Lysine-167 in *Escherichia coli* Alkaline Phosphatase May Be Involved in the Dissociation of Phosphate from the Active Site After Catalysis

A. Chow, G. Scofield, A. Dattillio, C. Volpe, J. Hustedt, C. Capp
Department of Chemistry and Biochemistry, University of Maryland, College Park MD 20742

Abstract

The role of the lysine residue at position 167 of *E. coli* alkaline phosphatase has not been previously studied in literature. However, its position near the active site of the enzyme suggests that it may play a role in the function of alkaline phosphatase. Comparative kinetic characterization of the K167A and K167S mutants indicate that the role of the lysine residue at position 167 is not required for catalysis, but significantly affects the rate of catalysis of alkaline phosphatase. No significant change in Km is observed between the wild type enzyme and the mutants in transferase or hydrolysis activity, implying that Lys167 is not involved in binding of its substrate. However, significant decreases in kcat and kcat/Km are observed between the wild type and the mutants, with K167A resulting in greater decreases than K167S, thus the side chain of residue 167 is involved in determining the catalytic efficiency. Based on the properties of lysine compared to serine and alanine, and the position of residue 167, the side chain of Lys167 may play a role in increasing the catalytic efficiency of the enzyme by hydrogen bonding to the product during dissociation from the enzyme.

Introduction

Escherichia coli alkaline phosphatase (AP) 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 an 80 kDa homodimer containing 449 residues each. The monomers of alkaline phosphatase contain an active site, which binds a phosphate ester substrate. There are three metal binding sites: M1 and M2 bind two Zn^{2+} ions, and M3 binds an Mg^{2+} ion. The zinc ions are involved in the binding of the phosphate ester substrate and help to coordinate the hydrolysis reaction.[2,3,4] Within the active site, the phosphate ester substrate is coordinated by two Zn^{2+} atoms in the M1 and M2 sites, as well as the basic side chain of Arg166.[2,3] Mutation of the Arg166 residue has been shown to drastically decrease alkaline phosphatase activity.[4,5] A neighboring residue, Lysine-167, may also have an effect on the binding of the phosphate ester substrate, due to its proximity to Arg166. Here we examine the exact role of Lys167 in the reaction of alkaline phosphatase by comparing the enzyme kinetics of wild type *E. coli* alkaline phosphatase to that of K167A and K167S mutant *E. coli* alkaline phosphatase, where Lys167 has been mutated to alanine or serine, respectively.

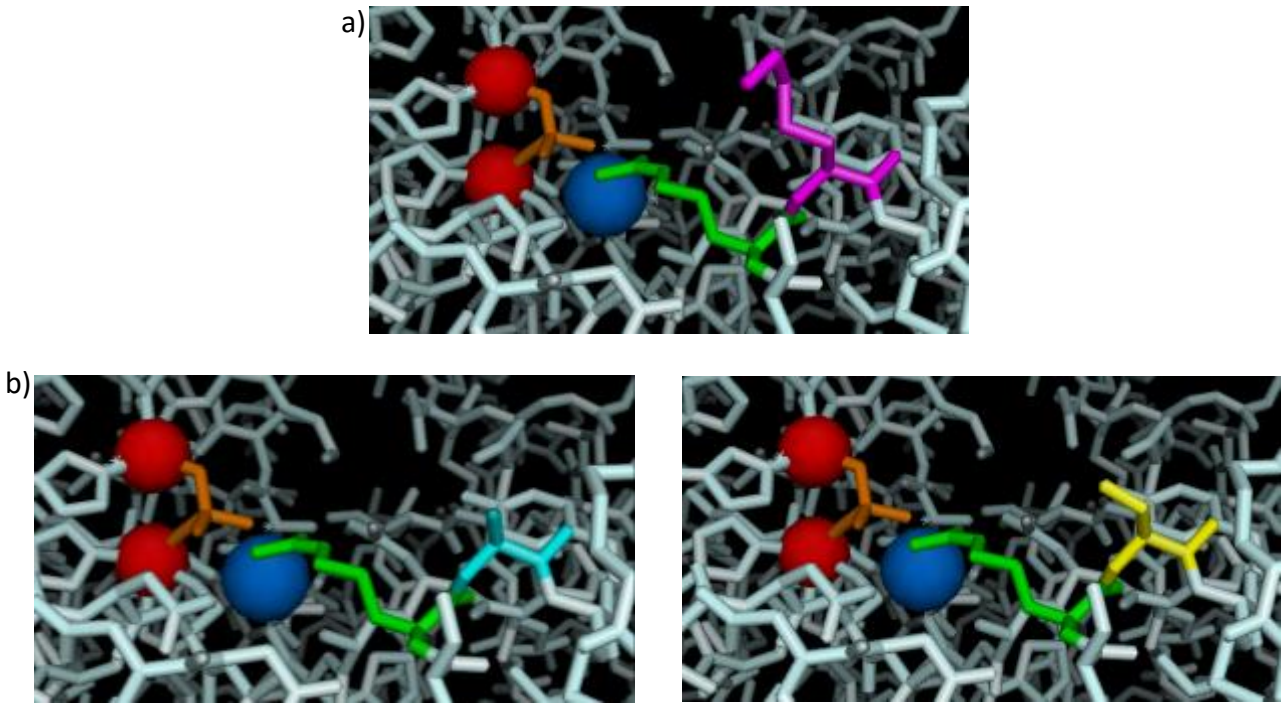


Figure 1. The active site of wild type E. coli alkaline phosphatase.[4] Arg166 is highlighted in green, a phosphate substrate is highlighted in orange, Zn2+ ions are highlighted in red, and a Mg2+ ion is highlighted in blue. a) Wild type E. coli phosphatase. Lys166 is highlighted in pink. b) Mutants of alkaline phosphatase. K167A is highlighted in cyan. K167S is highlighted in yellow.

