Exposure of Bovine Intestinal Alkaline Phosphatase to Calcium Ions Found to Increase Dephosphorylation of *para*-Nitrophenolphosphate

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Abstract

Bovine intestinal Alkaline phosphatase (AP) is a homodimeric enzyme that relies on one Mg^{2+} ion per subunit to function properly. We knew that Mg^{2+} has reversible association with the enzyme and we assumed that it could be replaced by Ca^{2+} because both ions have the same valence electron configuration. However, it was unknown whether this replacement would induce a large enough conformational change to affect enzymatic activity, or whether it would increase or decrease activity. We hypothesized that the Calcium-incubated enzyme would have a lower reaction velocity than the control, and we tested this hypothesis by comparing the average dephosphorylation rates of pNPP by the Calcium-incubated AP and by the control AP. Our results indicated a statistically greater reaction velocity for AP incubated with Calcium than the control AP. While these results could have indicated that Calcium can replace Magnesium and the resulting conformational change increased the enzyme's efficiency, it is possible that a precipitate formed during the assay which could have significantly influenced our absorption readings. Because of the flaw in our assay, we do not have enough confidence to either support or reject our hypothesis based on our results.

Introduction

Bovine intestinal Alkaline Phosphatase (AP) is an enzyme that cleaves terminal phosphate groups off a variety of substrates, and in the case of this study, para-nitrophenylphosphate (pNPP). Bovine intestinal AP is structurally similar to human placental

AP, and intestinal AP from rats, see figure 1 (*Homo sapiens* cover: 96%, E:0; Rattus *norvegicus* cover: 95%, E: 0) (NCBI 2017).

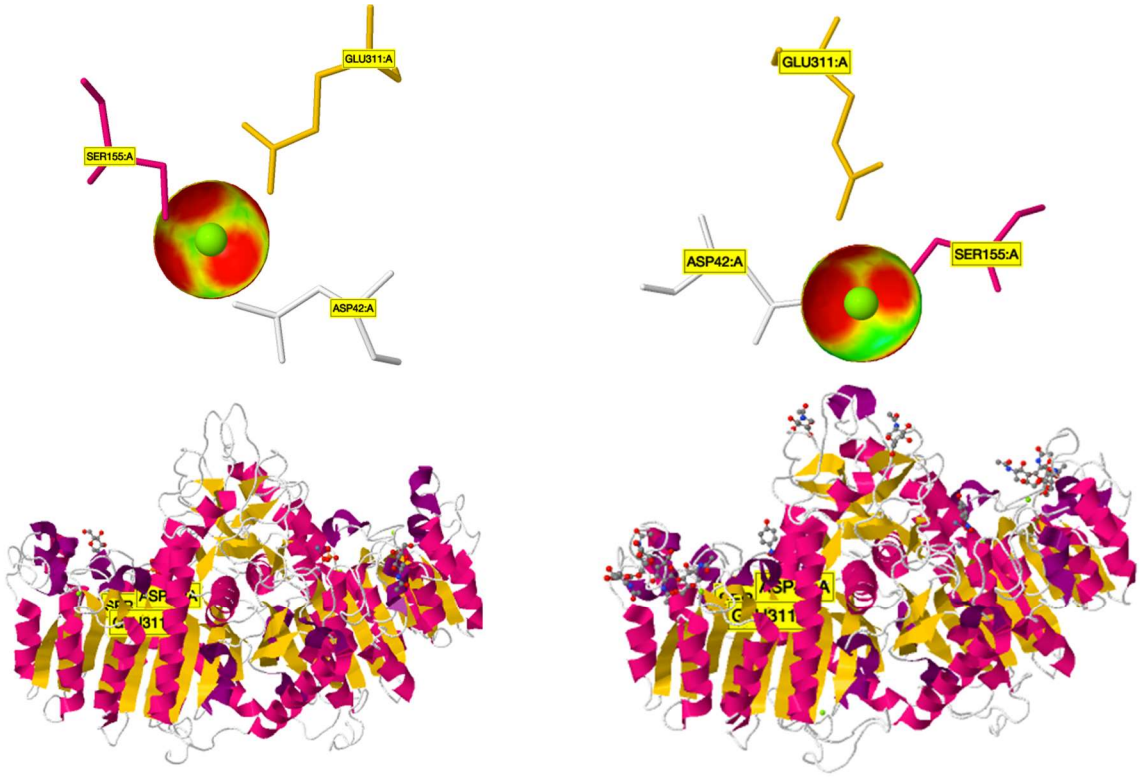

Figure 1: Mg coordination (top) and overall structure (bottom) of Human placental alkaline phosphatase (left) and Rat intestinal alkaline phosphatase (right) showing conserved Mg coordination site and overall structure between organisms.

