

The effect of calcium on conformational change of thimet oligopeptidase (EC 3.4.24.15)

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I. Introduction:

Calcium has become widely known as a necessary component in building strong bones. But many might overlook the fact that it is a type of metal, important not only in making us stronger, but also in a myriad of chemical reactions that happen in the human body. These chemical reactions are mostly very slow on their own, and would likely not happen if not for the presence of enzymes. Enzymes are responsible for catalyzing, or speeding up, chemical reactions, by lowering the amount of energy needed for the reaction to occur. Metals like calcium can interact with enzymes in many ways, sometimes helping, and sometimes slowing down, the enzyme's process of catalyzing a reaction.

The enzyme TOP (thimet oligopeptidase) is in the family of peptidase enzymes, which means that the type of reaction it catalyzes is the cleavage (or cutting) of peptides. TOP is unique in that, unlike many enzymes, it is able to work on many different substrates; most peptidase enzymes are limited by their shape, or conformation, to cleaving only a certain peptide. Studies about TOP are useful both in that they increase knowledge about the enzyme itself, and also because there are some analogous enzymes that the information may also apply to. TOP is known to cleave peptides that are from 5 to 17 amino acids in length. Many peptides in the body act as chemical messengers, which allow for cell-to-cell communication. By cleaving these peptides, TOP can effect if the chemical message is turned on or off.

The physiological roles of TOP are still under investigation. TOP has been found in the kidneys, lungs, brain, pituitary, testis, liver, and spleen. It is known to play a part in reproduction and homeostasis of the heart. Also, TOP cleaves an important peptide involved in Alzheimer's disease, so understanding this enzyme and how it works could be important in new findings about this so far incurable disease. Investigating the role of calcium on enzyme conformation and substrate specificity is important in investigating the mechanism and specific function of TOP.

The fact that TOP is effective on a variety of peptides has provoked much investigation into its mechanism for cleaving. The conclusions that have been made suggest that it functions like a clamshell. The active site of an enzyme is the region on its surface where the chemical reaction takes place, where the peptide binds to the enzyme. In its unbound form, the active site of TOP is like an open shell. When a peptide enters the active site, the enzyme closes around it. In this closed form, the chemical reaction takes place. The enzyme then opens, releasing two shorter peptides. This shape change that TOP undergoes is called a 'conformational change'. This mechanism is important to the enzyme's ability to be able to cleave different peptides.

TOP is secreted from cells and is seen in the extracellular environment. Previous studies have shown the calcium has an effect on its secretion from cells, and also on the cleavage site (between which two amino acids the peptide is cleaved). However, the exact role of calcium on the enzyme, and more specifically on its mechanism, is unclear. This research attempts to draw a correlation between calcium and TOP's conformational change, and expands to more generally investigate the mechanism by which TOP cleaves peptides. This was done by observing how the enzyme works on different substrates (activity assays), how it unfolds under different conditions (denaturation), and how its activity changes at different pH's.

II. Experimental Procedure:

A. Materials: Tris(hydroxymethyl) aminomethane, DTT, and TCEP, purchased from FisherBiotech (Fair Lawn, NJ, USA), Guanidine Hydrochloride purchased from MP Biomedicals (29525 Fountain Pkwy., Solon, Ohio, USA), Urea, Bis-tris, CHES, Potassium chloride, and Calcium chloride, purchased from Sigma-Aldrich (PO Box 14508, St. Louis, MO, USA), Glycerol purchased from Acros (New Jersey, USA), wild type TOP provided by Dr. Marc J. Glucksman at the Rosalind Franklin Institute (Chicago), inhibitors Angiotensin 1-7 and Neurotensin 9-13, and substrates MCA and MCA-Bk purchased from Bachem (4416 Bubendorf, Switzerland), substrate MCA-neurotensin (NH₂) purchased from Anaspec, Inc. (Fremont, CA, USA).

B. Equipment: 2000 and 100uL micro-cuvettes from Starna Cells, Inc. (Atascadero, CA, USA), Agilent 8453 diode array UV spectrometer, Eppendorf PhysioCare micropipettes and tips, fluorometer, Oakton pH/CON 150 series pH probe, and Perkin Elmer Luminescence spectrometer LS50B fluorometer.

C. Substrate preparation: All substrates were prepared by the same method. Dry stock was diluted with DMSO. Serial dilutions with DMSO made to prepare 6 to 8 different substrate concentrations. Concentration of the lowest found by measuring absorbance at 366 nm and calculating concentration based on the molar extinction coefficient $17.3 \text{ mM}^{-1}\text{cm}^{-1}$ (blank was plain DMSO). Subsequent concentrations back calculated based on how serial dilutions were carried out (usually a 50-50 dilution each time).