The mechanism of the alkaline phosphatase hydrolysis reaction proceeds through a ping-pong mechanism.[4] While the residue at position 167 is not directly involved in the mechanism and does not interact with the substrate, the neighboring residue, Arg166, is involved in coordinating the phosphate ester substrate before Ser102, the catalytic residue, completes a nucleophilic attack. The cleaved phosphate group is then attacked by another alcohol, undergoing transferase activity, or by a water molecule, undergoing hydrolase activity. Arg166, which is coordinated to the oxygens of the phosphate group throughout the reaction, is then involved in releasing the product after the reaction has been catalyzed.[4] At an acidic pH the second attack by an alcohol or a water molecule is the limiting step, while at an alkaline pH, the release of the phosphate is the rate-limiting step. As the optimal pH of the wild type enzyme is pH=8.0, the rate-limiting step is the release of the phosphate.[7] Here we use Tris-HCl, an alcohol, as the phosphate acceptor in order to examine transferase activity. We also examine hydrolysis activity by using MOPS to buffer the solution rather than Tris-HCl. MOPS is not an alcohol and does not accept phosphate, so water must be the phosphate acceptor in that case. Comparing transferase and hydrolysis activity would determine whether Lys167 affects the catalytic efficiency of alkaline phosphatase by interacting with the phosphate acceptor.

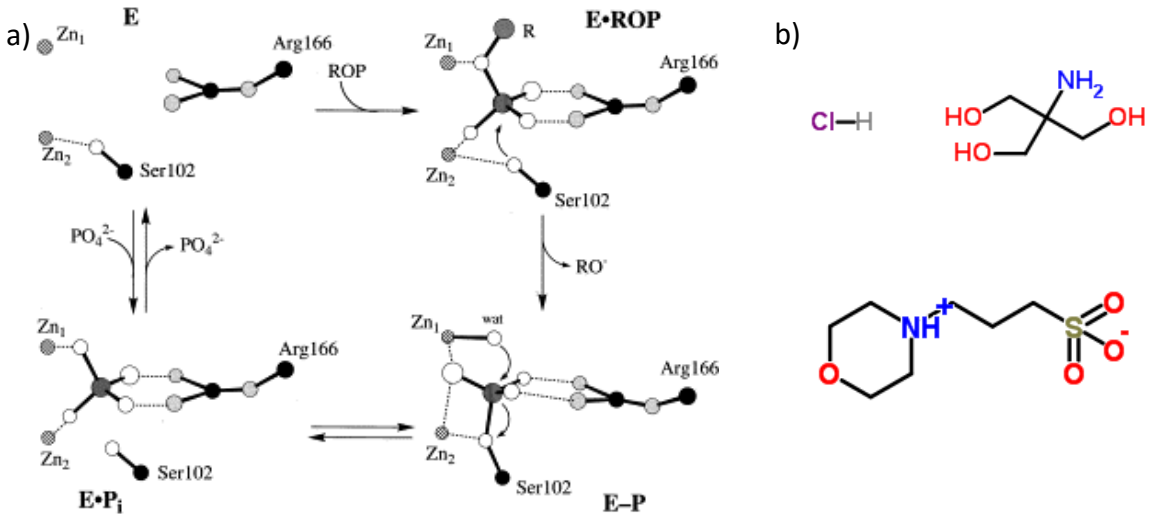


Figure 2. a) Mechanism of alkaline phosphatase. While Arg166 and a Zn^{2+} ion coordinate the phosphate, Ser102 catalyzes the reaction. Ser102 attacks the phosphate, causing the R group on the phosphate to leave. The phosphate is then attacked by a water molecule (shown in bottom right) or an alcohol (not shown), and then released by Arg166 and Zn^{2+}.[4] b) Structure of Tris-HCl (top) and MOPS (bottom). Tris is an alcohol capable of accepting a phosphate, while MOPS cannot accept the phosphate, meaning a water molecule must serve as the acceptor.[8,9]

Inorganic phosphate is a competitive inhibitor of the wild type enzyme. The catalytic residue is unable to attack the inorganic phosphate, as there is no group available to leave, and thus the phosphate is bound to the enzyme until a suitable alcohol or water molecule is able to accept it and release it from the enzyme. Thus, inorganic phosphate inhibits the enzyme from carrying out hydrolysis of a phosphate ester substrate.[10]

In this experiment, we determine enzyme kinetics by monitoring the initial rate of formation of deprotonated para-nitrophenyl (pNP-). Alkaline phosphatase catalyzes the reaction of para-nitrophenyl phosphate (pNPP) to para-nitrophenyl (pNP). At a pH above the pKa of pNP, 7.15, pNP becomes deprotonated. Deprotonated pNP absorbs light at wavelengths between 400-410nm. As the reaction proceeds, the amount of deprotonated pNP will increase, and this increase is proportional to the change in absorbance at 400-410nm. Beer's Law was used to determine the concentration of pNP from the absorbances. The molar absorptivities (ε) of pNP- at different buffer pH levels are given in Table 1.[11]

pH	ε_{400} (1/Mcm)	ε_{405} (1/Mcm)
6.5	4330	-
7	10100	-
7.5	14700	-
8	16900	18000
8.5	18400	-
9	19200	-
9.5	19300	-

Table 1. Molar absorptivities of pNP- at 400 and 405 nm.[11]

