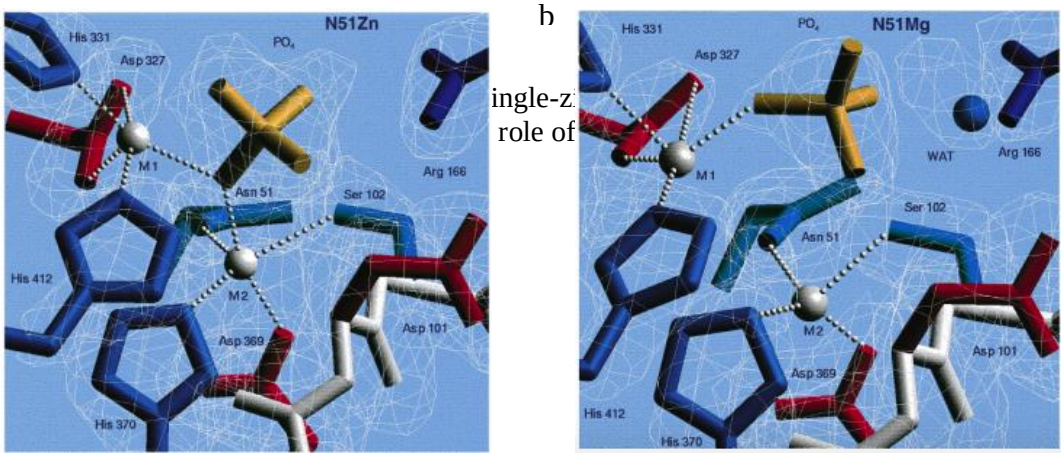

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Figure 6. (a) Zn2+ bound in the M2 site of the D51N mutant. (b) Mg2+ bound in the M2 site of the D51N mutant. Mg2+ is not coordinated to the phosphate; it is not able to stabilize the phosphate in the active site.

Interestingly, the M2 site only contains one histidine residue, as compared to two histidine residues in the M1 site. Therefore, the M2 site binds Zn^{2+} much weaker than M1, and the M2 site only has a slight preference for Zn^{2+} over Mg^{2+}. This suggests a possible role for the M3 site: to specifically bind Mg^{2+} in order to prevent Mg^{2+} binding in the M2 site. As a result, the M2 site would become much more specific for Zn^{2+}.[15]

The most important role for Zn^{2+} in the alkaline phosphatase mechanism is its ability to coordinate the oxygen in Ser102. The S102G and S102A enzymes lack the ability to bind an oxygen for the first step of the mechanism, however, the catalytic rate of these two mutants is still significantly greater than the rate of the uncatalyzed reaction. The rest of the residues involved in the active site shifted a minimal distance.[16] Therefore, the role of Zn_2 in these mutants must be to bind a water molecule for the first attack, in which the phosphate is attacked by the water molecule and results in the removal of the leaving group. It would have a similar role in coordinating the Ser102 oxygen to the phosphate in the wild type enzyme.

The M1 and M2 Zn^{2+} Binding Sites Are Highly Conserved

All alkaline phosphatases examined in the current literature contain highly conserved M1 and M2 metal ion binding sites, all of which bind Zn^{2+} for optimal catalytic activity.[1,7,8,9] This is highly due to the function of Zn^{2+} ions in the accepted mechanism of *E. coli* alkaline phosphatase. The well-supported mechanism proposed by Kim and Wyckoff specify direct and important roles for Zn_1 and Zn_2 in catalysis of the hydrolysis reaction.[6] Without these two ions, especially Zn_1, catalysis is extremely slow, if it occurs at all. Since these Zn^{2+} ions are important in the mechanism, the M1 and M2 binding sites are conserved throughout all alkaline phosphatases to ensure that Zn^{2+} can be coordinated successfully, and that catalysis can occur. The roles for Zn_1 and Zn_2 are clear within the proposed mechanism, but the role of the metal ion in M3 not so clear as it is not directly involved in the mechanism.

