Amino Acid Quenching

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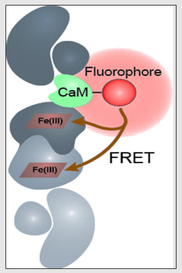
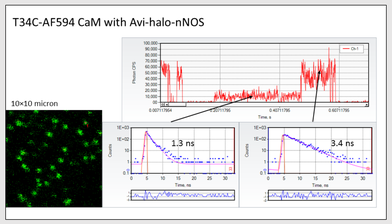
Introduction:

Researchers at NWC have been using fluorescence to study conformational changes that happen when a protein called Calmodulin (CaM) interacts with and activates a group of enzymes called Nitric Oxide Synthase (NOS). The illustration to the right shows a fluorescently labelled CaM complexed with a NOS dimer. The enzyme complex, whose role is to generate the molecule nitric oxide, functions through a series of large conformational or shape changes. Some of these conformations locate the fluorophore close to the Fe(III) centers on the enzyme while other conformations place the fluorophore further away from these iron atoms. The distance between the fluorophore (or dye) and the Fe(III) atoms matters since the iron atoms act as a fluorescence quencher, a process that is more efficient at short distances. Our measurements look at the intensity and lifetime of fluorescence coming from the fluorophores. In other words, we observe the number of photons coming from the dye molecules after excitation and how long it takes for those photons to be released by the dye. Our expectations are that those two parameters of intensity (I) and lifetime (tau) are correlated – fluorophores will yield small intensities and short lifetimes when close to the iron quenchers but large intensities and longer lifetimes when further from the iron atoms. This expectation, that fluorescence intensity and lifetime will track proportionately, is not perfectly followed by our experiments.



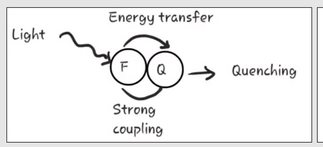
Expectation: dynamic quenching where fluorescence intensity and lifetime are proportional.
Observation: The fluorescence intensity and lifetime do not always correlate.

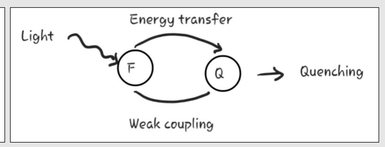
The image below illustrates how intensity and lifetime show up in our measurements. The data here was obtained from a single complex of CaM with a version of NOS. The top right panel of the image shows the fluorescence intensity as a function of time. The fluorescence lifetime for a low intensity region of the data is shown as is the fluorescence lifetime for a high intensity region. The lifetimes (1.3 ns and 3.4 ns) and intensities (10000 and 50000) do not rigorously follow the expected pattern. There is a 5X increase in the intensities but only a 2.6X increase in the fluorescence lifetime.



All this led us to be curious about types of fluorescence quenching and their impact on the relationship between fluorescence intensity and fluorescence lifetime. Were there quenching mechanisms at play in our samples that resulted in changing intensity with a smaller than expected change in lifetime? Other researchers had found that some amino acids quench the types of fluorescence dyes we use but did not report on the specific dye we most commonly employ. In this project, then, we explore the fluorescence quenching effect for three amino acids: Histidine, Tyrosine, and Methionine.

There are two types of quenching: static and dynamic. Static quenching occurs when the fluorescent molecule and the tagged amino acid form a complex, exhibiting strong coupling. Rather than emitting energy in the form of light, the fluorescent molecule-amino acid complex quenches the energy and there is no emission (left image). Dynamic quenching occurs when the fluorescent molecule and the tagged amino acid collide, exhibiting weak coupling (right image).





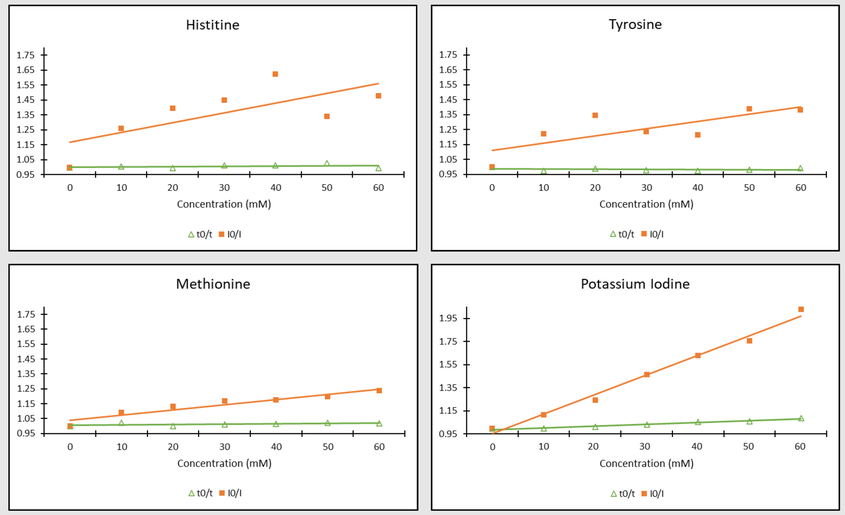
In this experiment, we study the quenching effects that these amino acids, Histidine, Tyrosine, and Methionine, have on the fluorophore molecule Alexa Fluor 594. We compare our results to the effects of Potassium Iodine, a dynamic quencher (2).