The Michaelis-Menten Equation is used to describe a best fit curve created by measuring the initial velocity (V0) of the enzymatic reaction versus an increasing amount of substrate. At low substrate concentrations, the initial velocity of the reaction is low, as the enzyme is limited by the amount of substrate available. At high substrate concentrations, the initial velocity depends only on the amount of substrate present, and the initial velocity will be equal to the maximum velocity of the enzyme (Vmax). The Km is defined as the substrate concentration when half of the Vmax is reached, and describes the affinity of an enzyme for its substrate.[11] Mathematica is able to create a best fit with this equation using experimental data, providing the values of Vmax and Km.

$$v_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Equation 1. The Michaelis-Menten equation, where [S] is the substrate concentration.[11]

The kcat describes the catalysis rate of the enzyme. A high kcat value implies that the rate of catalysis is high. It can be calculated using Equation 2.[11]

$$k_{cat} = \frac{V_{max}}{[E]}$$

Equation 2. Calculation of kcat, where [E] is the concentration of enzyme.[11]

. The kcat/Km is another useful parameter for describing the activity of the enzyme. It takes into account both the catalysis rate, as well as the affinity of the enzyme for a substrate.[11]

The Ki describes the affinity of an enzyme for an inhibitor, if present. In the presence of a competitive inhibitor, the enzyme will bind to either the substrate or the competitive inhibitor. When the Ki is high, the enzyme is said to have a high affinity for the inhibitor. The Kmapp, or apparent Km, is the Km that is determined when a competitive inhibitor is present. Using equation 3, the Kmapp and the actual Km of the enzyme can be used to calculate the Ki.[11]

$$K_{m_{app}} = \alpha K_m$$
$$\alpha = 1 - \frac{[I]}{K_i}$$

Equation 3. Calculation of Ki, where Kmapp is the Km obtained from the kinetic data done in presence of an inhibitor.

Here we characterize and compare the kinetic behavior of transferase activity of the wild type alkaline phosphatase with that of the K167A mutant alkaline phosphatase, produced via site-directed mutagenesis. Then, we compare the kinetic behavior of the hydrolysis activity of the two enzymes, allowing us to determine whether Lys167 interacts with the phosphate acceptor. We then examine the kinetic behavior of the two enzymes in the presence of an inorganic phosphate inhibitor. Lastly, we determine the optimal pH of the wild type and mutant

enzymes. The kinetic characterization of the K167A and K167S mutants from these experiments will be used to elucidate the role of Lys167 in the hydrolysis of phosphate esters.

Materials and Methods

Wild type AP and K167A mutant AP were purified from Mph44 E. coli cells. The wild type AP concentration (0.277 uM) was determined from concentrations given by the teaching staff (0.001u/uL, 38.36 u/mg). The absorbance of the K167A mutant was measured at 280 nm and using the extinction coefficient of the K167A mutant in Beer's Law, the concentration was determined to be 10.73 uM.[12] An experiment to determine the kinetic parameters for the transphosphorylation activity of alkaline phosphatase was first performed on both the wild type AP enzyme and the K167A alkaline phosphatase mutant. To do this, the rate of the change of the absorbance of pNP- was measured over the first 90 seconds of the reaction. The linear change in absorbance of pNP- was measured at 400 nm with a Shimadzu UV-1800 spectrophotometer. AP Assay Buffer (1M Tris-HCl, pH=8.0, 10mM MgCl2, 50 uM ZnSO4, 150mM NaCl) was prepared, then varying levels of pNPP was added just prior to the reaction being carried out. AP (final concentration 0.00139 uM) was then added to the AP Assay buffer containing pNPP in a cuvette with a 1 cm path length. The absorbance at 400 nm was measured for 90 seconds. The change in absorbance was obtained where the graph of absorbance vs. time was linear. This was done at multiple concentrations of pNPP (3.15 uM, 6.15 uM, 12.5 uM, 25 uM, 100 uM) in triplicate for each concentration of pNPP. The same was done for the K167A mutant using pNPP concentrations of 6.3 uM, 12.3 uM, 25 uM, 50 uM, and 200 uM. The final concentration of K167A mutant in the cuvette was 0.0536 uM.

The kinetic parameters of hydrolysis activity of wild type AP and K167A mutant AP was determined by using MOPS as the buffer, rather than Tris-HCl. AP MOPS buffer (100 mM MOPS, pH=8.0, 10mM MgCl2, 50 uM ZnO4, 150 mM NaCl) was added to the same concentrations of pNPP and enzyme as before, for both the wild type enzyme and the K167A mutant. The change in absorbance was determined using the same methods as above.

The effect of inorganic phosphate (Pi) on the kinetic parameters of the transphosphorylation reaction was then investigated. Pi (40 uM for buffer used with wild type enzyme, 80 uM for buffer used with mutant enzyme) was added to AP Assay Buffer containing pNPP (varying concentrations for wild type and mutant protein, same as above). The final concentrations of wild type and K167A alkaline phosphatase were the same as above, and the same procedures were used to determine the change in absorbance in triplicate.

The effect of pH of the buffer on the transphosphorylation activity of wild type and K167A mutant alkaline phosphatase was then measured. 0.2mM pNPP was added to AP Assay Buffer at pH levels of 6.5, 7, 7.0, 8, 8.5, 9, and 9.5. The concentrations of wild type and K167A were the same as above. The same methods were used to determine the change in absorbance in triplicate.

We received kinetic data on transferase and hydrolase activity of the K167S AP mutant from A. Dattillio and C. Volpe. The same procedures were used to find the change in absorbance

at 405 nm, also using a Shimadzu UV-1800 spectrophotometer. The final concentration of K167S mutant AP in the cuvette was 0.0232 uM.

All change in absorbance per minute (Abs/min) measurements were converted to initial velocity (uM/min) using Beer's Law and the extinction coefficients given in Table 1.

Results

The transferase activity initial velocities were plotted against the pNPP substrate concentrations, then the data was fit to the Michaelis-Menten equation.

