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2D Infrared Spectroscopy for the Study of Membrane-bound Proteins and Peptides

By

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Thomas Brunold, Professor, Chemistry
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Chapter for the General Public

I have included this chapter in order to communicate my research to the general public. The work we do in the Zanni group is meant to better understand the world and it is vital to communicate these ideas to the public who, through tax dollars, fund the work that we do and therefore have a stake in our results. Many thanks to the Wisconsin Initiative for Science Literacy at UW-Madison for the opportunity to communicate my work to the public.

1. Introduction

Consider a biological cell. They are little bubbles of life with machinery contained within a lipid membrane. The lipids are fatty acids that are flexible yet separate what is inside and outside the cell. These lipid membranes are considered to be bilayers because they are formed of two layers of lipids. The 'heads' of the lipids face outwards and the 'tails' face inwards. Membranes help to also maintain concentrations of ions, charged atoms and molecules, within the cell. Often, there is a charge difference between the inside and the outside of the cell, due to different concentrations of these ions on either side called the resting potential or voltage. However, cells need to be able to communicate with each other and import and export nutrients and waste. To do this, many proteins are embedded in the membrane. Typically, each membrane protein has one job to do:

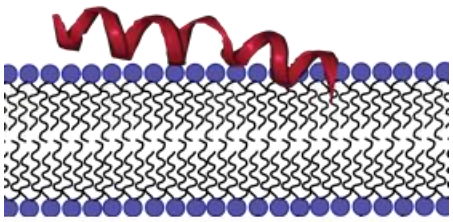

Figure 1. A section of lipid bilayer. The heads are in purple and the tails are in black lines. The red helix shows a membrane-associated protein sitting on top in a neutral position.

sense hormones, actively transport small molecules, or allow for the passive transport of ions across the membrane, causing a change in the membrane voltage. Proteins are made up of components called amino acids. Amino acids are bonded together through a peptide bond which create the 'backbone' of the protein like a string of beads. Each amino acid (there are 20 common ones) also has a section called the 'side chain' which dangles from the backbone and is unique to each amino acid. My Ph.D. work has been centered on figuring out how these membrane-bound proteins are moving in the membrane in order to do their jobs. Understanding typical protein function is important as a baseline to compare to proteins that cause disease, which ultimately can lead to rational drug design that specifically targets the disease-causing attribute of the protein. For example, in diseases where there are issues with ion channels not working properly due to mutations, like some forms of myotonia, leading to muscular contraction and seizures, channel blockers are used as a pharmacological tool to alleviate symptoms. The ability to block channels comes from knowing how healthy channels look and function.

2. An aside on the technique

The main technique that I have used to answer these questions is called two-dimensional infrared spectroscopy (2D IR spectroscopy). This technique involves using an ultrafast laser to

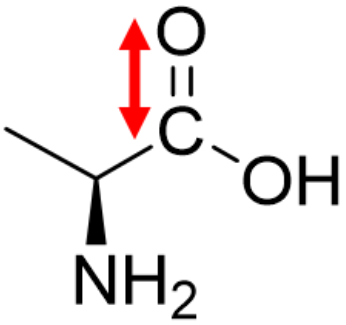

Figure 2. The chemical structure of an amino acid, alanine. We are mostly interested in the stretch between the C and the O as indicated by the red arrow. The amide I vibration that we study actually also includes the nitrogen atom and its hydrogens.