Abstract:

Alexa Fluor 594 is a fluorescent molecule commonly used to tag calmodulin, a protein that regulates a variety of cell processes. By measuring fluorescence, scientists can study calmodulin and its interactions with other proteins. However, fluorescence is not the only fate of Alexa Fluor 594. The molecule is also known to transfer energy to coupled amino acids through a mechanism called quenching. The purpose of this experiment was to study the quenching effects of Histidine, Tyrosine, and Methionine on the Alexa Fluor 594. These amino acids' Stern-Volmer plots suggest static quenching may be occurring. Moreover, the Stern-Volmer plot of Potassium Iodide (KI), a dynamic quencher, suggested that KI quenches the excitation Alexa Fluor 594 in part through a static mechanism. We plan to replicate our experiment and complete statistical testing to determine the significance of our data. Determining if these amino acids quench AlexaFluor594 will help researchers better characterize the calmodulin protein and its interactions.

Results:

Increasing the concentration of amino acid increased the fluorescence intensity with fluorescence lifetime staying constant. Similarly, increasing the concentration of Potassium Iodine (KI) increased the fluorescence intensity more than the fluorescence lifetime; however, fluorescence lifetime did not stay constant. Additionally, the slope of KI-AlexaFluor594 fluorescence intensity appears to change at around 700 mM KI.



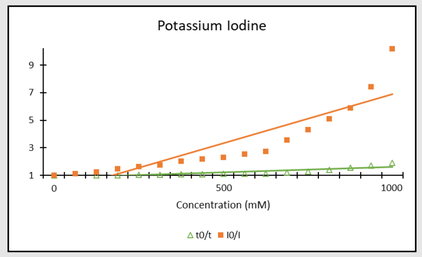
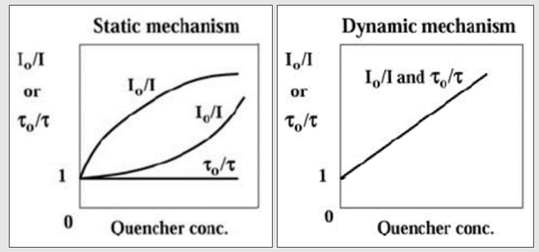


Figure 1: Stern-Volmer plots showing the correlation between intensity (I_0/I) and lifetime (τ_0/τ) and concentration. The variables I_0 and I represent initial intensity of AlexaFluor594 and intensity of couple, respectively. And the variables τ_0 and τ represent lifetime of AlexaFluor594 and lifetime of couple, respectively. (Top-left) Stern-Volmer plot of Histidine-CaM-AlexaFluor594, (top-right) Stern-Volmer plot of Tyrosine-CaM-AlexaFluor594, and (middle-left) Stern-Volmer plot of Methionine-CaM-AlexaFluor594. (Middle-right) Stern-Volmer plot of KI-AlexaFluor594, not including measurements at high concentrations; and (bottom) Stern-Volmer plot of KI-CaM-AlexaFluor594, including measurements at high concentrations.

Experiment:

Stern-Volmer plots are used to plot intensity (I_0/I) and lifetime (τ_0/τ) against quencher concentration. This allows for easy comparison of intensity and lifetime. If static quenching is occurring, the lifetime will quench at a different rate than the intensity. If dynamic quenching is occurring, the lifetime will quench at the same rate as the intensity (3).



Materials and Methods:

First, we placed a sample of Alexa Fluor 594 in a well, and using the confocal microscope, we measured the intensity and fluorescence lifetime after exciting the sample with a pulsed laser at 594 nm. We then added increasing amounts of the quencher (amino acids or KI) and repeated the intensity and lifetime measurements. We then created a Stern-Volmer plot of the data, plotting fluorescence intensity (I_0/I) and fluorescence lifetime (τ_0/τ) against quencher concentration.

Discussion:

For each amino acid, the fluorescence lifetime quenched at a different rate than the fluorescence intensity. Such correlations suggest the possibility that these amino acids use quench the fluorescent molecule AlexaFluor594 through a static mechanism. Because these correlations were not as strong as Potassium Iodine, we plan to replicate this experiment and complete statistical testing to determine the significance of our data.

Additionally, we plan to test these amino acids at higher concentrations as we did with KI. Not only are we are interested to see if the correlation will strengthen, but we are also curious if the fluorescence intensity will change slope around 700 mM as we saw with KI. Determining if these amino acids quench AlexaFluor594 will help researchers better utilize the AlexaFluor594 as a molecular tool and in turn better characterize the Calmodulin protein and its interactions.

References and Acknowledgements:

1. Huimin Chen, Syed S. Ahsan, Mitk'El B. Santiago-Berrios, Hector D. Abruña, & Watt W. Webb (2010). Mechanisms of quenching of Alexa fluorophores by natural amino acids. *Journal of the American Chemical Society*, 132 (21), 7244-7245. DOI: 10.1021/ja100500k
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3. Pete Crisalli & Eric T. Kool (2011). Multi-path quenchers: efficient quenching of common fluorophores. *Bioconjugate Chemistry*, 22 (11), 2345-2354. DOI: 10.1021/bc200424r

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