D. Enzyme preparation: Enzyme stock removed from -80°C freezer and allowed to thaw. 50uL buffer with 1mM TCEP added to enzyme stock, and this mixture centrifuged through filter. 10uL of this stock added to 110uL plain buffer. UV-vis taken of this mixture,

concentration calculated using the molar extinction coefficient $73.11 \text{ mM}^{-1} \text{ cm}^{-1}$ (blank was 120uL plain buffer with 1mM TCEP). Enzyme diluted with buffer to concentration needed for assay/ denaturation.

E. Activity assays: Appropriate buffer (either 125mM KCl or 85mM KCl & 20mM CaCl_2) prepared with 25mM Tris, 1mM TCEP, and pH 7.80. KCl was added to the CaCl_2 buffer to keep the ion strength constant, as ion strength can change enzyme activity. TCEP is a reducing agent, and keeps the protein from aggregating due to the formation of disulfide bonds, which inactivate the enzyme. To perform the assay, 1980uL buffer was mixed with 10uL of substrate. The enzyme was then added, for a final enzyme concentration of 0.0005uM, and data collection quickly started on the fluorometer. This procedure was repeated in duplicate at each substrate concentration. Parameters used for the fluorometer: excitation wavelength 325nm, emission wavelength 400nm, excitation slit 5.0nm, emission slit 6.0nm, duration 150 seconds, data interval 1 second.

F. Inhibitor studies: Inhibitors Angiotensin 1-7 and Neurotensin 9-13 were used in activity assays with the substrate MCA. The same procedure as (e) was used, except that 20uM NT9-13 or 25uM Ang 9-13 was added to the buffer before adding enzyme.

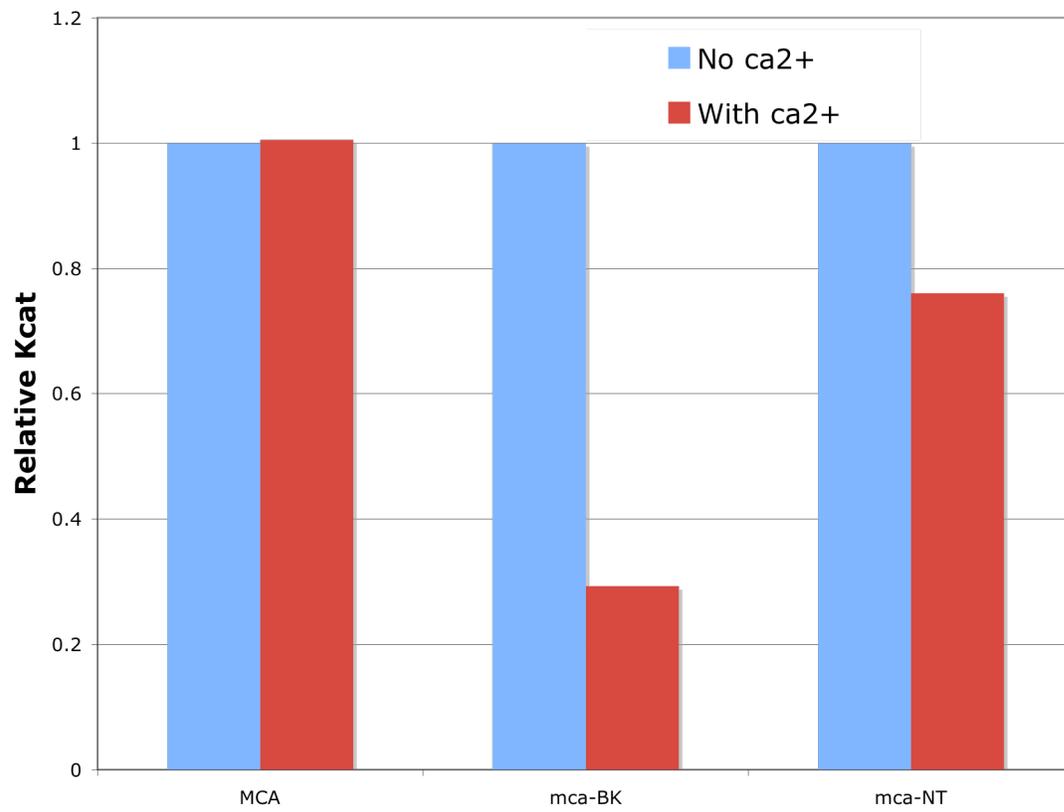
G. Denaturation: Buffer prepared with 25mM tris, 125mM KCl or 85mM KCl & 20mM CaCl_2 , 10% glycerol, 9M urea or 0M urea, and 5mM DTT, pH 7.80. 150uL samples prepared with varying ratios of urea and plain buffer. Enzyme was added for 0.5uM TOP in each sample. Blanks were prepared in the same manner, replacing the 5uL of enzyme with plain buffer. Samples were made in duplicate. Denaturation with inhibitor was performed in the same manner, with inhibitor added directly to each sample. Samples were allowed to equilibrate for 30 minutes, and then each ran on the fluorometer. Fluorometer parameters: excitation wavelength

