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Utilizing Cryogenic Ion Vibrational Spectroscopy to Investigate How Side Chains Affect Structure and Solvation of Small Peptides

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CHAPTER 8

Introduction into Fundamental Research Using Gas-Phase Spectroscopy To Probe Small Peptides

8.0 Preface

My whole life growing up my mother always stressed to me that education was important and told me that "education was the only thing that she was going to leave me behind." My mother had immigrated to this country from Argentina at 22-years old and my grandmother and aunt followed when I was 6-years old. Therefore, I grew up in a primarily Spanish speaking household and became the first person in my family to go to college in the United States and complete a double Bachelor's degree. And now, as I sit here and write my thesis, I will be the first in my family to earn a doctorate! Though we did not come from wealth, it was always important to them for me to get an education. My passion has always been with physical and analytical chemistry, and in general, physical chemistry has a lot of jargon which can be very daunting for a non-scientific audience. My family loves to be in the loop with what I am doing and does try to understand it, yet I know that even if they fully don't understand what I am studying, they are still pushing me to succeed. Therefore, I have written this chapter to explain my research to a broad, non-scientific audience and I wanted to dedicate it to my family.

Before I continue, I would also like to thank Professor Bassam Shakhashiri who created the Wisconsin Initiative for Science Literacy (WISL) at UW-Madison which is the program that gives graduate students the platform to be able to write this chapter. It is amazing that as a UW student, we are supported in our endeavors to create this chapter and it is recognized that describing our work to a non-scientific audience is actually important! I would also like to thank Professor Shakhashiri, Elizabeth Reynolds, and Cayce Osborne for dedicating their valuable time to read my chapter, provide valuable feedback, and also give encouragement in the quest to write this chapter.

8.1 Fundamental Chemistry—Why Should We Care?

When I began my graduate school experience and had to choose a group to do my PhD in, I had absolutely no knowledge of the field of gas-phase spectroscopy. There are many questions that I constantly get asked as I have gone through my PhD in this fundamental physical chemistry field: “Why does your research matter? Does this help discover something in pharmaceuticals? In the end will it help cure cancer?” The list goes on. Though fundamental in nature, my research has a larger impact when you look at the full picture; however, my particular research is far removed from practical applications.

In high school chemistry and biology classes, the teachers often tell students trends that are known to be true in chemistry without any of the facts or data behind it. For instance, one major example that teachers tell students is that the “structure of a molecule determines its function.” This is inherently true. These teachers might even tell their students particular trends, “If there is an electron withdrawing group then we expect the reaction to proceed in X way; however, there is an exception…” And as a high school student, we take this for granted, accept it as fact, and memorize the trends and exceptions. As we go on and delve further into the subject, we realize that there are a lot of reasons that these statements are true and why there might be exceptions to cases. But it isn’t until we go straight into the fundamentals, through quantum equations and derived principles that we can fully begin to understand why it is true on all levels. This is where my research steps in and helps guide scientists in many fields. We do work in trying to fundamentally understand what is occurring to then be able to explain the trends and exceptions that we see.

8.2 The Big Picture

When many people think of protein, they think of something they can ingest— food or supplements. Independent of food consumption choices, everyone knows that protein is necessary for a healthy diet in day-to-day life. Many people who do strenuous workouts supplement their diet with additional protein to help repair and grow their muscles. Besides being something that people ingest, protein is also found everywhere throughout the body and it plays many critical roles. Some of these critical roles are the proteins functioning as antibodies or serving as enzymes, which are molecules used to bring about specific chemical reactions in the body. However, there are many more roles that proteins play.[1]

There is much interest in understanding how proteins interact in the environment. However, before understanding how they interact in the environment, which can depend on many different factors, it is important to understand them in their simple state, i.e. without external influences, and then build up the complexity.

Proteins, which are very large peptides, are made up of a collection of amino acids. Each natural amino acid in general contains a carboxylic acid group connected to an amine, shown in **Figure 8.1**. A carboxylic acid group is where there is a central carbon atom (C) bonded to an oxygen atom (O) by a double bond and a hydroxyl group (-OH) by a single bond. Its last bond links the carbon atom to the rest of the protein chain. An amine group is where a nitrogen (N) is bonded to two hydrogen atoms (H). Its final bond links the nitrogen atom to the rest of the protein chain. All natural amino acids differ by their corresponding R-group, which is just an abbreviation for any group that has a carbon or hydrogen atom to attach to the "rest (R) of the molecule," that is tacked on the base, also shown in **Figure 8.1**. Therefore, every R-group gives each of the 20

Figure 8.1. The backbone of one of the 20 natural amino acids. It consists of a carboxylic acid attached to an amino by a single carbon chain. The R-group denoted in green changes based on the amino acid.

