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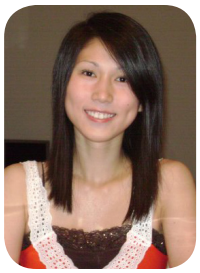

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Investigating a Conformational Change in the Enzyme Neurolysin

This summer I am working with Dr. David Rodgers in the Department of Molecular and Cellular Biochemistry at the University of Kentucky. I continue to investigate a proposed conformational change in the enzyme neurolysin, a zinc metallopeptidase that regulates peptide signaling molecules. My previous studies provided evidence that the enzyme undergoes a hinge-like motion during catalysis and demonstrated the ability of a novel inhibitor to interfere with that motion. I have expanded my work with neurolysin this summer using mutagenesis techniques to further investigate the conformational changes and the mechanism of inhibition. This gave me my first practical experience with molecular biology and genetics techniques. Dr. Rodgers and I have worked closely together for more than a year, and we discuss the project on a daily basis.

My goal for this summer is to use site-directed mutagenesis to alter the charge on the inner channel walls of the channel in neurolysin as well as to modify the binding site for the inhibitor C28. Since the channel walls have an overall negative surface charge, we hypothesize that increasing the charge will bias the enzyme toward an open conformation, which should reduce the activity of the enzyme. Furthermore, we hypothesize that C28 acts by inhibiting the hinge motion and that we can mimic this effect by using mutagenesis to fill the normal binding site of the inhibitor.

Progress

To begin my work, Dr. Rodgers and I used the crystal structure of neurolysin to find modifiable sites on the protein so as to change the surface charge, settling on the mutation of lysine 221 and arginine 248 to glutamate residues. We then designed oligonucleotide primers that could be used in site-directed mutagenesis to make the mutations and had the primers synthesized commercially. While waiting for the primers, I learned how to transform bacteria with the expression plasmid for neurolysin, and then I used a miniprep kit to make a stock of plasmid DNA. We then used the primers and plasmid DNA with the QuikChange mutagenesis system (Stratagene) to introduce the mutations by polymerase chain reaction (PCR) amplification.

We had a hard time making mutations on the wall surface of the enzyme at first. Because the mutagenesis protocol has been known to work properly, we had to sequentially eliminate possible causes of failure. The problem was eventually solved by repeating the protocol using freshly ordered reagents and by making fresh agar plates and media. Soon after solving the buffer problem, we realized that something was amiss with the cell stock, possibly mutations or damage due to long term storage. Whatever it was, the cell stock was newly cultured and all problems have been overcome. We believe we have introduced the mutations successfully, and we are awaiting final sequencing conformation. In the meantime we are proceeding with attempts to overexpress and purify the mutant proteins, and we are designing primers to introduce the mutations at the C28 binding site.

Future Plans

Assuming we have introduced the mutations correctly, we will then overexpress and purify the mutant enzymes according to well-established protocols in the group. The kinetic parameters of the wild type and mutant neurolysin constructs will then be determined by using fluorogenic peptide substrates (neurotensin and dynorphin A(1-8)). The conformational state of the mutant enzymes will also be determined by measuring rotational tumbling times using fluorescence polarization anisotropy measurements, and these will be compared to data for the wild type enzyme.

In parallel, we will mutate serine 146, leucine 69, and valine 119 to tryptophan residues in order to fill the C28 binding site. The idea is to increase the sizes of these amino acids to fill the C28 binding to mimic the inhibitor-bound state. We hypothesize C28 inhibits by stabilizing the open, inactive conformation of the enzyme and that filling the binding site should have the same effect. The expected result is that the mutated neurolysin would lose a significant amount of activity. I will also use fluorescence polarization to monitor the conformational changes of the altered enzymes and characterize the kinetic properties of the mutants on several substrates.