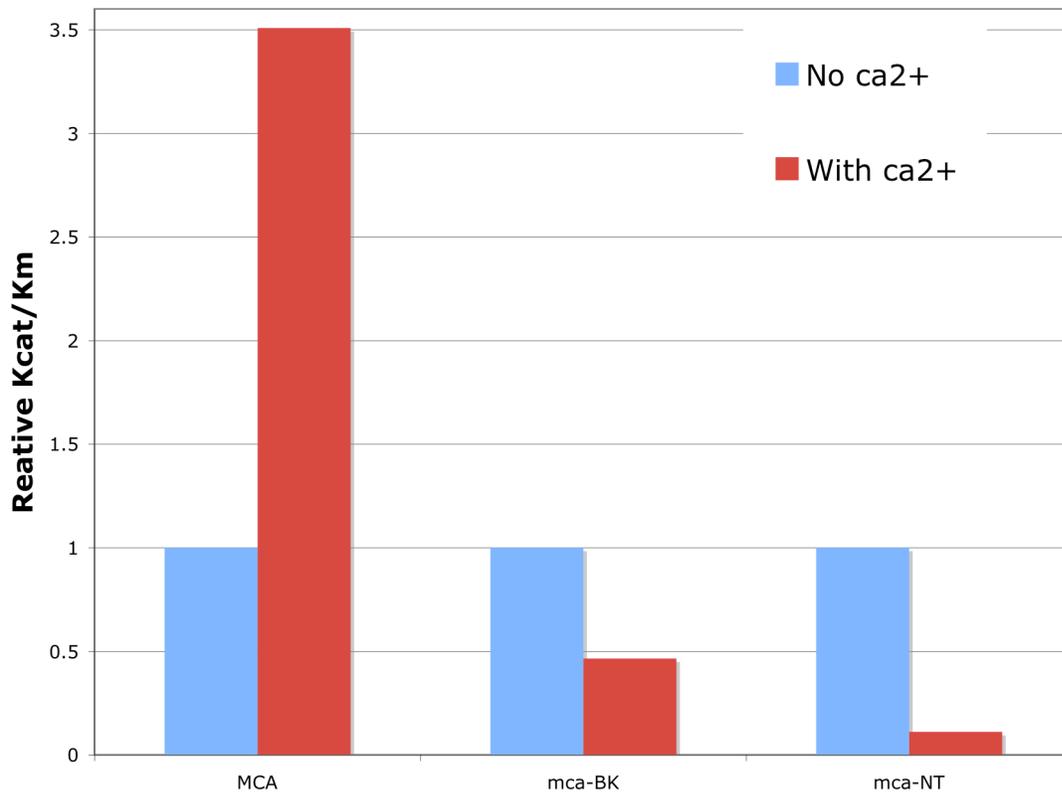
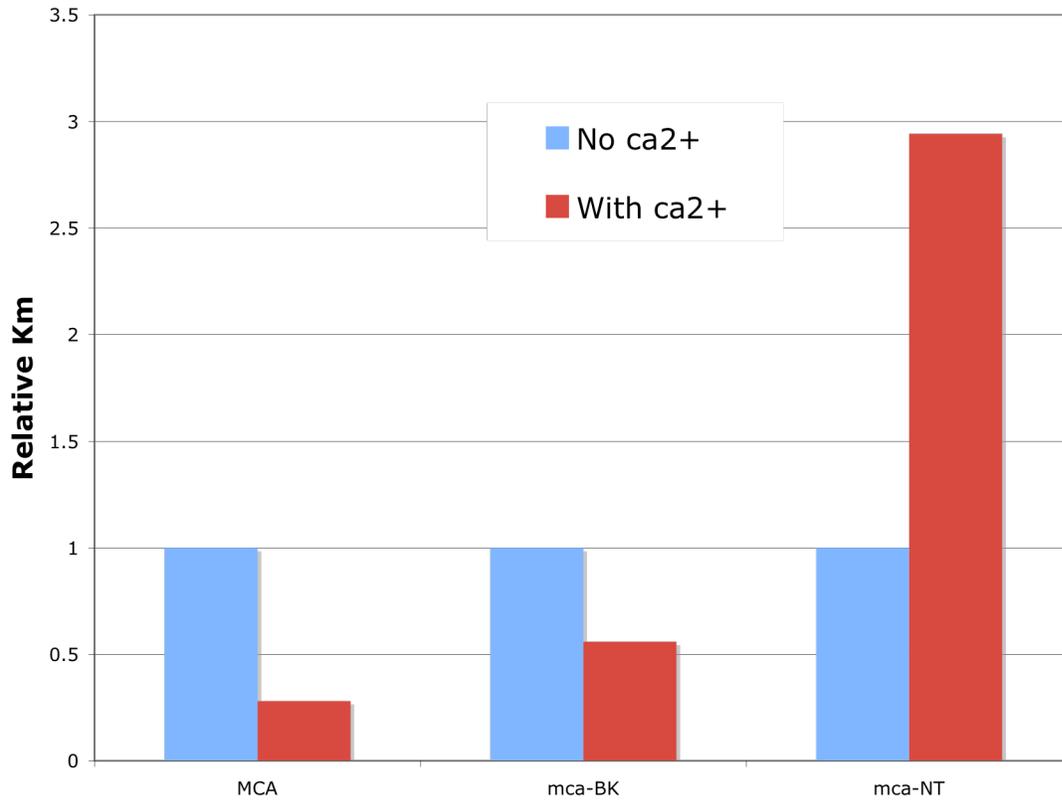
295nm, start @ 320nm, end @ 420nm, excitation slit 5nm, emission slit 10nm, scan speed 200nm/min, 3 accumulation scans. Urea concentration was found by taking the refractive index of each sample, and using the online urea calculator at <http://depts.washington.edu/bakerpg/guhel.html>, which converts refractive index of the sample to urea concentration.