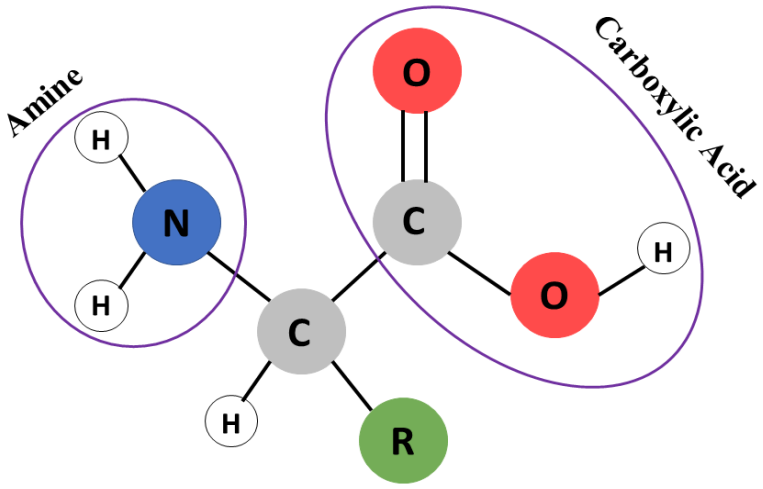
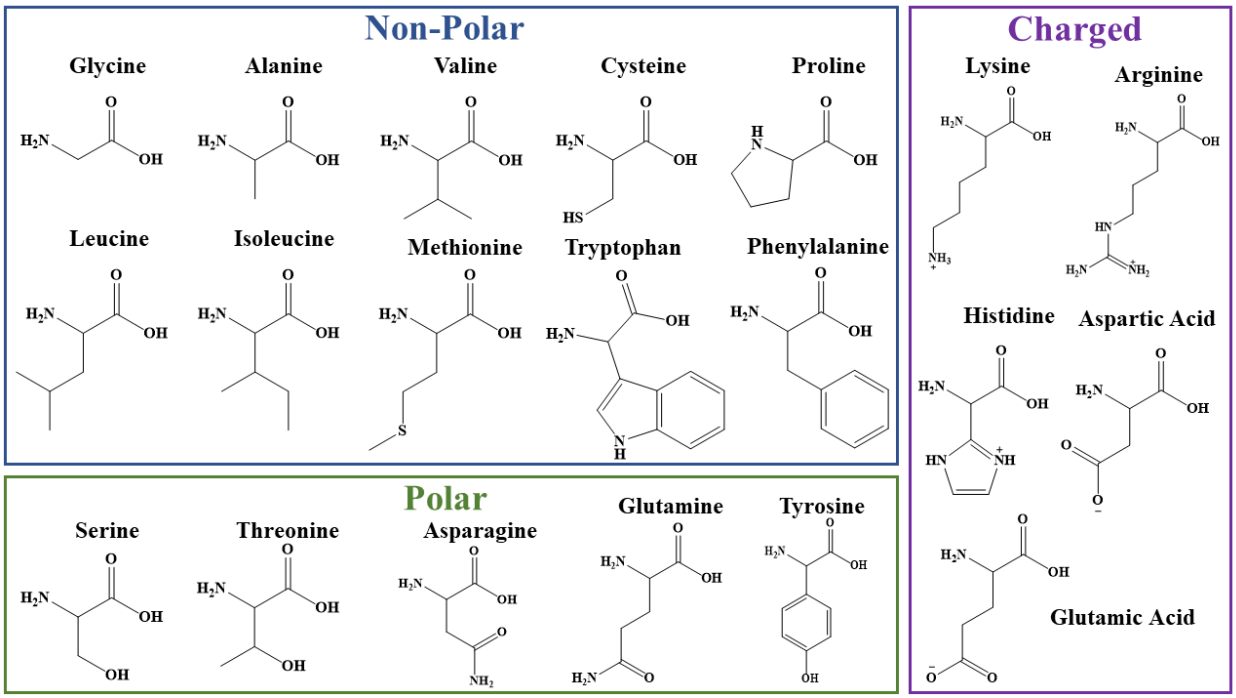


Figure 8.2. The 20 natural amino acids and their three distinct groupings.



natural amino acids their own specific properties. The natural amino acids can be divided into three distinct groups based on their side chains: non-polar (neutral molecule containing no positive or negative poles since all electrons are shared evenly), polar (neutral molecule containing positive and negative poles), and electrically charged (has a permanent positive or negative charge), shown in **Figure 8.2**. Amino acids attach together as building blocks for the peptide/protein. I think of amino acids like legos. Every different type of amino acid is a different size and might have a slightly different shape, just like the vast array of legos. Depending on how they are placed together, you might have a very different end structure (protein). And since we have heard in the past that the structure of the protein affects the function, it is important to understand why this happens. We can therefore use the amino acids like our own version of legos and build up our protein one block at a time to be able to see how every little change affects the structure and therefore the function.