look at the vibrational energy of molecules. Molecules are constantly in motion. They vibrate and rotate in space, even in materials that are solid. All of these movements correspond to a certain amount of energy. We are concerned with vibrational energies. These can be easily imagined by thinking about two balls on a spring. Each ball represents an atom: in our case one is a little heavier than the other. The heavier ball is oxygen and the lighter ball is carbon. The bond between the two atoms is the spring. Depending on the two atoms, the spring might be a little tighter or looser. Each bond, or spring, has a characteristic vibrational energy, which we think about in frequency. The characteristic frequency can be changed depending on the environment around the atoms we are looking at. For example, the hydrogens in water can push and pull on the oxygen. Or a positively charged ion, like a potassium ion, can interact strongly with the partial negative charge on the oxygen. These interactions will increase the 'heaviness' of the oxygen and will change the frequency. The energy of these frequencies is the same as infrared (IR) light. We shine our IR light on the molecules and we can measure how much of the energy is absorbed. By seeing the precise wavelength of light that is absorbed by our molecule, we can identify the atoms present, and also learn something about the orientation and environment of those molecules. Lastly, we use ultrafast laser systems (there are 1000 laser pulses per second, and each pulse is faster than one *trillionth* of a second long) so that we can see what is happening to these molecules over time. Molecules vibrate on the scale of picoseconds (one trillionth of a second) and proteins move on the scale of microseconds to milliseconds (one millionth to one thousandth of a second).

What makes this technique two-dimensional, is that we are actually using multiple pulses of light that interact with our sample. They are called the pump and probe pulses and that is how we label the axes. The pump pulse interacts with our sample first and puts energy into the vibration of interest. Then, after a designated amount of time the probe pulse interacts with the sample to

see what happened to the pump energy. Then we can calculate the change in the optical density which we call ΔOD which provides us with a spectrum. The Δ (delta) is used in many instances to mean 'change'. The 2D IR spectroscopy can be thought of as one friend calling another about their engagement. The friend is obviously excited from this interaction. Then, the new fiancée of the first friend also calls the friend right after, and sees how excited they are, compared to their normal state. The interactions between the pulse of light (new fiancés) and the sample (their mutual friend) tell us about the energy levels of the sample or friend.

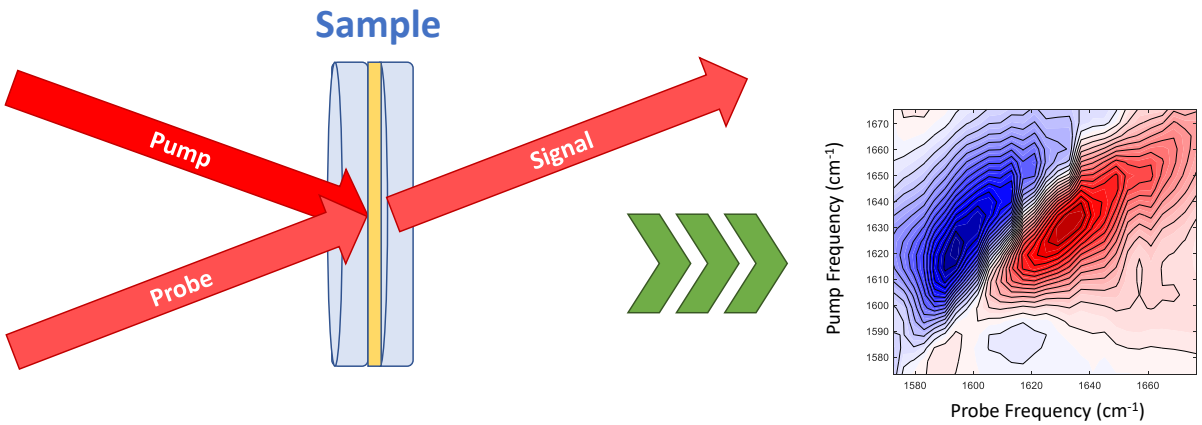

Figure 3. Graphic showing sample set up of 2DIR spectroscopy and a model spectrum representing the kind of data collected. The red arrows represent infrared light. The pump and probe overlap in time and space at the sample. Then we collect the signal beam and process the data. We then get a spectrum as shown here. This spectrum is of a voltage sensing domain discussed below.