X-Ray Crystallography of both Rat intestinal and Human placental AP indicate the same homodimeric structure, meaning that both APs are composed of two homologous peptide chains (Ghosh et al. 2013; Kim and Wyckoff 1991; Le Du et al. 2001). Crystallography also revealed that AP is a metalloenzyme that coordinates with two Zn^{2+} atoms and one Mg^{2+} at each active site (Ghosh et al. 2013; Kim and Wyckoff 1991; Le Du et. Al. 2001). Knowing that enzymes are so sensitive to their environment, and that AP coordinates with Zinc and

Magnesium, we seek to know whether the presence of metallic cations can inhibit the enzyme's function.

Enzymatic activity in alkaline phosphatase in pig kidney was found to be dependent on the presence of Mg^{2+} cation (Ahlers 1974). Because of metal cations ability to coordinately bond to multiple polar amino acids, they play a role in maintaining the structure of the enzyme, which is critical for its activity (Maret and Li 2009; Ahlers 1974). The structural support Zinc and Magnesium cations provide for AP through coordinate bonds to amino acid residues can be seen below (figure 2).

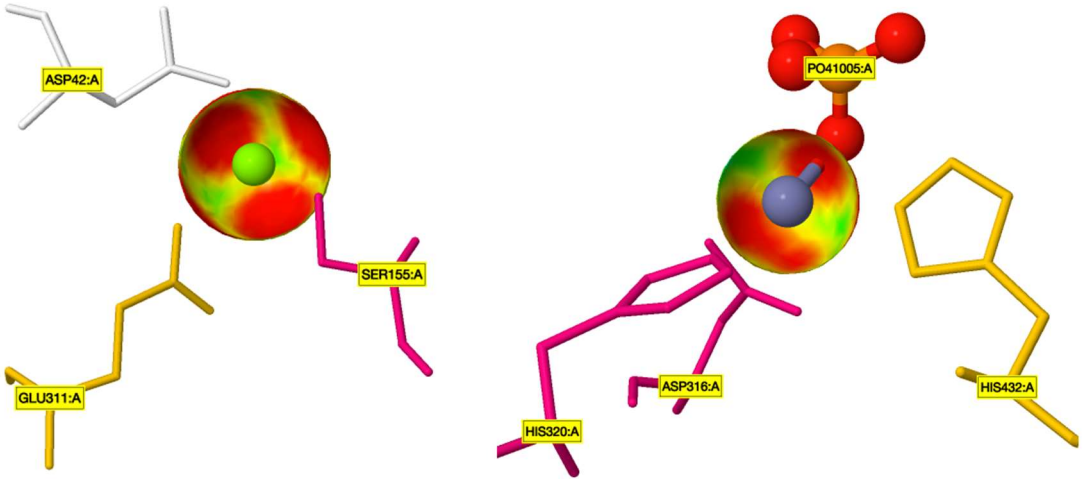

Figure 2: Coordination spheres of Mg (left) and Zn(right) in Human placental AP. Mg coordinates with SER, GLU and ASP, while Zn coordinates with ASP, HIS, HIS and an inorganic phosphate group (Le Du et al. 2001; RCSB 2017).