8.3 Why Probe in the Gas Phase?

As I said above, we want to start very simple. There are so many techniques to be able to look at these proteins. Many techniques probe them in the solution that you would find them in, such as water. Since our bodies are ~60% water, with some regions being a higher percentage and others being a lower percentage, it makes sense to want to probe these molecules in water. However, water is a tricky molecule. In many techniques, water leads to a broadening of experimental features so much so that features turn into a blob when recording spectra. It is not then possible to actually see every little wiggle in a spectrum, and therefore interpret it. Also, in water, there are many more interactions that can occur. The water can interact with the protein

through hydrogen bonding and the water molecules can interact with each other. Therefore, a simple molecule can get very complicated extremely quickly.

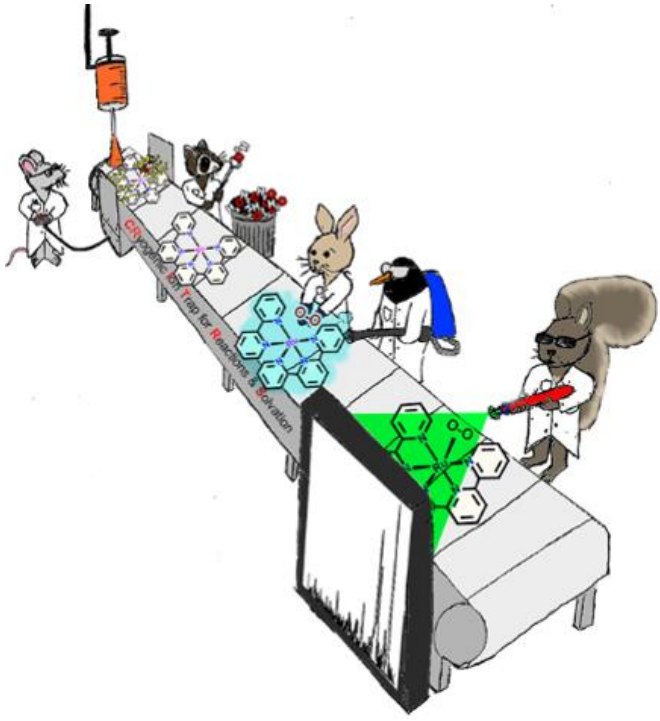
To be able to isolate the molecule outside of any solvent (a substance, which is normally a liquid, which is used to dissolve other materials in order to make a solution), we would need to put the molecule into the gas-phase. However, in the gas-phase, in order to detect your molecule, it would need to be charged in some way. Since all but five natural amino acids are neutral, which means that they do not contain a permanent positive or negative charge, it has to be manipulated for us to detect them and once manipulated, the charged species is called an "ion." Therefore, we can see the ion as it is without any solvent at all. And depending on instrumentation, it is possible to slowly solvate the molecule of interest one water molecule at a time and build up to bulk solvent. It is basically like putting salt in an empty glass and slowly adding one drop of water at a time. In the beginning, with such few drops you can see how the salt interacts with those drops, but as you keep adding more and more water, you build up to a glass of water where the salt is fully dissolved. After a certain point, it doesn't matter how many drops that you add, the extra water doesn't change the interaction between the water and the salt, this is called the bulk. This gives a better idea of the properties that the molecule might have on its own and how those properties change as a function of solvation.

8.4 The Machine

So how are we even going put our molecule into the gas-phase? We are able to probe the molecule on an instrument termed CIVS, which stands for Cryogenic Ion Vibrational Spectroscopy. If you walk into the Garand lab and see this "machine" as Etienne calls it, it looks like a daunting task, as a 3-meter instrument that has probably over 100 black cords crossing every

which way stares back at you. However, in its simplest form, it is basically an ion cooler and can be described by the cartoon that Jon Voss (PhD 2018) drew, and Erin Duffy (PhD 2017) colored in, shown in **Figure 8.3**. The figure itself is showing a ruthenium molecule going through the instrument, but the set-up is the same for a peptide. A peptide is the same as a protein since it is also a collection of amino acids, however it is usually smaller in scale.