Mg^{2+} M3 Metal Ion Binding is Not Directly Involved in Catalysis

Mg^{2+} binding at the M3 metal site is not directly linked to the phosphate at the active site, or directly involved in the mechanism of alkaline phosphatase. The crystal structure of ECAP has shown that Mg^{2+} is octahedrally coordinated in the M3 binding site to six ligands: Glu322, Thr155, Asp51, and three water molecules. Asp153, while not directly coordinated to Mg2+, has been shown to affect Mg2+ binding in the M3 site. Asp153 hydrogen bonds to two water molecules, two of which are ligands to Mg2+. The third water molecule is hydrogen bonded to Arg166, indirectly linking Mg2+ to the phosphate in the active site.

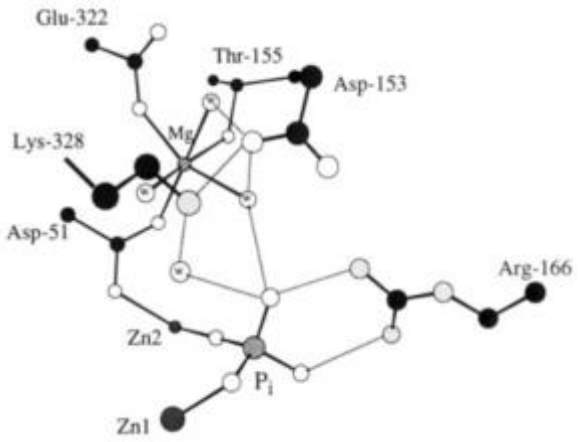

Figure 7. Mg2+ binding in the M3 site.

The Mg^{2+} ion is especially important for stabilizing the structure of the enzyme and increasing the rate of catalysis by stabilizing Zn_2. using both carboxyl oxygens. In the D51N mutant, which is only able to bind to a metal ion at either M2 or M3, no M3 binding was observed during catalytic activity, implying that asparagine was bound to a metal ion in the M2 site only.[15] The observation of catalytic activity despite no metal ion binding in M3 suggests that M3 binding is not directly linked to catalysis, but plays a lesser role in catalysis by being involved in enhancing catalytic activity through shifting M2 side chains to position Zn_2 in the correct geometry. Thus, the role of Mg^{2+} binding at M3 does not directly affect catalysis, but is important for stabilizing the enzyme in a geometry conducive to catalysis.

Another proposed role for Mg^{2+} in the enzyme is to accept a proton from Ser102, so that Zn_2 is able to coordinate to a negatively charged oxygen in Ser102 before the nucleophilic attack on the phosphate by the oxygen. The ability of Mg^{2+} to act as a proton acceptor depends on its position in the enzyme, which is determined by the M3 binding site. Therefore, the six ligands of the M3 binding site specifically coordinate and bind Mg^{2+} in an appropriate position to be a proton

acceptor for Ser102. Replacement of the Mg^{2+} ion by Zn^{2+} in M3 reveals that Zn is tetrahedrally coordinated by four ligands, rather than six ligands. The crystal structure of the tetrahedrally coordinated metal ion in M3 was found to shift the residues in M3, so that the metal ion in M3 would be too far from Ser102 to act as a proton acceptor. As a result, the *E. coli* M3 binding site is specific to Mg^{2+} so that the position of Mg^{2+} allows it to extract a base from Ser102.[18]

Another residue in the M3 metal ion binding site is Glu322, a ligand of Mg^{2+} in the M3 binding site. Its effect on Mg2+ binding was examined by mutating Glu322 to Asp322. The E322D mutant exhibited poor catalytic activity compared to the wild type, most likely owing to the inability of aspartic acid to coordinate Mg^{2+} in the correct position, since the side chain of aspartate is shorter than that of glutamate. The E322D mutant results in an intact but altered octahedral coordination sphere of Mg^{2+}, so that the position of Mg^{2+} in M3 is also altered. As a result, this reduces the ability of Mg^{2+} to position the side chains of the M2 site residues and to accept a proton from Ser102.[19]

Variation Between Alkaline Phosphatases Comes from Differences in the M3 Binding Site

While the M1 and M2 sites are highly conserved between alkaline phosphatases of different species as well as between structurally and functionally similar enzymes, the M3 site can differ greatly. The differences at the M3 metal ion binding site accounts for almost all the variation between enzymes similar to ECAP, as it is not directly involved in the catalysis of the reaction.