H. pH profile: Similar procedure used as activity assays, with mca-BK substrate. Assays were performed at a pH range 4.99-9.87. pH was measured both before and after the assays to check that the buffering system was effective. The assays at pH 4.99 and 6 were performed in 25mM bis-tris and those at 9.01, 9.5, 9.87 in 25mM CHES, as opposed to 25mM tris. These buffers were necessary to maintain pH at the higher and lower pH's.

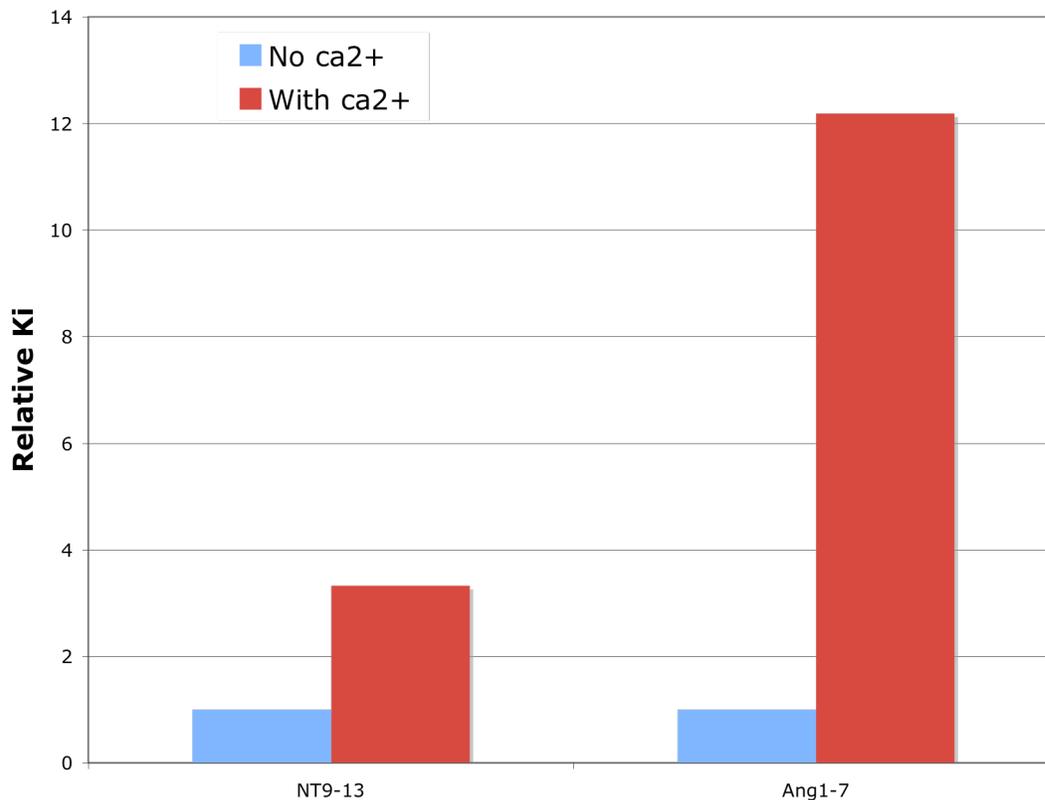
III. Results

A. Activity assays: Kinetic parameters were calculated from assays with and without calcium for the 3 substrates MCA, mca-BK, and mca-NT. The raw data was exported into Excel and average rates were calculated. The program Plot was used for non-linear curve fitting of the data points to a Michaelis-Menten curve, which is substrate concentration vs. rate. This curve fit gave values for K_{cat} and K_m (K_{cat}/K_m was easily calculated from there.) Values without calcium were