Neither Mg^{2+} or Zn^{2+} are permanently coordinated with AP (eq. 1), and have reversible association (Ahlers 1974).

$$E + Mg \underset{k_{-Mg}}{\overset{k_{+Mg}}{\rightleftharpoons}} E\text{-}Mg \qquad \textbf{(1)}$$

Mg has a disassociation constant of approximately $9.8x10^{-7}$ M at 30°C, this means that the enzyme is coordinated with the Mg^{2+} ion six orders of magnitude more than it is not (Ahlers 1974). Since this dissociation does happen at a measurable rate, it is reasonable to assume that another cation could take the place of Magnesium in the enzyme. In fact it has been found that the presence of various divalent and trivalent metals can inhibit the function of AP (Rej et al. 1980). Although the mechanism for inhibitions for the cations tested is not fully understood and disagreed upon by multiple authors, it is believed that some (Fe^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}) compete with Magnesium for coordination in the enzyme, some (Cr^{3+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}) interfere with the enzyme at other sites, and some ($[Be(OH)_4]^{2-}$ and $[VO_4]^{3-}$, formed after complexing with water) competitively compete for the active site since they resemble a phosphate group (Rej et al. 1980; Cathala et al. 1981).

While Rej et al. and Cathala et al. tested multiple metals, they did not test the divalent alkaline earth metal, Calcium. Calcium is the next period element in the same group that Magnesium is in, meaning it has the same valence shell configuration, but a larger ionic radius. Because it has the same valence shell configuration Magnesium, we assume that Calcium would be the most likely to compete with Magnesium for association in the enzyme. While it is known that metal ions can lower AP's efficiency, it is still unknown whether Calcium's larger ionic radius will change the conformation of the enzyme enough to inhibit the enzyme. We hypothesize that exposing AP to a Calcium containing salt will significantly lower the rate of the dephosphorylation reaction.

We will test this by comparing the average reaction rate of AP incubated with Calcium Chloride and control AP (no exposure to Calcium Chloride) on the dephosphorylation of pNPP to pNP

Methods

Pilot study

A series of pilot studies were conducted to determine a concentration that would significantly affect the activity of the AP enzyme to inform our primary study. Based on the results of the pilot studies, a concentration of 6.8mM $CaCl_2$ was chosen.

Experimental Protocol

Stock solutions of 10mM $CaCl_2$, .5M tris-HCl at pH 8.7, 2mM pNPP, and 1 M NaOH were prepared (Becker n.d.). Forty test tubes (twenty control, twenty experimental) were prepared with 5mL of solution containing .05M tris-HCl, pH 8.7, 125 µM AP, and DI water. The experimental tubes also contained 6.8 mM $CaCl_2$ (determined from pilot study) and were allowed to incubate for 20 minutes (to allow for dissociation of the Magnesium). After incubation, the tubes were placed in a 35°C water bath until the temperature equilibrated, then reaction was initiated by the addition of 76µL of the 2mM pNPP, resulting in a final concentration of .03mM. The reaction was allowed to run for 3.5 minutes and stopped with the addition of 1mL 1M NaOH. Absorbances were calculated for all tubes and reaction velocity was calculated via eq. 1. We also prepared control and experimental reagent blanks which contained 5mL of solution with .05M tris-HCl, pH 8.7, DI water and .03 mM pNPP (to correct for small absorbance of pNPP), as well as 6.8 mM $CaCl_2$ for the experimental blank.

$$v = \frac{A}{ab} * \frac{V}{t} \qquad \textbf{(1)}$$

Equation 1: Conversion of absorption to reaction velocity, where A is absorption at 400nm, a is the molar extinction coefficient of AP 18.4 (L/(mmol*cm), b is the path length (1cm), V is the volume of the solution (6.076mL) and t is the reaction time (3.5 min).

Data, research and calculations were all kept in accordance with our Data Management Plan (Appendix B).

Data Analysis

Descriptive statistics including mean and standard deviation were calculated for each group, then the statistical difference of the means was analyzed with a two sample bootstrap test (Appendix A). A quantile-quantile plot against a normal distribution as well as histograms of the distributions were used to assess the normality of the samples.

Results

Reaction velocity calculations revealed a control mean of .0235 µM min^{-1} with a standard deviation of .0023 µM min^{-1}, and an experimental mean of .0331 µM min^{-1} with a standard deviation of .0047 µM min^{-1}.