Mammalian alkaline phosphatases (MAPs), *Saccharomyces cerevisiae* (yeast) alkaline phosphatase, and ECAP are very similar, sharing 25-30% conservation and the same active site residues. Despite their similarities, MAPs are more active than ECAP, has a higher optimal pH, and is activated by additional magnesium. The yeast alkaline phosphatase exhibits a lower phosphate affinity than ECAP. Closer examination reveals that the residues corresponding to Asp153 and Lys328 in *E. coli* is replaced by histidine in MAP. In yeast alkaline phosphatase, only Lys328 is replaced by histidine.[7]

E. coli Asp153 is not a direct ligand of Mg^{2+}, however, it is hydrogen bonded to two water molecules, that are in turn coordinated to the Mg^{2+} ion at the M3 site. Asp153, therefore, is important for positioning Mg^{2+} within the M3 site.[4] Altering Asp153 to His153 in *E. coli* creates a mutant with preferential coordination of a Zn^{2+} ion at the M3 site, rather than the Mg^{2+} ion found in the wild type enzyme (Figure 8a and 8b).[20] The Zn^{2+} ion binding at M3 drastically decreases the catalytic activity of the D153H enzyme compared to the wild type enzyme. However, Mg^{2+} will replace Zn^{2+} at high concentrations of magnesium and will increase the catalytic activity of the enzyme, similar to the activation of MAP at a high magnesium concentration.[7]

The Lys328 and the Asp153 residues of ECAP form a salt linkage and has been shown to be important for binding of the phosphate (Figure 8c).The K328H mutation breaks the salt linkage between Lys328 and Asp153, and therefore the coordination of the Mg^{2+} and the phosphate. The phosphate group is thus displaced in this mutant, which correlates with the kinetic results which show a decrease for phosphate affinity. Similarly, the yeast alkaline phosphatase has decreased phosphate affinity compared to ECAP.[7] This one amino acid change important for the structure of the M3 site is responsible for the differences between yeast and ECAP.

Since MAPs have histidine residues at position 153 and 328, the double mutant D153H/K328H was examined. Again, the salt bridge between Lys328 and Asp153 was broken, and the phosphate shifted relative to wild type ECAP. The double mutant was found to exhibit many characteristics of the mammalian enzyme, such as increased optimal pH, increased activity compared to wild type ECAP, and activation by Mg^{2+} binding.[7] Because many of the active site residues are conserved between *E. coli* and MAPs, the differences in these alkaline phosphatases can be explained by the single amino acid changes to histidine at positions 153 and 328. These two residues are important in the M3 binding site, showing that the different characteristics between mammalian and ECAP mostly comes from variations in the M3 binding site.

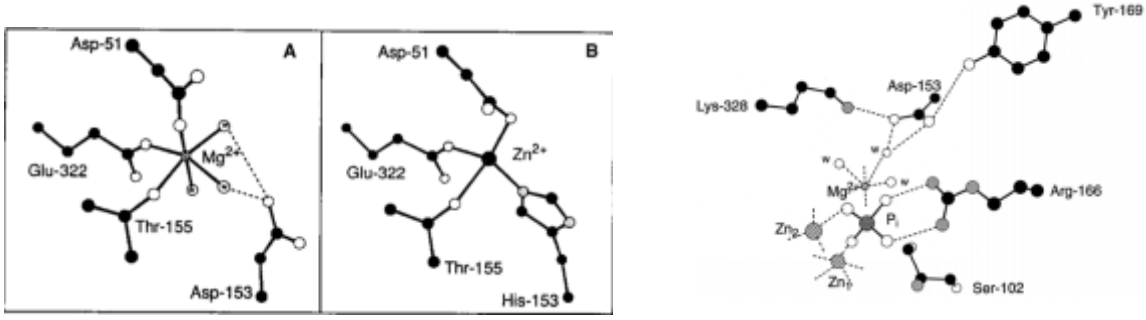

Figure 8. (a) Wild type ECAP. (b) The D153H mutant preferentially binds Zn^{2+} over Mg^{2+}. (c) Lys328 forms a salt bridge with Asp153 in wild type ECAP.