standardized to 1 in order to compare the effect of calcium on enzyme activity. Since the different substrates have inherently varying kinetic parameters, this standardization was necessary. Results are shown in the following charts.



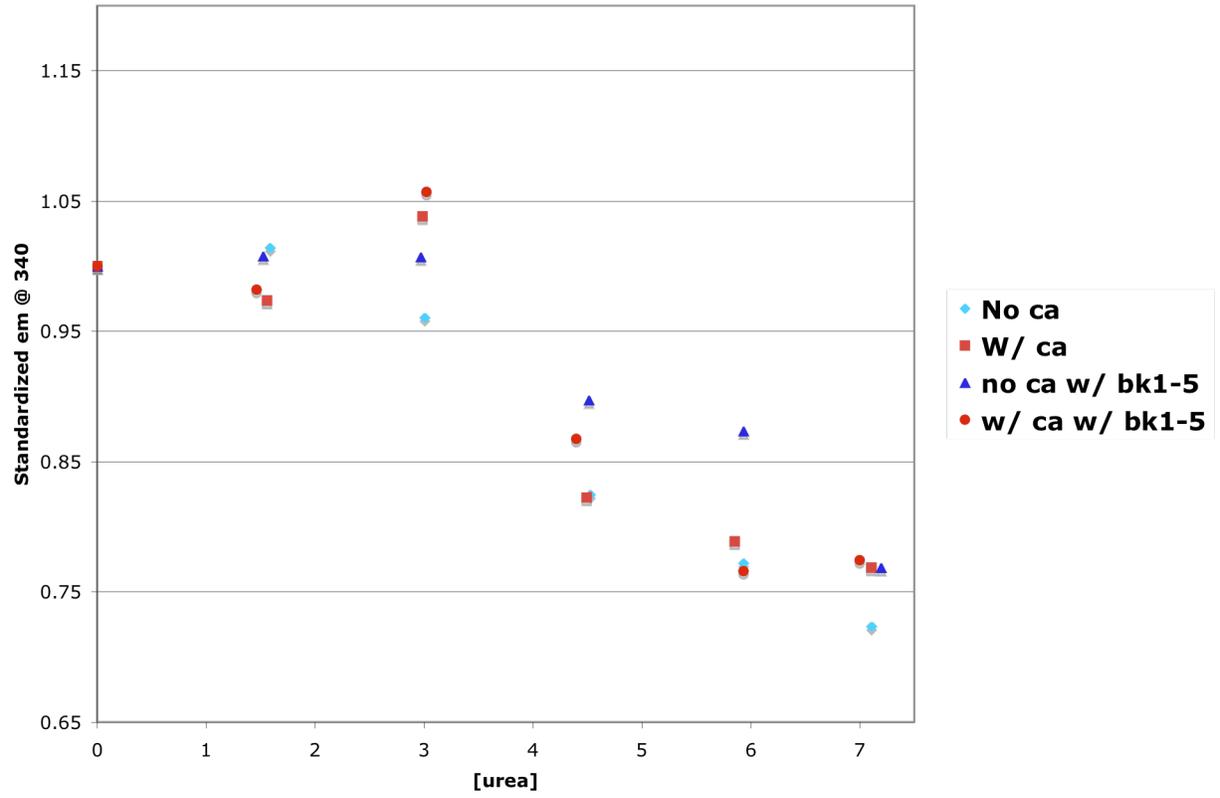


B. Inhibitor studies: Data from the assays without calcium were standardized to 1, similar to activity assays. The following chart displays relative K_i values. K_i is the inhibitor constant, which is the concentration of inhibitor required to decrease the reaction rate to half. Therefore, a higher K_i value means that a higher inhibitor concentration is needed, and therefore the inhibitor is not working as well. A lower K_i , conversely, means that the inhibitor is binding more strongly to the enzyme's active site. These data show that the NT9-13 inhibitor bind better in the presence of calcium than does the Ang1-7 inhibitor, although both bind less with calcium than without.