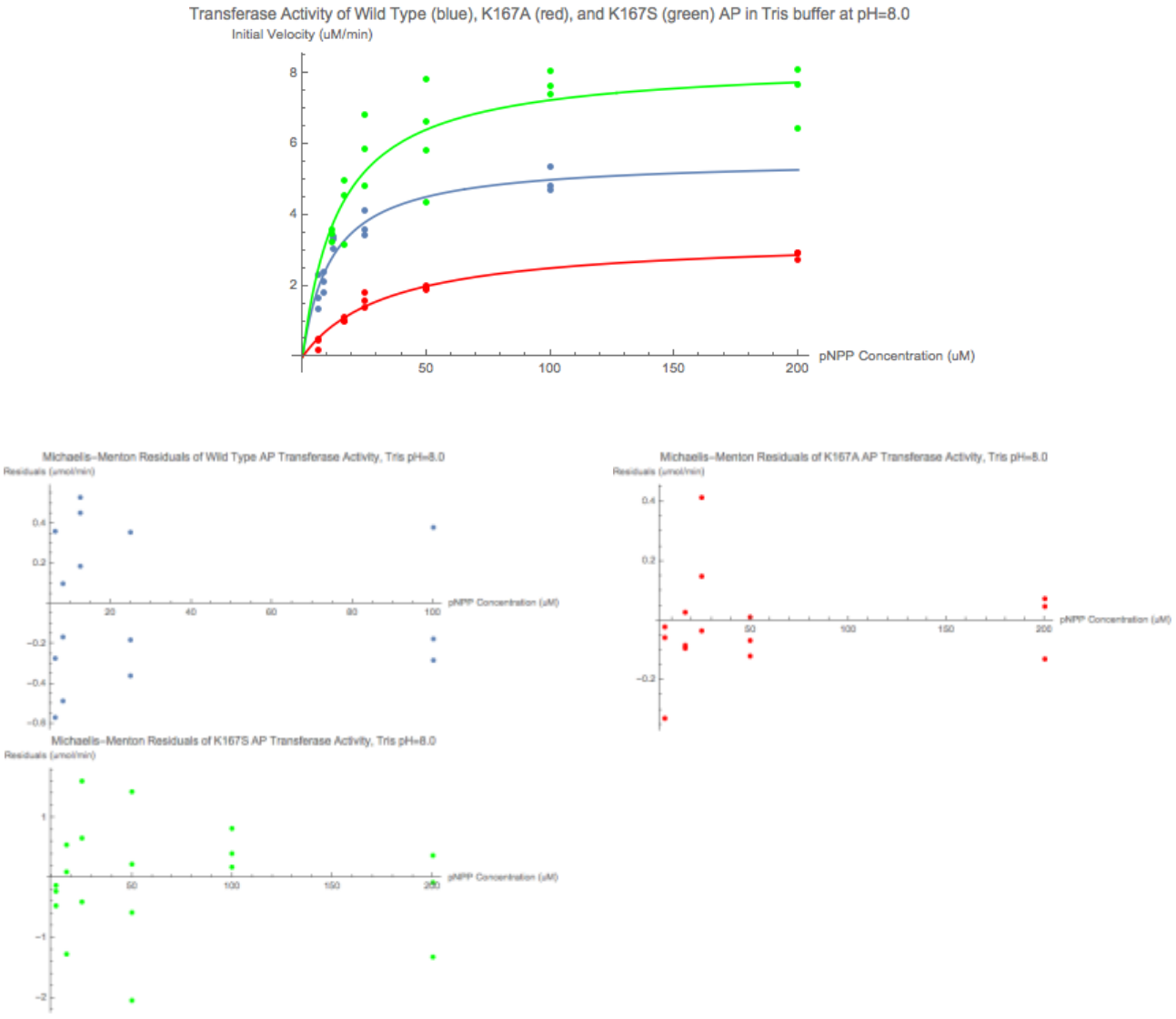


Figure 3. a) Michaelis-Menten plot of transferase activity of wild type AP (blue), K167A AP (red), and K167S AP (green) in 1 M Tris-HCl buffer, pH=8.0. b) Residual plots of the wild type AP, the K167A AP, and the K167S AP transferase data fitted to the Michaelis-Menten equation.

The transferase activity of alkaline phosphatase can be fitted to Michaelis-Menten curves. The residuals plot shows that the data is somewhat scattered around the x-axis, maKing the Michaelis-Menten equation a good fit for the data.

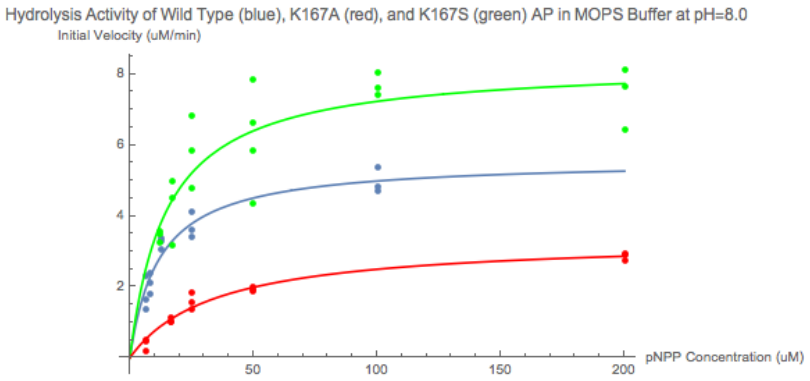
Mathematica was used to calculate the kinetic parameters Vmax and Km. Equation 2 was used to calculate the kcat.