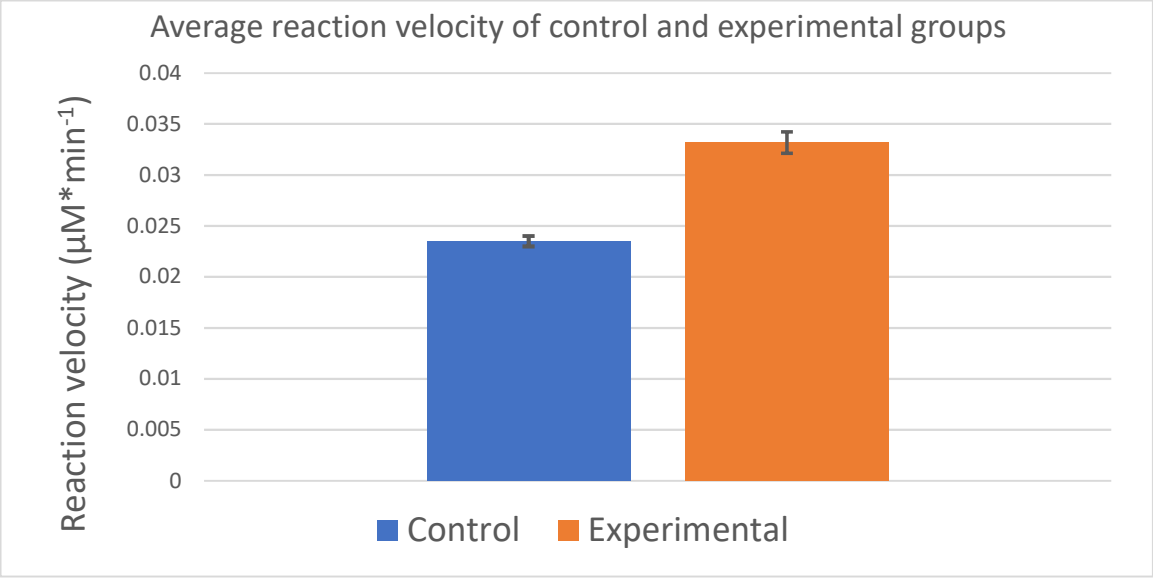

Figure 3: Average reaction velocity of AP exposed to 6.8mM $CaCl_2$ vs. control group with standard error bars. Control standard error +/- .000515 µM min^{-1}, Experimental standard error +/- .001052 µM min^{-1}.

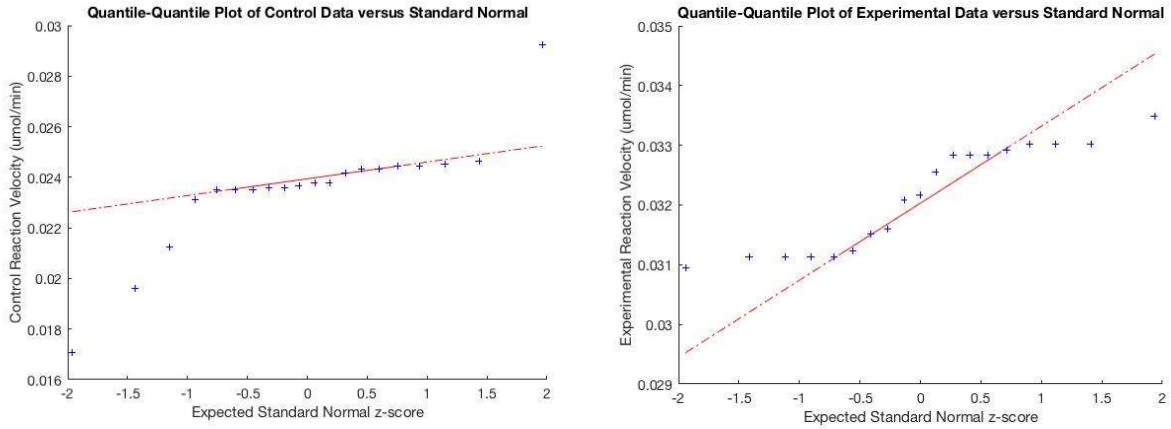

Figure 4: Quantile-Quantile plots of Control (left) and Experimental (right) samples vs Standard Normal Quantiles, reaction velocity values (y axis) were plotted against their expected z-score from a normal distribution. Data deviate significantly (almost systematically in the experimental data) from the ideal line (red) indicating the data are not normal.