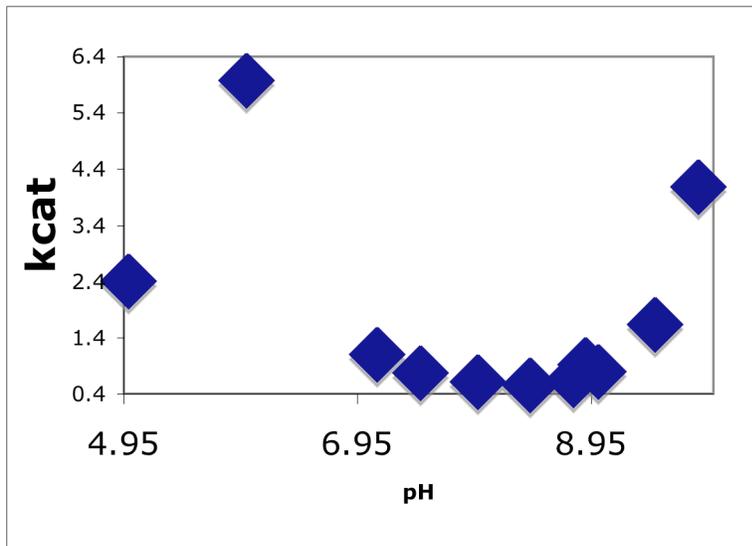
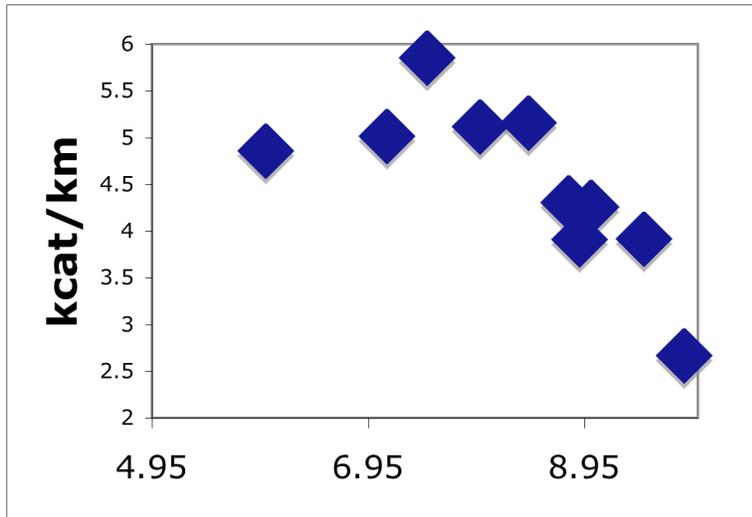


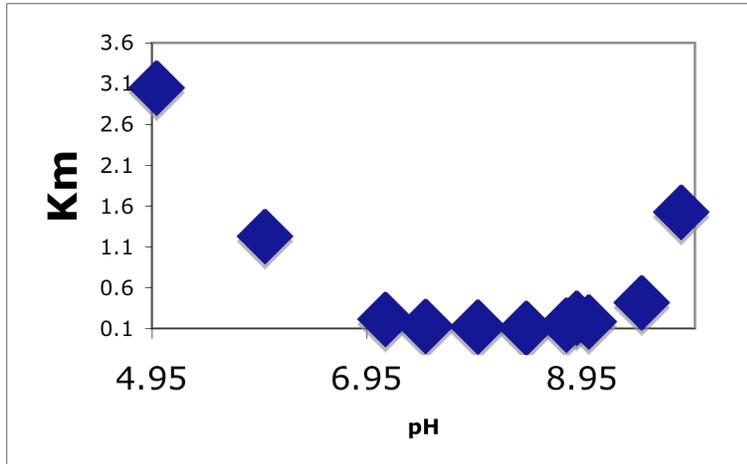
C. Denaturation: Data was plotted as concentration of urea versus emission @ 340. Data was standardized by enzyme concentration. Spectra of undenatured protein with and without calcium were identical, so the data was the standardized to 1, in order to compare the shapes of

the curves.



