Fluorescent Labeling of Calmodulin for Future Applications

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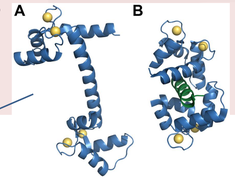
Abstract

Calmodulin (CaM) is a small dumbbell-shaped, intermediary protein. CaM binds with several hundred different molecules to help control bodily functions. At Northwestern, we use fluorescently labeled CaM to understand these functions further. The goal of this project is to successfully label CaM protein using the fluorescent dye Alexa Fluor 594. After labeling CaM, we will use buffer exchange chromatography to purify the sample. Following that, we will use three processes to verify the successful labeling of CaM: UV/VIS spectroscopy, mass spectroscopy, and fluorescent microscope imaging. Once labeled and purified, our CaM samples can be used for years to come by Northwestern students and professors to understand the unknown functions of bodily enzymes it interacts with.



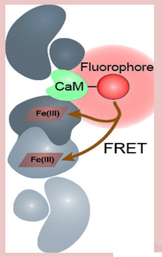
Structure of calmodulin bound to calcium ions (yellow) and a target peptide (green ribbon)

Image from https://www.researchgate.net/figure/Structure-of-Calmodulin-CaM-A-A-17-angstrom-ribbon-structure-of-free-CaM-blue_fig18_41487625

Introduction

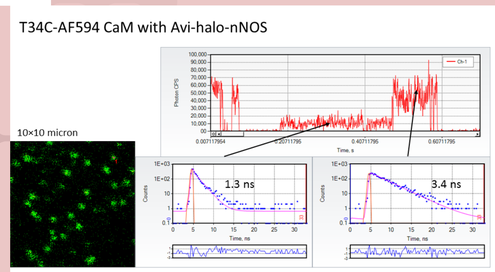
What is calmodulin? Why would we want to label it? And what will we use the finished product for?

Calmodulin (CaM) is a protein that binds to calcium and regulates different processes in cells by being a second messenger in signal transduction pathways. CaM plays a role in activating various calcium-sensitive enzymes, ion channels and other proteins. Here at NWC, we have been studying fluorescently labeled calmodulin and some of the enzymes it interacts with. Most recently, we have been using calmodulin to learn about the conformations adopted by nitric oxide synthase enzymes once calmodulin binds to it. The key to all these studies is that the dye on CaM can interact with target proteins in such a way that its fluorescence is changed.



(left) Example of labeled calmodulin interacting with an enzyme target. In this case, the fluorophore (dye) interacts with an iron-heme in the enzyme (nNOS), causing the fluorescence lifetime of the dye to change in a way that depends on the structure of the complex.

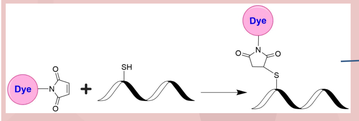
(below) An image of calmodulin nNOS complexes collected on our fluorescent microscope. The image insets show how a calmodulin molecule changes fluorescence lifetime over time as the structure of the nNOS changes.



Materials & Methods

Binding Chemistry

The dye we chose for this project contains a maleimide linker that covalently binds to a sulfur atom on the cysteine amino acids in the protein. Calmodulin is naturally free of cysteine, so a threonine was mutated to a cysteine.



Example of maleimide dye labeling chemistry.

Image from AATBIO.com

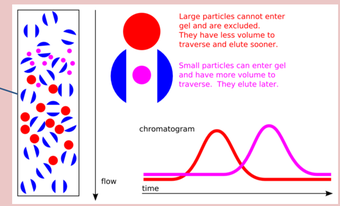
The dye labeling itself was carried out based on a protocol developed over time by the Johnson group at the University of Kansas. Briefly, 125nmoles of CaM-T34C (Medical College of Wisconsin, Milwaukee, WI) dissolved in 500nmoles of TCEP (TCEP Concentration) and mixed for 5 minutes. 1mg of AF488-C5 maleimide (ThermoFisherScientific Waltham, MA) dissolved in 1mL HEPES buffer. 4L of HEPES buffer made from 9.5304 g HEPES, 29.823g .1M KCl, .81324g of 1mM MgCl2, and .058808g of .1mM CaCl2 adjusted to pH 7.4. 2 mmoles of NaCl added to HEPES then mixed with CaM/TCEP solution for 5 minutes. AF488 added drop wise into CaM/TCEP/NaCl solution and was stirred for 1 hour in dark.