3. The problem and our proposed solution

We started this project by thinking about how much is still not known about ion channels in membranes. Ion channels are a class of membrane-bound proteins that open and close after a physiological trigger and passively transport ions like potassium, sodium, and calcium across the membrane. In humans, they play a vital role in nerve cells. The electric signals down nerve cell axons are perpetuated by voltage-gated (voltage controlled) ion channels which open when the voltage across the membrane changes. The continued opening of the channels successively down the axon of the nerve cell keeps triggering the voltage for the next ion channel, eventually signaling

the release of neurotransmitters to the next neuron. The signal moves just like a series of dominos down the nerve cell. What is very interesting about ion channels is that they typically only allow one ion to flow through, but the ions flow through at a rate that is almost equivalent to just an open hole in the cell membrane. We want to know: what is it about the structure of these proteins that allow this to be possible? How are these proteins moving after a stimulus? How are the ions flowing through the channel? And finally, can we use our ultrafast laser systems to be able to 'watch' the movements in these channels in real time? Many of these experiments have not been done yet, but we are currently working on techniques to make them possible.

2D IR is an excellent technique to make these measurements. We are able to measure the amide I vibration, which is largely composed of the stretching vibration of the carbon and oxygen in the backbone of a protein. This vibrational frequency is very well studied and characterized. Proteins can take on different secondary structures like helices which look like hair ringlets and sheets which look like stacked lasagna noodles. Since these structures create different environments for the atoms we are interested in, the frequencies of these vibrations are also different. 2D IR also allows us to look more in depth at the way that vibrations interact with each other. This is a phenomenon called coupling, and it can be thought of as walking a dog on a leash. The speed of the dog when it stops to sniff causes a change in the speed of its human due to tension through the leash which couples them. We see cross peaks in the spectra when this occurs and we are able to see what parts of the sample are interacting with each other. Cross peaks are shown as the peaks not on the diagonal in figure 4. The shapes of the peaks in the 2D IR spectra also give us information about the environment of the vibration. Elongated ovals along the diagonal mean that there is less structure or slower molecular motions, and more circular peaks on the diagonal indicate more structure or faster molecular motions. There are experiments that we do that help

parse the difference between the motions and the structure. The last piece of data that 2D IR offers is the ability to extract information about orientation in space, when we have a sample that is on a surface. For example, when we have a single lipid bilayer with membrane proteins in it, we can calculate the angle of insertion of the protein by using our spectra! This means that our technique yields a lot of information when we are asking questions about environment, movement, and orientation of our proteins.

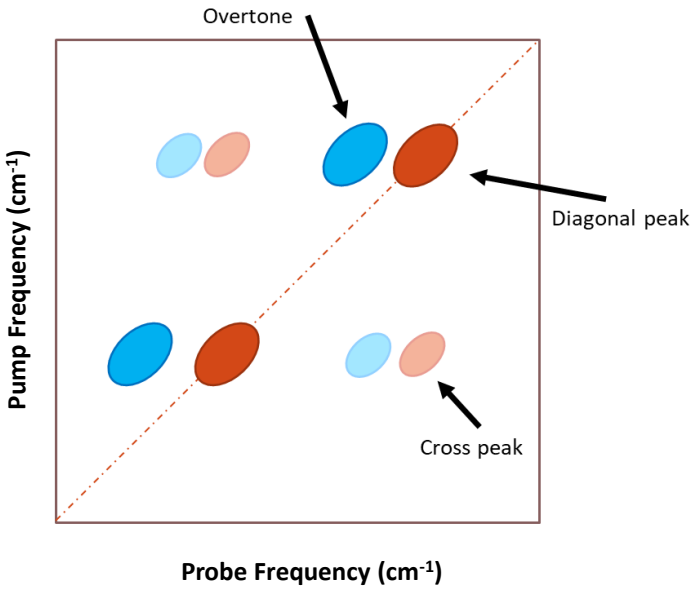

Figure 4. A cartoon 2DIR spectrum of two, coupled vibrations. Shades of red represent positive peaks and shades of blue represent negative peaks. The diagonal peaks fall on the diagonal line and represent the main vibration. The overtones next to them are the next higher in energy vibration. Cross peaks between the two peaks show the coupling between the two vibrations.