	V_{max} (uM/min)	K_m (uM)	k_{cat} (1/sec)	$\frac{k_{cat}}{K_m}$ (1/Msec)
WT	5.57983	11.8882	66.90443645	5.63E+06
K167A	3.35681	34.4287	1.043784204	3.03E+04
K167S	8.31099	14.8735	5.961606247	4.01E+05

Table 2. Kinetic parameters of wild type and K167A mutant alkaline phosphatase transferase activity in 1 M Tris-HCl buffer, pH=8.0

Because the concentrations of wild type enzyme and mutant enzymes in the reaction were different, the Vmax cannot be directly compared to each other. However, the Km and kcat are both independent of enzyme concentration. While the Km of the K167A mutant was greater than that of the wild type enzyme by only threefold, the kcat of the mutant decreased around 67-fold. The kcat/Km was decreased in the K167A mutant by two orders of magnitude. The Km of the K167S mutant was about the same as the Km of the wild type enzyme. The kcat of the K167S mutant was decreased from the wild type about 11-fold. The kcat/Km was decreased in the K167S mutant by one orders of magnitude.

The hydrolysis activity of the wild type and the mutant was then fitted to the Michaelis-Menten equation.



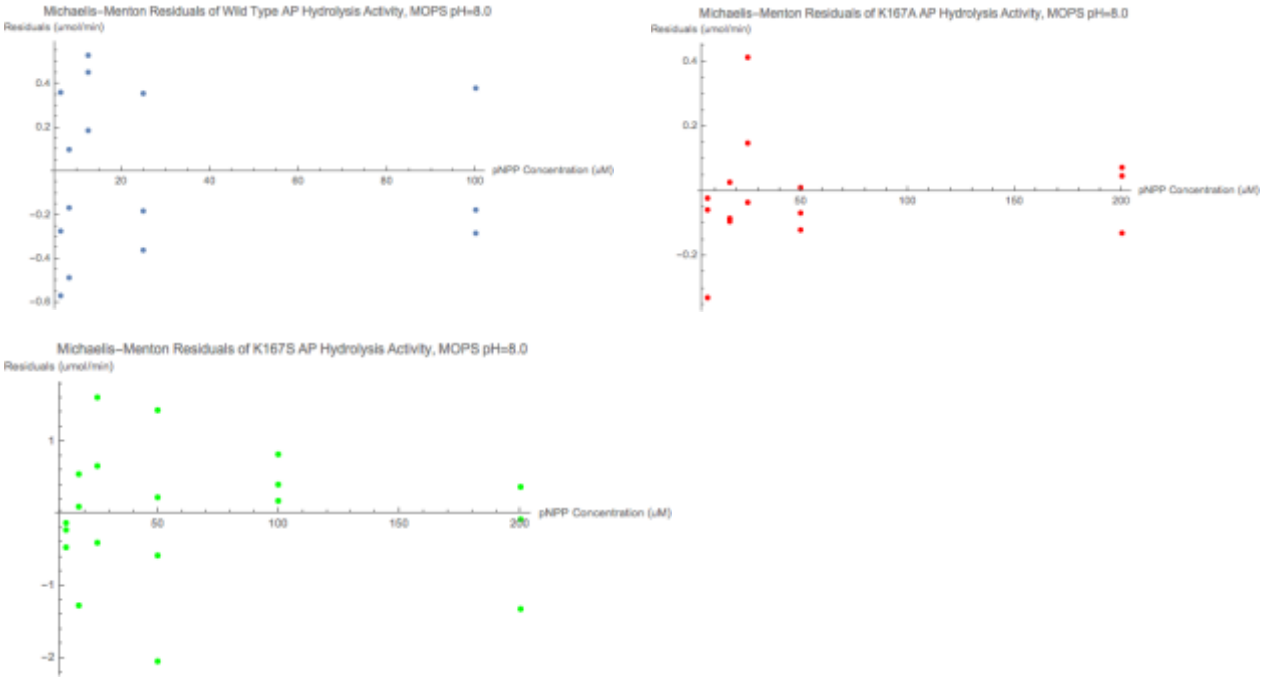


Figure 4. a) Michaelis-Menten plot of hydrolase activity of wild type AP (blue), K167A AP (red), and K167S AP (green) in 100 mM MOPS buffer, pH=8.0. b) Residual plots of the wild type AP, the K167A AP, and K167S AP hydrolysis data fitted to the Michaelis-Menten equation.

The hydrolase activity can be fitted to the Michaelis-Menten equation. The maximum velocity of the hydrolase activity wild type enzyme is higher than that of the mutant. The residuals are moderately evenly scattered around the x-axis, making the Michaelis-Menten fit a moderately acceptable fit for the hydrolase activity data.

The kinetic parameters were determined in the same way as above.

Enzyme	V_{max} (uM/min)	K_m (uM)	k_{cat} (1/sec)	$\frac{k_{cat}}{K_m}$ (1/Msec)
WT	0.544566	3.45215	6.529568345	1.89E+06
K167A	0.284438	3.16556	0.088444652	2.79E+04
K167S	0.715425	6.55792	0.513185812	7.83E+04

Table 3. Kinetic parameters of wild type and K167A mutant alkaline phosphatase transferase activity in 100mM MOPS buffer, pH=8.0

Similar to the kinetics of the transferase activity, the Km of the hydrolysis activity stayed constant for both mutants, while the kcats decreased drastically. The kcat of the K167A mutant decreased from the wild type kcat about 73-fold, and the kcat of the K167S mutant decreased 12-fold. The kcat/Km of both mutants were two orders of magnitude lower than the kcat/Km of the wild type enzyme.

The transferase activity of the wild type enzyme and K167A mutant in the presence of a competitive inhibitor, inorganic phosphate, was then examined. The transferase activity with the inhibitor present was fit to the Michaelis-Menten equation.