FPLC separation

Once the CaM was labeled, we used FPLC to separate the labeled protein from the excess free dye. The method we used was size exclusion chromatography, specifically gel filtration. We attached two HiTrap Desalting columns with a Sephadex G-25 resin. We attached two of the 5 mL columns together to better our separation. The larger dyed protein molecules will move through the separation column more quickly than the smaller free dye particles. The separation cutoff for these columns is 5000 grams/mole. This is one way we can purify our sample in preparation for verification and future use.

Illustration of size-exclusion chromatography.

Image from creative-biolabs.com



Mass Spec verification

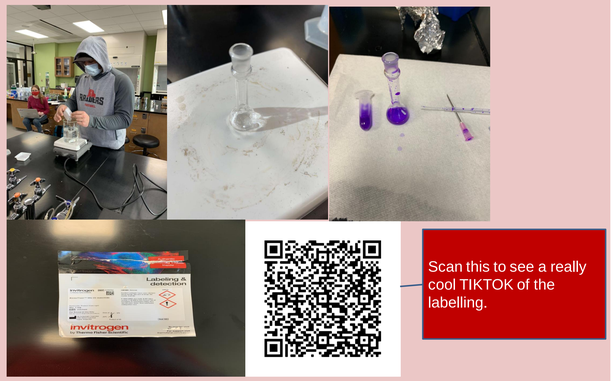
A solution of purified and labeled CaM was mixed with a standard matrix recommended by our local MS experts. It was then allowed to dry on the mass spectrometer plate. Once dry, we used MALDI mass spectroscopy to verify if CaM had been dyed. Our CaM protein and the dye both have known masses of 16750 daltons and 819 daltons, respectively. We can detect both masses on the Mass Spectrometer. When we see a peak at what would be the dye and protein masses added together. When then know we have successfully labeled our protein.

UV-VIS verification

We can use UV-Vis Spectroscopy to see how much of the dye we labeled. Proteins often absorb UV light at a wavelength of 280 nm, while the dye we are using absorbs light at 590 nm. By running an absorption spectrum of our sample, we can take the absorption peaks of the protein and dye and calculate the concentration of protein in our sample.

Results

Reaction

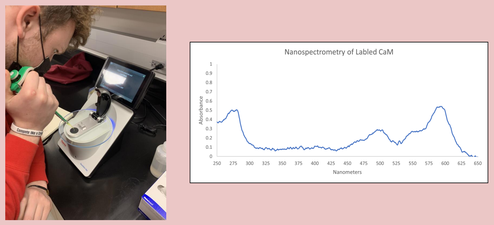


Scan this to see a really cool TIKTOK of the labelling.

Separation and Purification

Mass Spec Images

UV-Vis Images



Summary & Conclusions

This project was full of ups and downs. We started with a cheaper dye-protein system and were remarkably successful at labeling Hemoglobin protein with fluorescein.

Our first attempt at labeling calmodulin led to some curious results. We mixed CaM with a reducing agent so that the sulfurs on cysteine would be available to the maleimide dye. We then mixed in the dye and attempted to separate labeled protein from free dye. This was unsuccessful and we were unable to get a clear separation between the protein and the free dye. Mass spec analysis of this sample did indicate that we had some labeled protein in the sample, but the sample was overwhelmed by free dye.

Our second attempt at labeling calmodulin was much more successful. The labeled protein separated from free dye on the FPLC. UV/VIS analysis suggested, however, that the labeling efficiency was fairly low – about 10-20%.

It is important to use the proper materials that have been known to be successful. We had some difficulties in the beginning stages of research. In the future we plan to perform mass spectrometry to further verify that we attached the dye to CaM. We plan to find more efficient ways to link the dye to the protein as well as a more successful purification. In the future we take our labelled CaM and attach it to a glass slide to view it under a fluorescent microscope.

What have we learned

This project was part of the lab for the instrumental analysis class and it provided a real-life context to learn about instruments like the FPLC, UV/VIS spectrometer, and the mass spectrometer.

As we progressed, we found out how difficult it was to get the results we were looking for. It took resilience because we kept running into bumps in the road. We had the goal of finishing the project and that is what kept us going. We also learned how to use all the instruments involved and the math behind them.

Acknowledgements:
We want to thank Carey Johnson and Lexi Gambill from the University of Kansas for consulting with us regarding dye-labeling protocols. We also want to thank Brian Smith and Sarah Wynia-Smith at the Medical College of Wisconsin for expressing and purifying the calmodulin. Lastly, we want to thank Noah Gritters for help with the HPLC.