4. Developing a proof-of-concept to measure voltage-gated proteins

The first step in starting to study ion channels was to develop a method to work with a single bilayer and our protein of interest. We needed to use a single bilayer for two main reasons: we wanted to have a membrane that was oriented in space in order to see how the sample protein moved relative to the membrane, and we also wanted to put a voltage across our sample to mimic

the biological context of our proteins. Even if we had an oriented, but stacked, bilayer, it would be dangerous to apply a voltage since with each successive layer added, more of a voltage would have to be applied for the proteins to 'sense' the appropriate voltage due to the inherent electrical characteristics of a lipid membrane. However, if we just have a single bilayer of lipid and protein, there are not enough molecules to generate a nice signal. To counteract this, we used gold nanoparticles to enhance the signal. The gold nanoparticles are tiny drops of gold—only about 3-10 nm in size. This is incredibly small; a red blood cell is 6000 nm across. The gold nanoparticles are sometimes called nanoislands because under a microscope they look like little islands on an ocean. When IR light shines on the nanoislands, they create 'hot spots'. The hot spots are formed due to the IR light causing the electrons in the gold to bounce back and forth across each island inducing areas of highly concentrated energy. By placing our sample on these nanoislands, we are able to 1) enhance our signal, 2) tether our membrane to the gold by using lipids with sulfur attached to their heads, which readily bond to gold, allowing us to orient our sample and 3) use the gold as an electrode to create our voltage!

To test the viability of these methods, we used a smaller membrane protein. Alamethicin is a small membrane-associating protein that is produced by a fungus *Trichoderma viride*. The fungus creates alamethicin because it is an antibiotic. Alamethicin is drawn to a lipid membrane and will aggregate into groups of 6-8 individual proteins and form pores in the membrane. These pores are devastating to cells as they interfere with cell homeostasis, or stable conditions: hence its antibiotic effect. Alamethicin was a good candidate for our experiments because it is known to be triggered to aggregate and insert into the membrane based on either a change in pH or in voltage. We could then verify our ability to measure a change of voltage by comparing the results to the pH experiments.

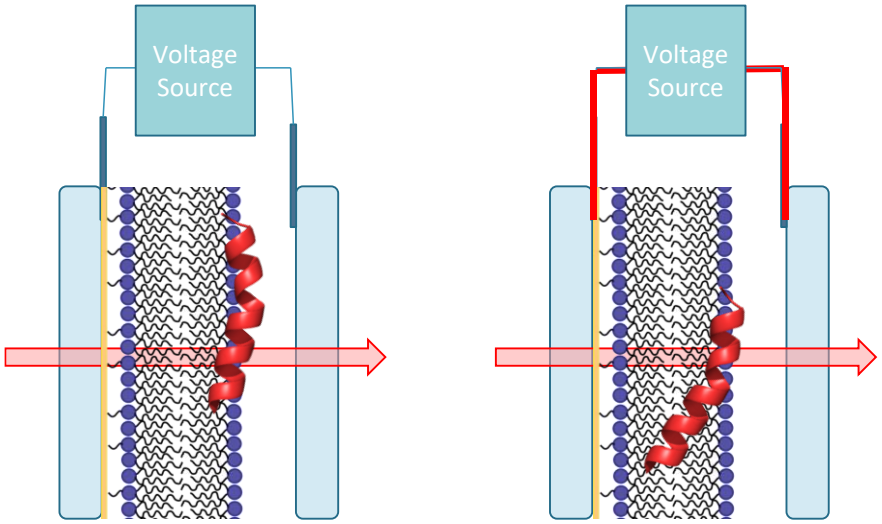

Figure 5. Schematic showing the sample cell with alamethicin on a bilayer tethered to gold (yellow line). Before the voltage is turned on (blue wires), the alamethicin is laying on top of the membrane. Spectra are taken represented by red arrow. Then voltage is turned on (red wires). Then alamethicin will insert into the membrane and more spectra are taken in order to compare.