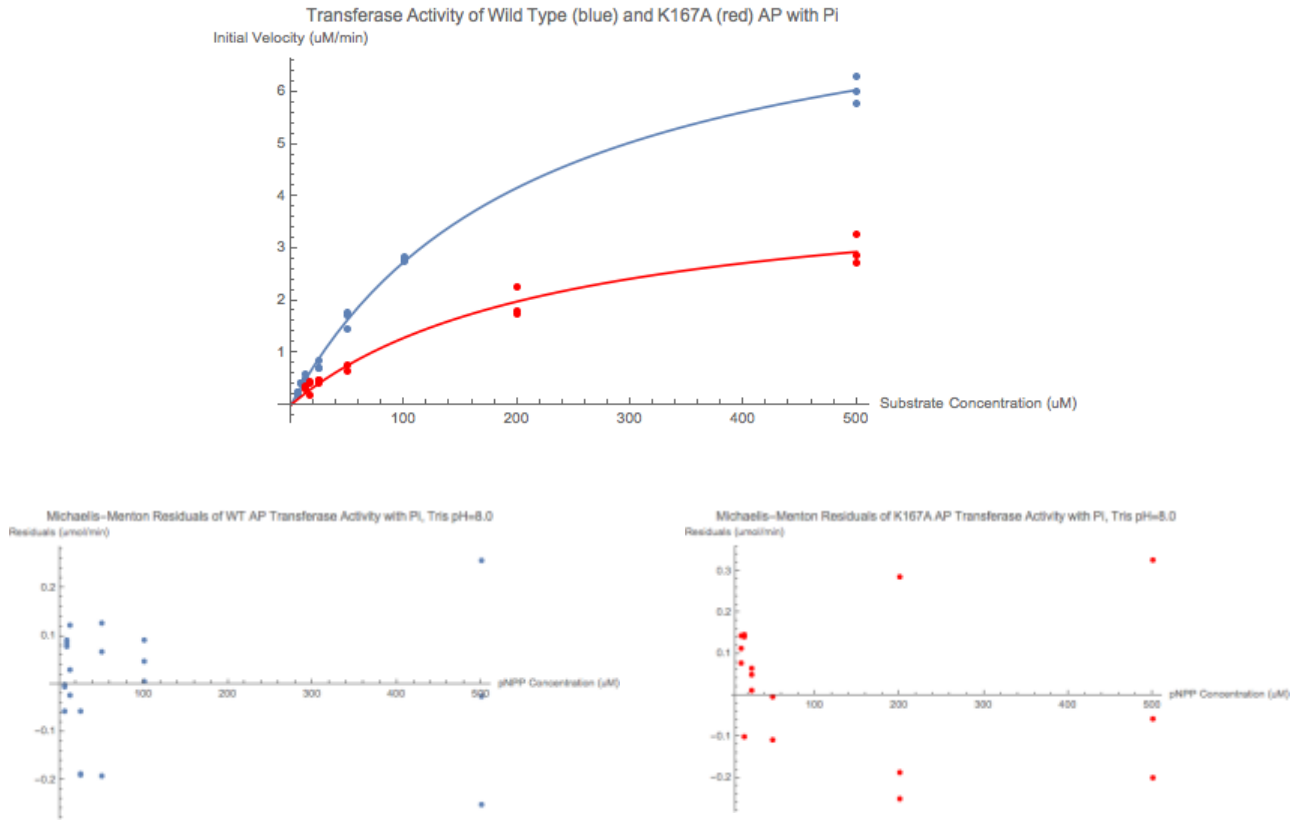


Figure 5. a) Michaelis-Menten plot of transferase activity in the presence of inorganic phosphate of wild type AP (blue) and K167A AP (red) in 1 M Tris buffer, pH=8.0, with Pi (40 uM for wild type, 80 uM for K167A). b) Residual plots of the wild type AP and the K167A AP transferase activity in presence of inorganic phosphate fitted to the Michaelis-Menten equation.

The wild type showed more activity than the mutant in the presence of an inhibitor. The residuals are scattered evenly around the x-axis, showing that the Michaelis-Menten equation is a good fit for the data.

	V_{max} (uM/min)	$K_{m_{app}}$ (uM)	K_i (uM)	k_{cat} (1/sec)	$\frac{k_{cat}}{K_m}$ (1/Msec)
WT	8.63013	214.282	2.349518612	103.478777	4.83E+05
K167A	4.31713	235.671	13.68646651	1.342391169	5.70E+03

Table 4. Kinetic parameters of wild type and K167A mutant alkaline phosphatase transferase activity in the presence of inorganic phosphate (40 uM Pi for wild type, 80 uM Pi for mutant)

The Km remained constant for the wild type and K167A mutant. The Ki was increased about 6-fold for the mutant compared to the wild type enzyme. The kcat for the mutant enzyme

was significantly lower (two orders of magnitude) than that of the wild type enzyme. The kcat/Km was also two orders of magnitude lower for the mutant enzyme than for the wild type enzyme.

Lastly, the effect of pH of the buffer on the initial velocity of the enzyme was examined. The initial velocity was plotted against the pH of the buffer.

