Kaleidoscope

Volume 10 Article 8

June 2012

Screening Synthetic Combinatorial Protein Libraries for Changes in Calmodulin Binding Specificity

Donald E. Dick

Faculty Mentor: Dr. Luke Bradley

Summary

Through this research, the importance of the calmodulin (CaM) central linker region in binding specificity is be explored. Using a novel technique known as high-throughput thermal scanning (thermofluor), this research is beginning to provide valuable insight into the binding specificity of CaM to the myosin light chain kinase (MLCK) family of physiological target proteins involved in muscle contraction. In order to determine the importance of the CaM central linker region, the use of a hydrophobic-binding dye (SyproOrange) was used to compare binding affinity towards specific target proteins. Research performed during the current summer has identified the binding affinities of several target proteins. Upon identity completion, correlations between binding specificity and binding affinity will be understood. Current methods of spectroscopy and traditional binding assays will validate the hypothesis that thermofluor is a technique capable of determining binding specificity. If the traditional binding assays confirm the thermofluor results, the discovery of a high-throughput thermal scanning device would be generated. This would prove beneficial by requiring less sample to perform protein melts (for a variety of purposes) and the generation of these results would be expedited as compared to previous methods. By establishing both the importance of the central region of the CaM helix and the binding specificity of CaM, numerous medical breakthroughs are possible. The study of any disease resulting from a calmodulin mutation could then be studied to determine possible mechanisms of CaM alterations for treatments.

Introduction

A viable approach towards advancing our understanding of the nature of molecular interactions conferring protein binding specificity in the healthy and diseased cell, is to use protein engineering to alter the specificity of natural proteins. We have selected the eukaryotic cell's primary calcium signaling transducer, calmodulin (CaM) as a model to examine protein binding specificity. In the cell, the calcium signal is transmitted to over 300 target proteins by CaM. In the presence of calcium, CaM undergoes large conformational changes throughout the protein to present a structurally dynamic surface to recognize and bind to its numerous target proteins (Shifman and Mayo). Most of these structural changes occur in the protein's unique central linker region. Numerous studies have led to our hypothesis that the interactions provided by this region are primarily responsible for CaM-target binding affinity and, as a result, the CaM pervasive binding specificity (Bradley et. al.).

To test this hypothesis, and to explore the molecular basis that provides CaM-target specificity, we proposed to utilize a novel high-throughput system to screen synthetic protein combinatorial libraries of the CaM central linker for alterations in melting point. Following addition of an indicator dye that binds to exposed hydrophobic surfaces, the sample mixture was incubated at increased temperatures, 1 degree Celsius per two minutes, and monitored for dye binding (upon release of bound target protein) by fluorescence spectroscopy at one minute intervals. In other words, the melting of a Calmodulin (CaM) protein exposed hydrophobic surfaces of the molecule, allowing it to retain a dye (SyproOrange) and emit a fluorescence. The temperature

that allows CaM to open and bind to the SyproOrange will be of importance in determining the shape of the molecule, thus the melting temperature presented in the graphs below are of significance. A higher temperature required to get a fluorescence, suggests a protein that possesses stronger bonds. Relative changes in binding affinity and specificity for the MLCK family of target proteins, compared to wild type CaM, will be quickly determined. After obtaining these results, our experiment was repeated numerous times as a form of validation. Through the comparison of binding values (measured through thermofluor) to each target, the specificity of CaM to certain protein targets can be determined. However, the process of doing this long and complex portion was not completed during the summer. The continuing dedication I will place within the lab this semester will be crucial in determining these specificities.

In order to verify that the results from the thermofluor experiment are accurate, it will be essential to perform an alternative method that will also give the relative binding affinity of CaM. Upon completing the high-throughput thermal scanning experiment, traditional binding assays and spectroscopy need to be used to validate thermofluor as a new technique. It is expected that the results will be similar and will validate thermofluor as a new laboratory technique used in identifying target proteins.

Within the experiment, the use of a wild type protein will be essential. The wild type protein (WT), as mentioned above, served as a control, and is essential in comparing the changes in binding affinity and specificity for the MLCK family of target proteins. Through the comparison of the wild type and target proteins, the ability to understand the protein specificity of CaM will be understood.