I took 2DIR spectra of alamethicin at a neutral pH and at a basic pH. I also took 2DIR spectra at no applied voltage and an applied voltage. What we saw was surprising! We expected that there would be a change in the frequency of the vibrations that would correspond to a change in the structure of alamethicin, which had previously been hypothesized. However, we saw instead that there was just a change in intensity of the three main peaks in our spectra. This was the case in both the voltage and the pH data. By using a piece of software developed by a previous graduate student in my group, we were able to model different secondary structures and orientations to determine what was happening to alamethicin when we either increased the pH or applied a voltage across the bilayer. We found that alamethicin structurally is composed of two helical elements connected by a kink in the protein. When alamethicin inserts into the membrane the kink angle changes along with the angle of insertion! We were excited that we were able to not only prove

that we could take quality data using this platform, but also that we were able to contribute to the understanding of alamethicin.

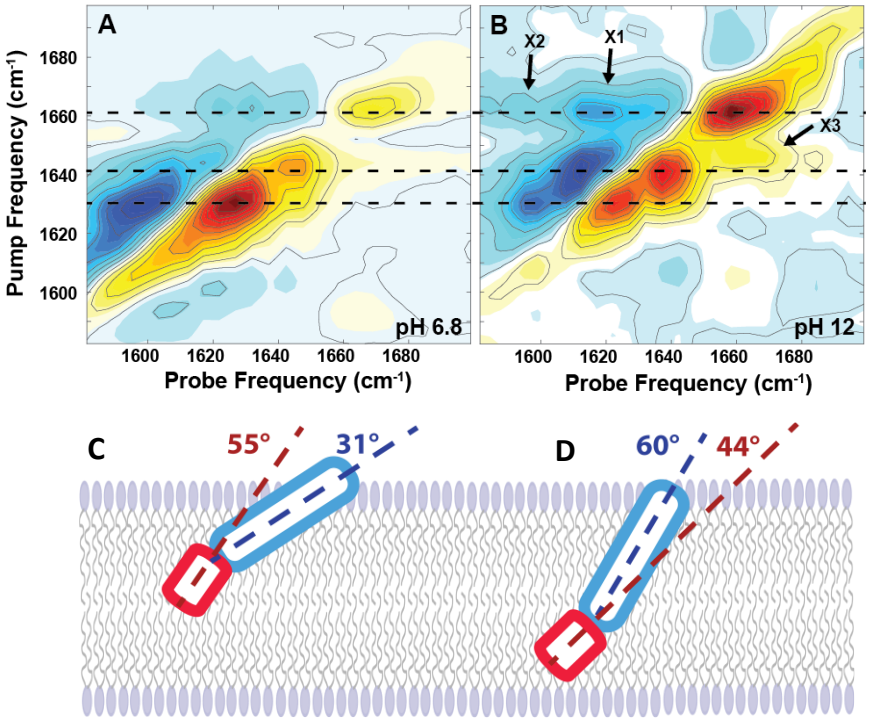

Figure 6. Representative data from my alamethicin project. A) shows the neutral pH spectrum, which is similar to the neutral voltage experiment. B) show the basic pH spectrum which is similar to the applied voltage spectrum. We saw these three peaks shown on the dotted lines are consistent across the spectra. Using modeling to recreate these spectra, we could back calculate the insertion angles of the two sections of alamethicin shown in C) (neutral) and D) (basic/applied voltage).