The data show a significant difference between means, however, each sample has a significant number outliers and the data do not appear normal. Because the data were not normal, we chose to run a two-sample bootstrap test (see Appendix A). After running the bootstrap test, we found a statistical significance between the two means (p=0, T=15.608).

The control and experimental reagent blanks were found to be negligible, so we did not use them to correct the absorbances of either group.

Discussion

In this study, we were attempting to characterize the effects of Calcium ions on Bovine Intestinal Alkaline Phosphatase activity, namely inhibition. Our data showed an apparent statistically significant *increase* in dephosphorylation reaction velocity of AP in the presence of a Calcium salt. While this is not the effect we expected, this data could still support our assumption that Magnesium can dissociate from AP and that Calcium can take its place. Secondly our data also suggests that the substitution of Calcium for Magnesium in the active

site of AP can affect the enzymatic activity of AP, most likely through a structural change in the enzyme (Maret 2009).

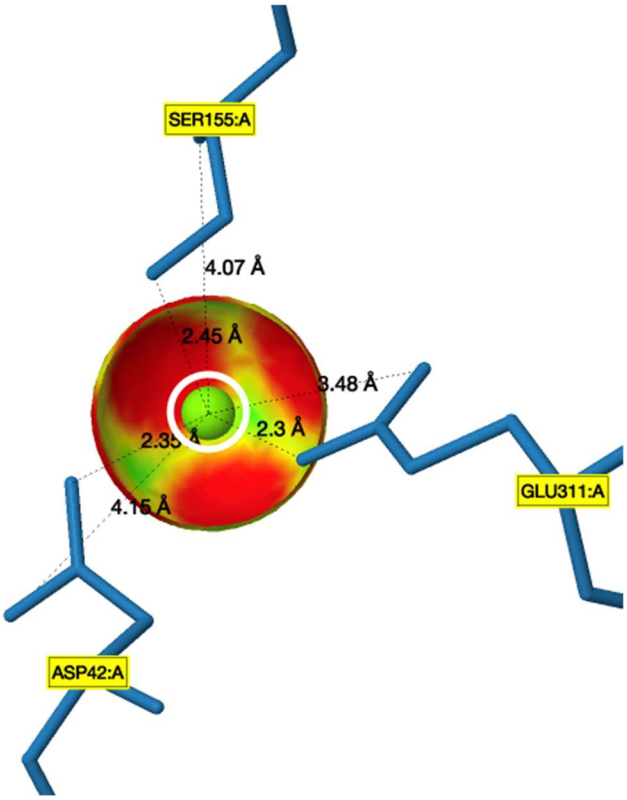

Figure 5: Coordinative bond distances determined by X-Ray crystallography between amino acid residues and Mg^{2+} (green) ion in Human placental AP (Le Du et al. 2001). Calcium ionic radius shown in white outside of Mg^{2+} sphere.

The ionic radius of Magnesium is approximately .72 Å, whereas the ionic radius of Calcium is approximately 1.0 Å and the radius of Zinc is approximately .74 Å. We attribute the change in enzymatic activity to the conformational change that can be seen in figure 5: Since Calcium is larger than Magnesium, the liganding amino acids must lie further away from Calcium than Magnesium, thus changing the immediate tertiary structure of the enzyme around the Calcium site (Katz et al. 1996). We believe that this change in conformation lowered the transition sate energy of the substrate, thus lowering the reaction's activation energy and increasing the reaction velocity.

While it is impossible to know from our data whether it was truly Magnesium being replaced by Calcium in AP instead of the Zinc ions, it is more likely that due to the electronic similarity of Calcium and Magnesium that the Calcium was indeed replacing the Magnesium.

Future work would need to be done to more fully support our assumption as well as determine whether the increase in enzymatic activity in the presence of Calcium is associated with Calcium ions replacing Magnesium or Zinc ions. Specifically, after a long incubation period for a solution of AP in aq. Calcium chloride, the solution could be assayed for the presence of Magnesium and Zinc.