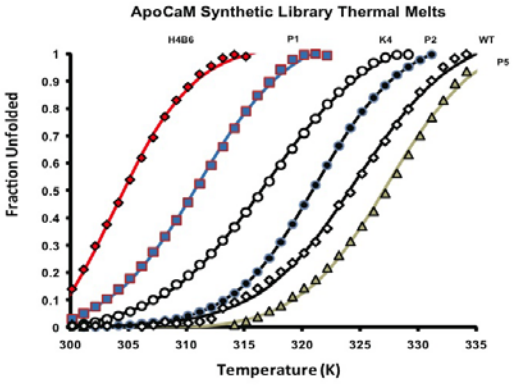
Results

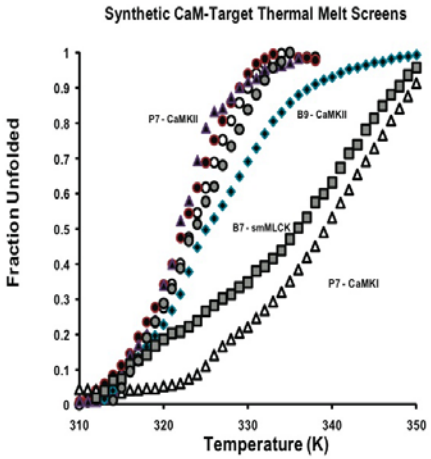
Using the thermoflor experiment, the melting temperatures of the following high-quality library members were recorded.

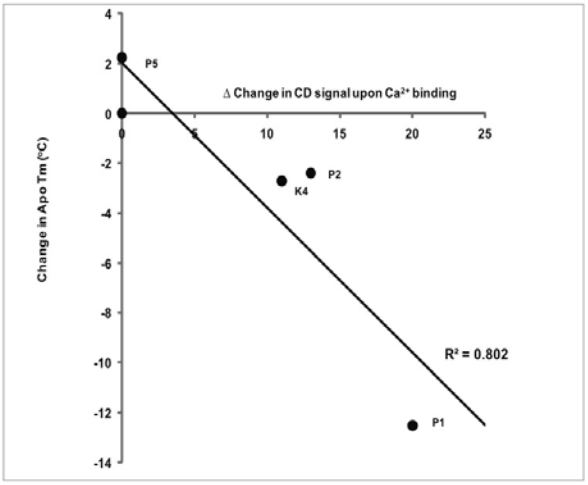
Library Member	Melting Temperature (Tm) Celsius
P1	311.21
P2	321.34
K4	317.27
WT (Control)	323.75
P5	**325.98**

P5 was shown to have a slightly higher melting temperature than WT, thus has a greater stability. This will be important in continuing my research when determining binding specificity of these randomly selected library members. Future studies will determine if P5 is more stable, as our prediction with thermal stability indicates.

Upon completion of the experiments, we found results that yielded the following graphs:







Central Linker libraries generate a range of proteins that have differential effects on backbone conformational changes upon calcium binding, and changes in the stability of the apoprotein.

A relationship is observed between the changes in apoprotein stability and the changes in the backbone conformational changes (as determined by CD spectroscopy) relative to WT.

Focusing our search of sequence space on the central linker region and in particular results in destabilizing the apoprotein stability , using high-quality protein library design, we have a library rich in not only folded, soluble protein but rich in those with altered specificities to known peptide targets. Thus this reverse engineering approach, when applied to the appropriate protein scaffold, results in a library of protein rich in novel & specific binders.

The top left graph represents the melting temperatures found using thermofluor. It was found that our mutated form of CaM (known as P5) has a greater melting temperature than WT CaM (control). The top right graph displays the results for our binding thermal melt screens to various MLCK and Kinase I and II targets. The bottom graph shows the linear relationship of calcium-induced changes to the Apo form of CaM, and the changes in melting temperatures. These experiments were repeated numerous times, and the graphs depict the averages of all trials.

Through this data, we would be able to determine which protein (WT, H4B5, H4B10) is more specific to each type of myosin light chain kinase target. By comparing each protein type, it would be numerically possible to calculate what protein strain has the greatest specificity. By determining target specific proteins, it may be possible to create therapeutics that alter only that specific target. These developments are in the very early stages, and a great deal of research will need to be completed before any extreme biotherapeutics are engineered.

Literature Cited

Bradley, L. H., Bricken, M. L., & Randle, C. (2011). Expression, purification, and characterization of proteins from high-quality combinatorial libraries of the mammalian calmodulin central linker. Protein Expression & Purification, 75, 186-191.

Lavinder, J. J., Hari, S. B., Sullivan, B. J., & Magliery, T. J. (2009). High-Throughput Thermal Scanning: A General, Rapid Dye-Binding Thermal Shift Screen for Protein Engineering. Journal of the American Chemical Society, 131, 3794-3795.

Shifman, J. M., & Mayo, S. L. (2002). Modulating Calmodulin Binding Specificity through Computational Protein Design. Journal of Molecular Biology, 323, 417.

Acknowledgments

Represented by the abundance of information reported above, it was an honor to continue my research through the summer. Dr. Luke Bradley has proven to be an exceptional mentor, and the University of Kentucky Office of Undergraduate Research supported our research this summer. Brian Chaffin, undergraduate at Georgetown University, also aided tremendously in advancing this research.