5. Looking at ion channel structure

We now had solved half of the problem of figuring out how we were going to study voltage-gated ion channels. However, we were working with a small protein, where we could look at every section of backbone at once and learn about the structure and orientation. The ion channels we want to study have 12 times the number of amino acids compared to alamethicin. This means that instead of seeing individual peaks for different protein structures, all of the peaks bleed together

into one broad peak. We want to be able to learn about what is happening at an individual amino acid, or at least at a small collection of amino acids so that we can best interpret our data.

We are interested in two parts of an ion channel. One is called the voltage sensing domain which, as the name suggests, is the part of the protein that senses a change in voltage. The voltage sensing domain only exists in voltage-gated ion channels and contains many positively-charged amino acids that react to changes in charge, whether it be being attracted to negative charges (opposites attract!) or being pushed, or repelled, by other positive charges. This work on the voltage sensing domain is very exciting though not the main thrust of the work I will present here. Though some preliminary work done on the voltage sensing domain shows us that something is in fact moving when we apply a voltage.

The other part of the ion channel that we are interested in looking at is called the selectivity filter. This is the part of the channel that only allows the particular ion of the channel to move through. The ion channels we study are potassium channels which are especially interesting. When you picture a filter, you probably think of something like a sieve. For example, when I am baking, when I sift my dry mixture of flour, baking powder, and salt, sometimes the salt does not pass through the filter because it is too large in size. The selectivity filter in these proteins actually does the opposite. In the particular ion channel we study, called KcsA, the filter lets potassium through, but prevents sodium, which is smaller, from entering the channel!

As I mentioned above, the 2DIR spectroscopy we do on proteins looks at how a carbon and oxygen bond vibrates. We are sensitive to changes in this vibration that can occur when an ion or