Our data were independent between groups and mostly independent within groups. Measurements were taken with the exact same protocol and calibrated spectrophotometer between groups, but within groups, we staggered each reaction by 30s meaning that the first tubes had a shorter incubation time (approx. 20 min) while the last tubes had a longer time (approx. 30 min). However, we did not see a significant change between the first and last tubes in either the control or the experimental groups, and the in-group variation was very low.

By using a spectrophotomer calibrated to DI water and using the same cuvette for every measurement, we significantly reduced bias and dependence in our data collection. Our measurements relied on a quantitative source, rather than a qualitative one, which eliminated bias in human interpretation, and by using the same cuvette every time we ensured each measurement was independent of each other.

While our data did show a statistical significance between treatments, it is difficult to rely on these results being accurate because of a flaw in our assay. The base we used to quench the reaction, NaOH, can form a precipitate with Calcium: $Ca(OH)_2$. While we didn't

observe a precipitate in our experimental test tubes, it is impossible to know to what extent this precipitate formed and influenced our absorption readings. Because of the possible inaccuracy of our data, it is difficult to make any biological conclusions based on the interpretation above.

Our study compared 20 replicates of control reactions with 20 replicates of experimental reactions, had minimal bias and all the data were independent, but because of the possibility of the precipitate influencing our absorbance and therefore reaction velocity results, we cannot support or reject our hypothesis.

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Appendix A: Two sample Bootstrap test supplemental MATLAB code

```
function [ pval ] = bootstrap2pop(dat1,dat2,numBoot)
%bootstrap2pop: 2 sample bootstrapp t-test
%   dat1=sample 1 input, dat2=sample 2 input, numBoot=number of resamples
%   returns p-val that sampling dist. would take on a value as extreme or
%       more extreme than the observed T-val
truemean = mean(dat1)-mean(dat2);
    n1 = length(dat1);
    n2 =length(dat2);
tObs = abs((mean(dat1)-mean(dat2))/(sqrt(var(dat1)/n1+var(dat2)/n2)));
disp(tObs);
low = [];
high = [];
for i=1:numBoot

    samp1 = datasample(dat1,n1);
    samp2 = datasample(dat2,n2);
    samp1mean = mean(samp1);
    samp2mean = mean(samp2);
    samp1var = var(samp1);
    samp2var = var(samp2);
    thisObs = (samp1mean-samp2mean-truemean)/(sqrt((samp1var/n1)+(samp2var/n2)));
    if thisObs<(-tObs)
        low = [low thisObs];
    end
    if thisObs>tObs
        high = [high thisObs];
    end
end

pval = (length(low)+length(high))/numBoot;
end
```

Figure 1: MATLAB code to reproduce bootstrap simulation method. Resample a size of n1, n2 respectively for the two samples numBoot times. Generate a T-score from each sample, with >5000 resamples, a sampling distribution is approximated. Pval is the number of resamples that generate a T-score more extreme than the observed T-score divided by the number of samples.

Appendix B: Data Management Plan

Data files will be saved as XLSX files with raw data kept separately from calculations and

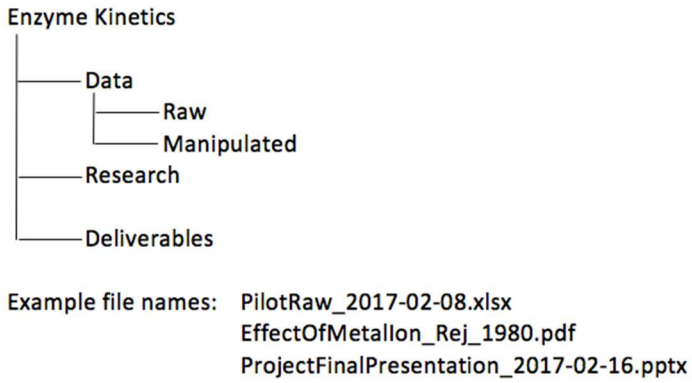

Figure 1: Outline of file structure for project and example file names.

data manipulation. Files will be stored on each group member's computer, as well as on my backup flash drive and on OneDrive provided through UW-Madison. The file structure for the project as well as example file names can be seen in figure 2. Raw data files will be kept separate from interpreted data and statistical tests. Raw data and interpreted data will be kept in XLSX format and converted to a non-proprietary CSV upon completion of the project.