Metallopeptidases are kinds of Zn-dependent enzymes that are involved in the cleavage of bonds between peptides, chemical messengers that are intermediates in the communication between cells (1). This interaction between cells mediates life processes in multicellular organisms. The enzyme, thimet oligopeptidase (TOP), is one type of metallopeptidase that directs the turning on and off of these cell-to-cell communications. It is known that TOP is of high concentrations in the brain, pituitary and testes, and impacts many physiological processes in the human body like reproductive and cardiovascular homeostasis (2). It has also been discovered that TOP is important in controlling the build-up of peptides that cause Alzheimer’s disease. (2) To add to that, the structure of TOP is very similar to other enzymes which have not yet been fully characterized. Thus, studying TOP is not just important for knowing its own physiological roles, but also for knowing the roles of other enzymes in the human body. (2)

The focus of this project is to investigate the conformational change of thimet oligopeptidase. TOP is a three-dimensional structure made up of multiple amino acids, and a zinc atom in its active site. Its 3D structure is very important to its function. Based on X-ray crystallography and molecular modeling, TOP is a clam like structure that has an “open” and “closed” conformation.

Figure 1. The structure on left is the open and substrate free enzyme. The structure on the right shows the closed enzyme based on homology modeling to E. Coli dipeptidyl carboxypeptidase (2)
What triggers the conformation of the enzyme to change from its open to its closed form is the entry of the substrate into the enzyme. Additionally, it is only in its closed form that the enzyme can break peptide bonds, and the process of closing can limit what peptide molecules the enzyme could cleave. Previous data shows that conformational changes in TOP are part of its mechanism and are key in influencing substrate selectivity (2). In other words, TOP’s 3D structure influences what molecules it will react with and its precise physiological role.

With regards to the mechanism of TOP’s conformational change, a previous student in Professor Sigman’s lab attempted to show that substrate binding triggers the change in conformation of TOP. The student did this by attaching a fluorescent tag called N-((2-(iodoacetoxy)ethyl)-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANDB) on the enzyme and observing how much light the tag emitted. IANDB specifically binds to cysteine, an amino acid that can be found in the inner part of TOP once it is in the closed conformation. Cysteine 425 or 175 would be good attachment points for the tag. Fourteen other cysteines are found in surface of the enzyme and to remedy the complications that could arise if the IANDB attaches to one of those, a blocking agent called iodoacetate (IA) was used to block these cysteine molecules. A substrate was first introduced to the enzyme so it would go to its closed conformation. Then, the IA was used to block the outer cysteine molecules. Next, the substrate was taken out so the enzyme would open up once more and IANDB could be attached to the inner cysteine molecules.

The fluorescent tag is sensitive to the environment it is in: as the polarity of its environment decreases, the greater the tag will fluoresce. In the open conformation, the tag would have been exposed to the polar environment that the enzyme was situated in, while on the closed conformation, the tag would have been inside the enzyme and segregated from the polar environment. Because of this, it was predicted that tag would fluoresce once the enzyme went to its closed conformation. However, the tag did not produce the great amount of light that was expected from it and not enough data was collected to truly support the conformational change.

We wish to accomplish two goals by the end of the research, the first being to further develop the data from the past experiment done by past students by following their methods and accumulating enough of the tagged enzyme to determine the location of the tag on TOP. Knowing the location of the tag will allow us to judge the method’s effectiveness at monitoring the open to closed structure change. It will also be necessary if we are to make mutations in the enzyme as a means to change the location of the tag. The second and more important goal is to investigate other methods for attaching fluorescent tags to TOP. This will involve changing the

![Figure 2](image-url). This is a figure of the open conformation of TOP. Seen are seven to ten cysteine molecules, including Cys 425 and 175 (2).
buffer conditions, the block agents, or substrates in order to attach the fluorescent tag in a more sensitive spot on the enzyme. This includes finding tags of different polarities that might attach to different parts of the enzyme, changing the order of some of the steps in the synthesis, changing the environment that the enzyme would be in, etc. Furthermore, molecular modeling will be used to provide support for the experimental findings. These two parts of this project will be done simultaneously.

As stated before, the first part of the project involves following up on the past work of students regarding fluorescent tagging. We will go through the procedures they did, as mentioned earlier. We will add an inhibitor to the enzyme solution and this will act as the substrate. We will then add the iodoacetate and attempt to block the cysteine molecules on the surface of the TOP. Then, we will detach the inhibitor from the active site and introduce the enzyme to IANDB and tag the remaining unblocked cysteine. These IANDB tagged enzymes will then be sent to UCSF for Mass Spectroscopy (MS) to confirm the number of tags attached to them. Next, we will perform tryptic digestion to the tagged enzyme to break it down into small peptides and then use High-Performance Liquid Chromatography to purify and isolate the peptides. These past two steps will be repeated until enough of the tagged peptides are accumulated to obtain their mass by MS (performed by UCSF). Based on the mass of the peptides determined from the MS, we will identify the location of the tag on the enzyme. Lastly, we will use molecular modeling software to establish possible mutation sites on the enzyme that could be new possible attachment points for the tag.

The second part of the project involves investigating alternative methods for attaching the fluorescent tag in a spot on the enzyme that can better detect the conformational change from the open to the closed form. First, we will investigate and order fluorescent tags that have less polar natures when compared to the previous tag used. Professor Sigman is currently in possession of a possible tag that we can use, and we will look into other options. Next, we will develop methods to attach the newly ordered tags to the enzyme. With the use of a UV-Vis spectrometer, an apparatus that is currently present in Brousseau Hall, we will then measure the number of tags attached to the enzyme. We will also test how effective the new tag is in detecting the conformational change by performing two experiments. We will measure the fluorescence of the tag as the enzyme is denatured by urea and guanidine hydrochloride. We will also titrate the enzyme with a substrate that will trigger the enzyme to close and monitor any fluorescence that is taking place. The success of this experiment will indicate if the tag is a good indicator for the conformational change. After all this, we will go back and modify the experiments to further improve our results and send the enzyme to UCSF for Mass Spectroscopy to confirm the number of tags attached to the enzyme.

All of the experiments will be done in Brousseau Hall of St. Mary's College with the guidance of Professor Sigman, except for the Mass Spectroscopy, which will be done by scientists at UCSF.
Time Frame

Week 1
- Attachment of fluorescent tag to enzyme using past student’s methods
- Sending of the enzyme modified through past student’s methods to UCSF for Mass Spectroscopy
- Investigation and ordering of other possible fluorescent tags

Week 2
- Tryptic digestion of tagged enzyme using past methods

Weeks 3-4
- Purification of peptides by HPLC and isolation of tagged peptide using past method
- Development of methods for attaching the new tags to the enzyme
- Use of UV-Vis to determine the number of tags on the enzyme using the new method

Week 5
- Determining of the digest pattern and peptide’s mass to identify the location of the tag in the past method
- Determining the effectiveness of the new tag in detecting conformational change using chemical denaturation and titration

Week 6
- Using molecular modeling software in establishing possible mutation sites that would allow for new points of attachment for the tag

Week 7
- Development of new enzyme tagging method to improve results

Week 8
- Sending of enzyme prepared using the new tagging method to UCSF for Mass Spectroscopy

References

2. Sigman, Jeffrey. “NSF-RUI Grant”. 2010