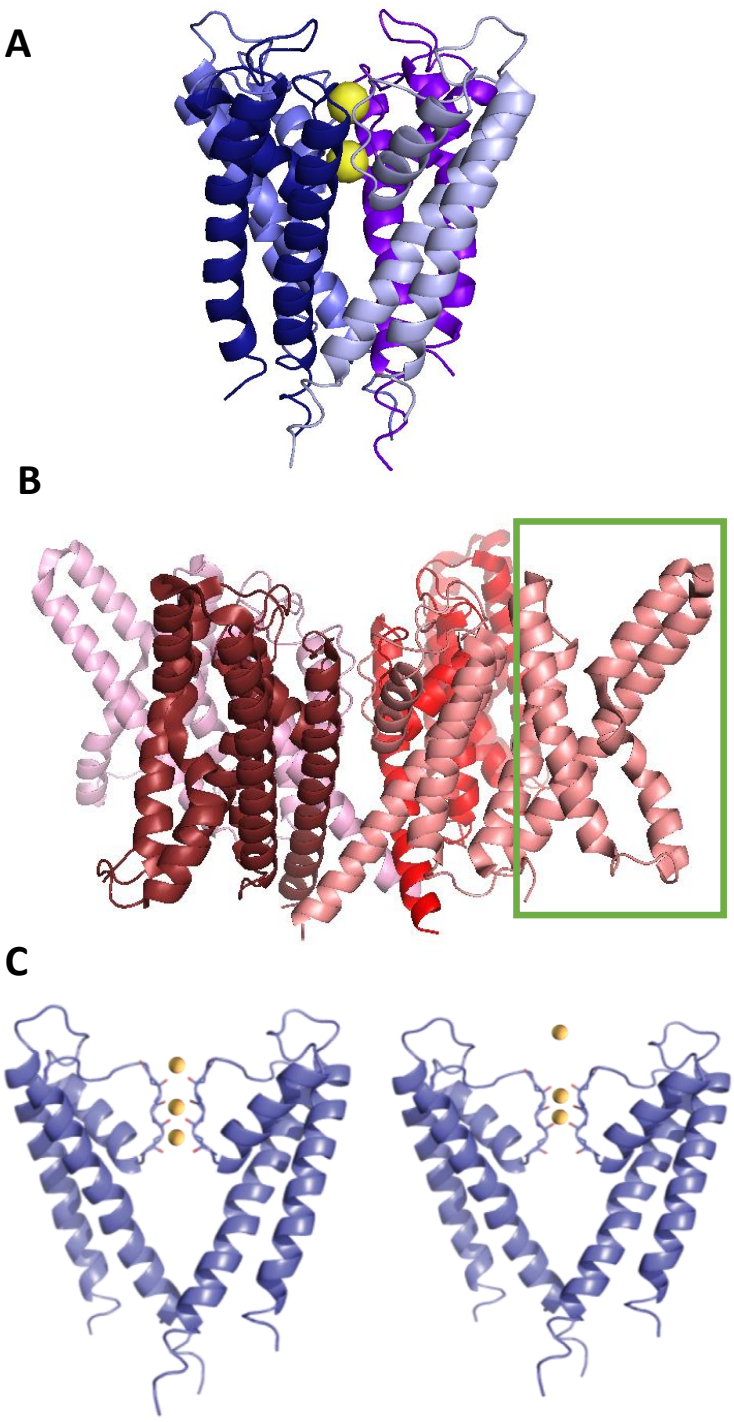

Figure 7. Cartoon images of ion channels. A) Shows KcsA with the four segments shown in different shades of blue and potassium ions in yellow moving through the channel. PDB 1Bl8. B) Shows KvAP, a voltage-gated ion channel with the four segments in different shades of red. The green box indicates the voltage sensing domain of one of the segments. PDB 6UWM. C) Shows different possible potassium configurations in KcsA. Only two of the four sections are shown to see how the selectivity filter interacts with the ions. The inward facing sticks are the carbon and oxygens bonds that we are measuring.

are impacted by the environment it is in, we need a way to be able to see one site away from the rest of the protein. We do this by creating labels. Labels can be used to change the frequency of the site of interest in order to see it on its own in the spectrum. One way to make a label is to replace the carbon and the oxygen with heavier isotopes. We are still measuring the same amide I vibration, but the carbon weighs 13 atomic mass units instead of 12 atomic mass units, and the oxygen weighs 18 atomic mass units instead of 16 atomic mass units. Increasing the mass of the carbon and oxygen makes the amide I vibrate more slowly and allows us to look at just the amino acids where we replaced those atoms. A benefit of the isotope labelling technique is that there is no change in the protein structure when this label is added. Another method for labelling is just changing the amino acid to something else. The difference between an amide and an ester is that a nitrogen is being swapped for an oxygen. The ester stretch is higher in energy and also allows us to look directly at the site, but there is a small structural change. We can look at it because it moves the peak out of the bulk, like wearing a neon shirt at the state fair, so your family can find you more easily.

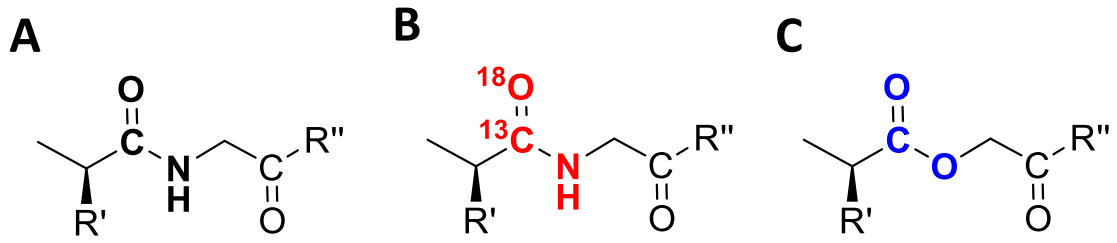

Figure 8. Showing a bonded pair of amino acids with different labelling schemes. R' and R'' are stand-ins for the side chains. A) is the unlabeled sample. B) Is the isotope label sample. Shown in red are the atoms contributing to the vibration. The weight is increased by the isotope labels of the carbon and the oxygen. C) shows the mutations from an amide in A) and B) to an ester (in blue). This will also cause a shift in the vibration.