Variations in the residues of the M3 binding site can also confer different metal specificities. Although the metal binding sites are conserved between *Thermotoga maritima* alkaline phosphatase (TMAP) and ECAP, TMAP activity is dependent on Co+2 coordination in the M3 site, not Mg^{2+} as in ECAP. The main sequence differences, again, are the residue at position 153 and 328 – in *T. maritima*, Asp153 is changed to histidine and Lys328 is changed to tryptophan. The difference between *T. maritima* and ECAPs can be explained by changes in only two residues, which are, again, in the M3 binding site. This may suggest that evolutionary changes to the M3 site specializes alkaline phosphatase for the environment or needs of each species. *T. maritima* is an extremophilic organism isolated from geothermal heated marine sediment, and its proteins are noted for its stability under high temperatures. While *E. coli* and other related organisms continued to use Zn^{2+} and Mg^{2+} in their metal ion binding sites, *T. maritima* may have adapted to using Co^{2+} instead, for survival in its unique environment.[8]

Alteration of metal ion binding sites are also seen in the structure of alkaline phosphatase from Antarctic strain TAB5 (TAP). This enzyme also has high sequence homology compared to ECAP, and the residues at positions corresponding to 153 and 328 in *E. coli* are again replaced by histidine and tryptophan in TAB5. Although the changed residues in TAP relative to ECAP are the same as the changed residues in TAP relative to ECAP, it is unclear whether TAP utilizes Mg^{2+} or Zn^{2+} in its M3 binding site. While TMAP mutants preferentially bind Zn^{2+}, wild type TMAP has not been shown to preferentially bind either Zn^{2+} or Mg^{2+}. The orientation of the M3 binding site in TAP is more similar to M3 in ECAP than the D153H/K328W mutant of ECAP, which

suggests that TAP binds Mg^{2+} at M3. Several mutations of TAP at the M3 site also confer cold-adaptive properties to TAP. As a result of these mutations, the optimal temperature of TAP was found to be 25°C, which is considerably lower than that of ECAP. The most noticeable difference in TAP is the addition of M4 and M5 metal ion binding sites, both occupied by Mg^{2+}, which may help to increase catalytic activity of the enzyme. Changes in the M3 site in TAP allow TAB5 to better adapt to its cold environment.[8,21]

Complete eradication of the M3 binding site is observed in the alkaline phosphatase of *Sphignomonas sp.* strain BSAR-1 (SPAP). The residues corresponding to Thr155 and Glu322 in ECAP are replaced by Ala174 and Gly295 in SPAP; these changes no longer allow SPAP to coordinate a metal ion using these two residues. Instead of a metal ion, Lys171 in SPAP is hydrogen bonded to Asp49 (Asp51 in ECAP) much like Mg^{2+} in ECAP, and performs a similar function as the proton acceptor in SPAP. This is consistent with the proposed role of Mg^{2+} in M3 of ECAP, in which Mg^{2+} extracts a hydrogen from Ser102. Despite being within the same enzyme family as ECAP, the M3 site of SPAP is nonexistent, and illustrates the role of Mg^{2+} in ECAP more clearly. Because the structure of SPAP is more similar to other enzymes within the alkaline phosphatase superfamily than to ECAP, it is possible that SPAP is a relatively distant relative of ECAP, and convergent evolution has resulted in two different methods of extracting a proton from the nucleophile. This suggests the M3 binding site is a relatively newer adaptation of alkaline phosphatase, and is less conserved than the M1 and M2 sites.[9]

Conclusion

The purpose of this review is to examine the roles of each metal ion binding site within *E. coli* alkaline phosphatase, and assess how each metal ion contributes to the function of alkaline phosphatase. Comparison of the metal ion binding sites across the alkaline phosphatase family revealed that Zn_1 and Zn_2 are crucial for the catalysis of the hydrolysis reaction catalyzed by alkaline phosphatases. They are directly involved in the mechanism of the enzyme, and as a result, the M1 and M2 sites of all alkaline phosphatases are highly conserved across different species. In contrast, the M3 site is less conserved. In E. coli alkaline phosphatase, M3 binds a Mg^{2+} ion, which helps to position the residue side chains in M2 and helps with coordination of phosphate within the active site. Depending on the species, the M3 site can be modified, giving the alkaline phosphatase of each species different characteristics, such as in mammalian and yeast alkaline phosphatases. The M3 site can be adapted to suit certain environments of a particular species, as seen in *T. maritima* and *Sphignomonas sp.* Therefore, the binding sites of metallic cofactors that are directly involved in the mechanism of an enzyme are conserved throughout evolution. The binding sites of metallic cofactors that do not have a direct impact on catalysis can vary among species and allow for environmental adaptations. This suggests the M3 binding site of alkaline phosphatases are subject to evolutionary changes.

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