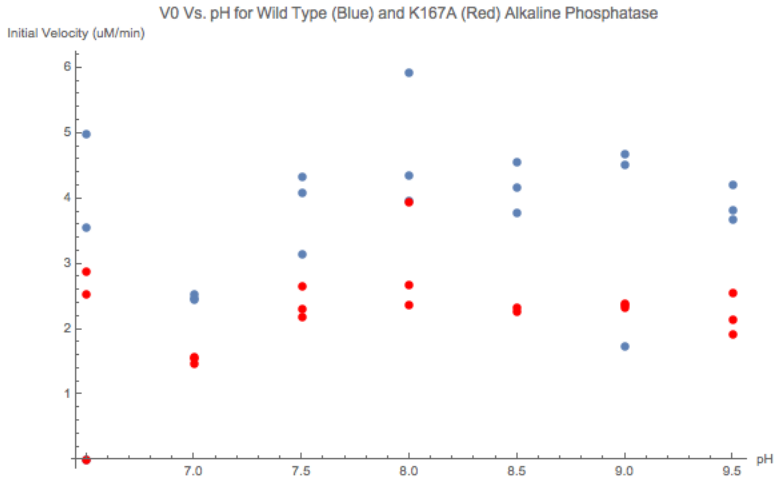


Figure 6. Initial velocity vs. the pH of the buffer. Wild type AP is shown in blue and K167A mutant AP is shown in red.

The plot of initial velocity vs. pH shows that the optimal pH for both enzymes is around pH=8.0-8.5. The optimal pH for the buffer remained the same for the buffer the wild type enzyme and the K167A mutant.

Discussion

The kinetic behavior studies of the mutant K167A AP and K167S AP showed that these mutants are effective catalysts in the hydrolysis of pNPP, although they do not catalyze the reaction as quickly as the wild type AP does. Therefore, Lys167 is not an essential residue for the function of alkaline phosphatase. The kinetic behavior of the transferase activity, shown in Table 1, showed that the Km stayed relatively constant between the wild type AP, the K167A mutant AP, and the K167S mutant AP. As the Km describes the affinity of the enzyme to the substrate, we can conclude that the Lys167 does not significantly affect the affinity of the enzyme for the substrate, and therefore, may not be involved in the binding of the substrate to the enzyme. However, the kcat of both mutants decreased significantly compared to the kcat of the wild type AP. The kcat, which describes the rate of catalysis of the enzyme at saturated substrate concentrations, is higher for enzymes that have a high catalysis rate. Therefore, the significant decrease in the kcat suggests that Lys167 must have a role in the catalysis of the transferase reaction. The kcat/Km, or catalytic efficiency, of the K167A mutant, was about two orders of magnitude smaller than the catalytic efficiency of the wild type enzyme. The kcat/Km of the K167S mutant was one order of magnitude smaller than that of the wild type enzyme. Therefore, Lys167 does not alter the catalytic efficiency by participating in substrate binding, but by its role

in helping the catalysis of the transferase activity. Since the catalytic efficiency of the K167A mutant was lower than that of the K167S, the serine must allow for some function in catalysis that alanine does not allow for. Both lysine and serine have an electronegative atom bonded to a hydrogen at the end of their side chains, while alanine only has a methyl group in its side chain. The presence of this electronegative atom and the hydrogen must be essential for reaching maximum enzyme activity. These atoms are capable of hydrogen bonding with other molecules, so the ability to hydrogen bond may have a role in catalysis.

The kinetic parameters of hydrolysis activity of the K167A were similar to that of the transferase activity. Changing the phosphate acceptor from Tris to water would determine whether Lys167 affects catalysis by interacting with the phosphate acceptor. Our results showed that the Km did not vary significantly between the wild type enzyme and the mutant enzymes. Both the K167A and K167S mutant kcat values were significantly smaller compared to the wild type kcat. The decrease observed in the mutant kcat values of transferase activity was similar to the decrease observed in the mutant kcat values of hydrolase activity. The K167A mutant resulted in around a 70-fold decrease in the kcat in both types of activities, while the K167S mutant resulted in about a 12-fold decrease in the kcat, also in both types of activities. This consistency implies that the rate of catalysis in the mutant enzymes is not affected by the type of reaction that alkaline phosphatase catalyzes; the change in the kcat is due only to the amino acid change. Therefore, the side chain of Lys167 must be involved in catalysis. The side chain of serine retains hydrogen bonding abilities, while the side chain of alanine cannot hydrogen bond at all. This explains why K167S has a higher catalytic efficiency than K167A, although the catalytic efficiency of K167S is still lower than that of the wild type enzyme.

Addition of the inorganic phosphate inhibitor to the Tris buffer again showed similar changes in the kinetic parameters between the wild type enzyme and the K167A mutant. Introducing the inorganic phosphate would allow us to deduce whether Lys167 affects the catalysis of the substrate, as inorganic phosphate binds to the active site of AP in the same way as the substrate but does not allow for transferase or hydrolysis activity while it is in the active site. A 77-fold change in the kcat is also observed in the transferase activity even in the presence of the inhibitor, which is consistent with the change in the kcat observed in the transferase and hydrolysis activity. The kcat/Km of the mutant is also two orders of magnitude less than that of the wild type enzyme. The introduction of the inhibitor holds the binding of the inorganic phosphate constant, so that a difference in the kcat/Km is solely due to the rate of catalysis of the enzyme. Interestingly, the Ki of the K167A mutant increased 7-fold compared to the Ki of the wild type enzyme, therefore the K167A mutant binds to the inhibitor weaker than the wild type enzyme does. This may have to do with the change of shape in the space above active site caused by the substitution of alanine for lysine. Lysine has a longer multi-carbon side chain that protrudes somewhat into the path that the substrate or inhibitor would take into entering the active site, whereas alanine has a short side chain consisting of only one methyl group. The mutant would have a larger space for the inhibitor to diffuse in and out of, leading to weaker binding of the inhibitor as compared to the wild type enzyme. Thus, Lys167 may play a role in allowing the diffusion of the product out of active site. Despite the difference in the Ki, the Kmapp remains unchanged between the two enzymes, implying that Lys167 does not affect the diffusion of substrate into the active site.

The optimal pH of both enzymes was found to be between 8.0-8.5; this is the pH at which the enzyme can catalyze the reaction the quickest. At the optimal pH of the enzyme, the side chain of lysine is protonated as the pKa of the side chain is 10.5. Therefore, Lys167 stays protonated throughout the reaction, allowing the side chain to hydrogen bond with other molecules.