D. pH profile: In order to compare results with the pH profile previously done on MCA substrate, plots were made of pH vs. K_{cat} , pH vs. K_m , and pH vs. K_{cat}/K_m .





IV. Discussion

A. Activity Assay:

The kinetic parameters being considered are K_{cat} , K_M , and K_{cat}/K_m . K_{cat} is the turnover number; the number of substrate molecules converted to product per unit time (reported in s^{-1}). K_m is related to the affinity of the enzyme to the particular substrate. A higher K_m indicates that the affinity is less. K_{cat}/K_m is a second order rate constant that is often used to compare catalytic efficiency of the same enzyme under different conditions. It has been previously assumed that the substrate mca-BK does not require the enzyme to undergo its conformational change in order to be cleaved, whereas MCA substrate does. This means that the enzyme is able to use difference mechanisms for cleaving for different substrates. This was used as the original basis upon which to compare enzyme activity with and without calcium, in attempt to draw a correlation between the mechanism and the presence of calcium. The activity data show that for MCA, the presence of calcium increases binding affinity and overall activity. For mca-BK, calcium still increases the binding affinity, but it decreases the turnover rate, which leads to an overall lower activity. For the mca-NT substrate, however, calcium decreases binding affinity and turnover rate, which makes the enzyme efficiency much less. The differences in the kinetic parameters between MCA

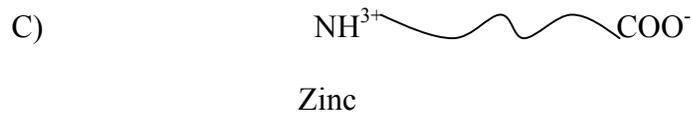
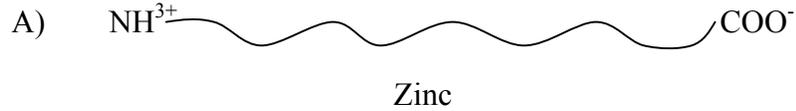
and mca-BK offer a possible correlation between calcium and enzyme conformation. Under the same conditions, the overall enzyme efficiency is opposite for the different substrates, while the effect of calcium on binding affinity does not change. The mca-NT substrate is a special case. The C-terminal of this peptide has been modified from a COO⁻ to NH₂, which changes how it interacts with the enzyme's active site. The previous negative charge is now neutral. A new version of this substrate with the normal COO⁻ on the C-terminus has been ordered, and assays with this substrate will show specifically how the C-terminus interacts with the enzyme, and how changing this influences the kinetic parameters. These assays have yet to be performed.

Molecular modeling on a computer program can also be used to predict how this C-terminus change will affect how the substrate fits in the enzyme, and how repulsions or interactions help of hinder activity. Based on what was seen in the assays using this substrate, it is possible to hypothesize that changing the C-terminal to an uncharged residue is unfavorable, as both binding affinity and enzyme activity decreased. In order to confirm this, however, the unmodified substrate must also be used in order to compare the kinetic parameters between the two. Another possibility has to do with the residues of the mca-NT substrate. Both MCA and mca-BK are relatively non-polar and have mostly positively charged residues. Since calcium is found in the form Ca²⁺, it would only interact with negatively charged residues. Therefore, the MCA and mca-BK would not be inhibited by the calcium, because all residues are available to interact with the active site like normal. The mca-NT, on the other hand, has a negatively charged glutamate residue, which has the potential to interact with the calcium. When the substrate binds without calcium, the negatively charged residue has the ability to interact with a positive portion on TOP's active site. In the presence of calcium, then, the negative charge would be neutralized by calcium, and would not interact as well with the active site, therefore not binding as effectively

as before. This possibility can also be examined on a molecular modeling program to see which regions of the active site each substrate residue has the capability to interact with.