In the work that I am presenting here, we use the ester label. Not only would this be the first time that an ester in a protein backbone is studied using 2D IR spectroscopy, but we also are able to glean information about the structural change. Previous information using different

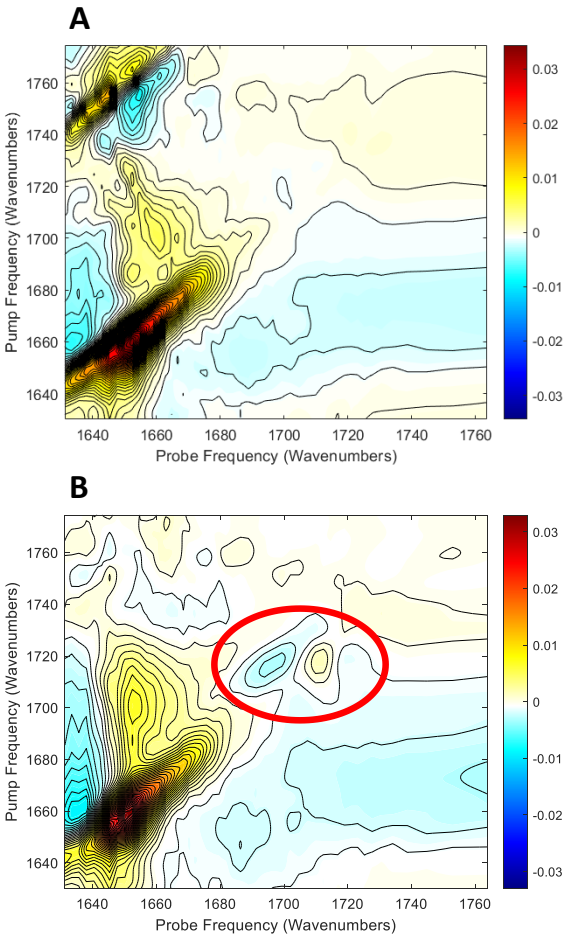

Figure 9. 2DIR spectra demonstrating the use of an ester label. A) NaK2K (a relative channel to Kcsa) without a label. The amide I vibration is the large peak in the lower left corner of the spectrum. B) NaK2K with an ester label. The peak corresponding to the ester vibration is circled in red. Now this specific site in the protein can be analyzed separately from the rest of the protein.

biochemical techniques show that the ester insertion in the selectivity filter has caused the ion distribution to rearrange and that ions stop flowing through the channel. By systematically changing which amino acid in the channel was switched to an ester, those scientists were able to determine which amino acids are responsible for stopping ion flow. We are currently taking 2D IR spectra of KcsA, our potassium channel, with the ester label and are taking spectra at different time points to learn about how ions are influencing the carbon and oxygen bond. Then we hope to interpret our data with the help of calculations that will allow us to see how the ion channel could be moving.

6. Conclusions

Most of the work done in my Ph.D. focused on finding ways to make new and exciting measurements on membrane-bound proteins. Many of the problems I have needed to solve have come from figuring out how to make measurements of difficult to work with samples and then being able to take data that allows us to learn something. Furthermore, this work is a leap in

progress in method development. Much of the work presented here is ‘basic science’ which means that we are trying to figure out how the world around us works. Although it is harder to see the impact of this work on things like human health, for example, I think it is similar to the fact that before you can understand how to fix a broken car engine, you also need to know what a working one looks like and understand how it runs. I like to think that the impact of this work, maybe decades down the road, will help us better understand and treat the root of nervous system diseases, based on my creating a useful tool to study ion channels and membrane-bound proteins over the last five years. Next, I will be working at the Smithsonian Institution as a post-doctoral fellow using vibrational spectroscopy, along with other techniques, to study how display cases and other gallery and storage environments interact with the objects on display so that the objects can stay healthy for generations to come.