The properties of Lys167 plays an important role in determining the catalytic efficiency of the enzyme. K167A had the lowest catalytic efficiency out of all enzymes studied. K167S had a higher catalytic efficiency than K167A, but was still not as efficient as the wild type enzyme. This difference is due to the differences in the properties of the side chains of lysine, serine, and alanine. The side chain of Lys167 is capable of hydrogen bonding and protrudes into open space near the active site of the enzyme. The side chain of serine, also capable of hydrogen bonding, protrudes less into this open space, and the side chain of alanine does not hydrogen bond and protrudes very little. Therefore, the length of the Lys167 side chain and its ability to hydrogen bond plays an important role in catalysis. The location of Lys167 near the active site implies that Lys167 is possibly involved in hydrogen bonding with the product during the its release, since it was concluded that Lys167 does not interact with the phosphate acceptor and does not play a role in binding the substrate. Lys167 is also too far from the active site to hydrogen bond to water molecules or the phosphate during the reaction. The only function for Lys167, based on its hydrogen bonding capabilities and its location, is to catalyze the dissociation of the phosphate group after the nucleophilic attacks. Lys167 is positioned right above the active site of the enzyme, where the product must travel past in order to dissociate from the enzyme. The side chain of Lys167 create a hydrogen bond to the product in place of Arg166, helping the product diffuse out of the active site after it has been hydrolyzed. This function of Lys167 would increase the diffusion of the product out of the active site, resulting in a higher rate of catalysis and increasing the catalytic efficiency of alkaline phosphatase. This is consistent with our results, as a serine or alanine in the place of Lys167 would be less efficient in this role – the length of the serine side chain is not as long as the side chain of lysine and would not be as efficient at hydrogen bonding the product, and an alanine side chain would not hydrogen bond at all, leaving the product to diffuse out of the active site on its own. Therefore, the side chain of Lys167 plays an important role in the increasing the rate of catalysis of alkaline phosphatase by encouraging the hydrolyzed product to diffuse out of the active site quickly.

Conclusion

Examination of the kinetic parameters of wild type *E. coli* alkaline phosphatase, K167A mutant AP, and K167S mutant AP indicate that Lys167 plays a role in determining the rate of catalysis of hydrolysis of a phosphate ester. Experiments comparing the kinetic parameters of transferase and hydrolysis activity of wild type, K167A, and K167S mutant alkaline phosphatases suggest that Lys167 does not participate in substrate binding and does not interact with the phosphate acceptor. The optimal pH of alkaline phosphatase is around 8.0-8.5, implying that the side chain of Lys167 is constantly protonated, allowing it to hydrogen bond to other molecules. The ability of the side chain of Lys167 to create hydrogen bonds and its position near the active site allows it to help the phosphate product diffuse out of the active site. In the case of K167A, alanine is unable to hydrogen bond to the product, lowering the rate of catalysis. In K167S, serine is able to hydrogen bond to the product, but cannot do so as effectively as lysine

because the side chain of serine is shorter than the side chain of lysine. Despite the mutations of this residue, both K167S and K167A are able to catalyze hydrolysis of a phosphate ester, suggesting that Lys167 is not a necessary residue for catalysis, but greatly increases the rate and efficiency at which alkaline phosphatase is able to function.

References

1. Koutsioulis, D., Lyskowski, A., Maki, S.; "Coordination sphere of the third metal site is essential to the activity and metal selectivity of alkaline phosphatases."; *Protein Sci.*; 2010.

2. Holtz, K., Kantrowitz, E.; "The mechanism of the alkaline phosphatase reaction: insights from NMR, crystallography and site-specific mutagenesis."; *FEBS Letters*; 1999.

3. Coleman, J.E.; "Structure and Mechanism of Alkaline Phosphatase"; *Annu. Rev. Biophys. Biomol. Struct.*; 1992.

4. Kim, E.E., Wyckoff, H.W.; "Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis."; *J. Mol. Biol.*; 1991.

5. Butler-Ransohoff, J.E., Kendall, D.A., Kaiser, E.T.; "Use of site-directed mutagenesis to elucidate the role of arginine-166 in the catalytic mechanism of alkaline phosphatase"; *Proc. Natl. Acad. Sci. USA*; 1988.

6. Chaidaroglou, A., BrezinsKi, D.J., Middleton, S.A., Kantrowitz, E.R.; "Function of Arginine-166 in the Active Site of Escherichia coli Alkaline Phosphatase"; *Biochem.*; 1988.

7. Wang. J., Kantrowitz, E.R.; "Trapping the tetrahedral intermediate in the alkaline phosphatase reaction by substitution of the active site serine with threonine"; *Protein Sci.*, 2006.

8. Chemspider; TRIS hydrochloride; http://www.chemspider.com/Chemical-Structure.84470.html; 2016.

9. Chemspider, MOPS; http://www.chemspider.com/Chemical-Structure.63972.html; 2016.

10. Hull, W.E., Halford, S.E., Gutfreund, H., Sykes, B.D.; "Phosphorus-31 nuclear magnetic resonance study of alkaline phosphatase: the role of inorganic phosphate in limiting the enzyme turnover rate at alkaline pH"; *Biochem.*, 1976.

11. Capp, C.; "Alkaline Phosphatase Activity: Determination of Michaelis-Menten Parameters (Km, kcat, Vmax)"; *BCHM464 Lab Manual*; 2016.

12. Gasteiger E., Hoogland C., Gattiker A., et al.; "Protein Identification and Analysis Tools on the ExPASy Server"; *The Proteomics Protocols Handbook, Humana Press*, 2005.