B. Inhibitor study

Inhibitors are basically chopped up substrates, which bind to the active site to prevent the enzyme from cleaving full-length peptides. When the inhibitor is bound, the enzyme is in its closed shape, and therefore inactive. In this study, the inhibitors used were either the first or second half of a substrate. The reason that there is a difference between the two is because of how the different inhibitors bind in the active site, and more specifically which end of them is interacting with the zinc molecule, which allows the inhibitors to bind. Each inhibitor also has an N-terminus and C-terminus, just like any peptide. Since the inhibitor Ang1-7 is the first half of the Angiotensin substrate, its C-terminus will interact with the zinc. Conversely, the NT9-13 (second half of the Neurotensin substrate) will interact with the zinc at its N-terminus (see representation below). The reason that calcium will have an effect on this is because it interacts with the negatively charged C-terminus, but not the positively charged N-terminus. In using an inhibitor like Ang1-7, there is potential for calcium to interact with this negative charge, making it so that the inhibitor can no longer bind as well to the active site. For NT9-13, this is not a problem, as the C-terminus of this inhibitor is free and not required to interact with the zinc. This is what was observed based on K_i values. The K_i for Ang1-7 with calcium is significantly higher, which means that it is not inhibiting as well. Although the K_i for NT9-13 with calcium goes up, suggesting that the inhibitor doesn't bind as well, there is a much larger increase for Ang1-7. In order to verify that this reasoning is correct, another inhibitor, like BK1-5, could be used. One would expect the K_i for this inhibitor to go up significantly, since it too has to interact with zinc at the C-terminus.



Generic representation of A) normal substrate, B) inhibitor (like Ang1-7), and C) inhibitor (like NT9-13), interactions with zinc. Note that if the C-terminus reacts with Ca^{2+} it will no longer be available to interact with zinc in the active site.

C. Denaturation

Analyzing denaturation data is different from activity assays because denaturation measures the fluorescence of the enzyme itself, rather than the substrate. TOP contains the fluorescent amino acid tryptophan, which will fluoresce differently under different conditions, as is observed in a denaturation curve. After standardizing the denaturation data, the important difference to note is

between 1 and 3M urea. Previous studies have shown denaturation curves with a slight increase in intensity at low urea concentrations, followed by a downward slope. This was thought to be due to a conformational change that the enzyme undergoes before denaturing. It might be speculated that this is the same conformational change that the enzyme undergoes in its cleavage mechanism. Note that both curves without calcium (blue) increase slightly around 1.5 M urea. Those with calcium, on the other hand, originally decrease, then increase at around 3M urea. It is also important to notice the difference between the blue points (no calcium) at 3M urea. The enzyme with inhibitor fluoresces significantly more at this urea concentration. This suggests that both calcium and inhibitor delay the conformational change, as it takes a higher concentration of urea to see this change. It is known that the addition of inhibitor serves to hold the enzyme in a closed position, which supports the idea that the conformational change in denaturation is delayed by the presence of inhibitor. Since this delay is also seen with calcium, it can be speculated that calcium too holds the enzyme in its closed conformation. To further investigate this possibility, it would be necessary to perform the denaturations at intermediate urea concentrations in order to get more detailed curves.

D. pH profile

The pH profile was performed more as a supplement to a previous study than an investigation involving calcium. Its results, however, are important to the assumption made for activity assays that the substrates MCA and mca-BK are cleaved by TOP through different mechanisms. A pH profile with TOP and MCA substrate has been performed previously. This pH study used the mca-BK substrate, and the pH plots made can be compared to those with the MCA substrate (see references; the paper “pH dependence studies provide insight into the structure and mechanism of thimet oligopeptidase (EC 3.4.24.15)” contains these plots). The most significant difference is

that of the influence of K_{cat} and K_m on K_{cat}/K_m for the different substrates. Both substrates show the same general curve for K_{cat}/K_m , with an increase around the pH of 7. Since K_{cat}/K_m is dependent on both K_{cat} and K_m , these two charts can be analyzed to see which is the major influence. In the case of this report, K_m seems to be a bigger factor in this increase. The K_{cat} is actually decreasing around pH 7, so in order for the K_{cat}/K_m to show the increase that it does, it must be because of the influence of decreasing K_m . In the previous report with MCA, however, it can be seen that the increase in K_{cat}/K_m is more influenced by K_{cat} . K_m values stay more constant, while K_{cat} shows a large increase. This difference may suggest a difference in the mechanism. While MCA has more of a pH dependence on K_{cat} , the turnover rate, pH changes using mcaBK are more influenced by K_m , the binding affinity. While one cannot draw an absolute correlation between this change and the mechanism, this potential can be seen as further evidence to back up the hypothesis that the substrates MCA and mca-BK are cleaved by a different mechanism.

V. Conclusion/Acknowledgement

The preceding data and analysis gives some possible explanations for what has been observed in the different studies. As has been mentioned various times throughout the discussion, however, a more thorough analysis including more experiments is needed in order to draw more definite conclusions. What has been observed so far strongly suggests a connection between calcium and TOP's conformational change. There are, however, many factors to consider and more experiments that should and will be performed in the future.

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