Figure 8.3. Cartoon instrument schematic drawn by Jonathan Voss (Ph.D. 2018) and colored by Erin Duffy (Ph.D. 2017).

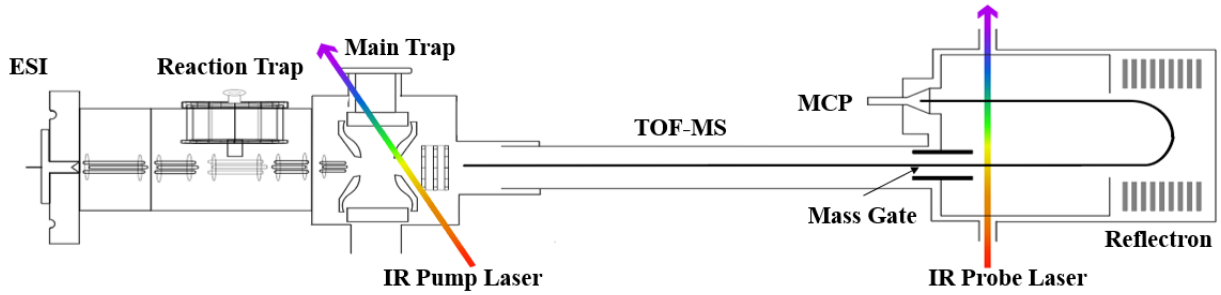


The instrument starts when the user (the lab rat) adds a liquid sample of the protonated peptide (a peptide with an additional hydrogen attached to it forming a charged species, peptide-H^+), which is our ion, being probed into a syringe that has a voltage applied to it to allow the sample to spray and go into the gas phase. We call this electrospraying the solution into the instrument. The ions then get guided through the instrument and stop at the reaction trap (the

racoon). At this stage, the user can add a solvent into the instrument through one of the gas lines. Even with one drop of water, for instance, the gas can flow over the drop and bring water molecules into the instrument to cluster around the peptide. Once clustered, the molecules get transferred from this stage into the main tagging trap (the bunny and the penguin). Here the clusters get cooled to 10 Kelvin, which is -263 degrees Celsius (very cold!), by a cryostat and a tag molecule, usually D_2, gets added to the cluster. D_2 is deuterium gas, which is an inert gas (it does not interact or chemically react with other species) and deuterium is an isotope of hydrogen; it has one additional proton than a hydrogen leading it to have a molar mass of 2 g/mol while hydrogen is only 1 g/mol. We utilize a D_2 tag for the laser stage of our instrument so we can observe a change of mass whenever something interesting is happening to our molecule through interaction with a laser. From here, the tagged cluster is taken into the last stage of the instrument, which is the laser stage (the squirrel). Whenever the laser is resonant with a vibration of the molecule, the molecule begins to move by a certain mode (bending or stretching of certain bonds). The vibrations are then redistributed by intermolecular vibration redistribution (IVR) and this motion allows the D_2 tag to pop off the cluster. As I sit here and write this next to my golden retriever, all of what I described above can be related and visualized as a human petting a dog. The dog is like our molecule and the human is like the laser. As the human is petting the dog, they move their hand from the top of the dog's belly to down near the legs, kind of like the laser scanning a certain frequency range. When you hit that "sweet spot" on a dog, the dog's lower legs start going crazy. This "sweet spot" on a dog can be related to a "resonance" of the molecule. If we imagine that there was something weakly attached to the dog's leg, as the D_2 tag is to our molecule, when the dog starts to move his legs, the weakly attached substance can be dislodged! All species are separated and hit a detector. We now then have two different masses reaching the detector when the laser is resonant (the untagged

and tagged cluster) and only one mass reaching the detector when the laser is not resonant (just the tagged cluster). By monitoring the appearance of the untagged cluster as a function of laser wavelength as we scan the laser, we can produce an IR spectrum of the cluster that we are interested in. The actual instrumental set-up is shown in **Figure 8.4**.

Figure 8.4. Real CIVS instrument schematic.



8.5 Understanding the Infrared (IR) Data

As stated above, the data that we get is basically how the peptide vibrates. Since each peptide is just a collection of amino acids, there are a couple of vibration features that we know that all the IR spectra will have. Each peptide has an O-H on the carboxylic acid (carbon double bonded to an oxygen and singly bonded to an -OH) and N-H features on the amine (nitrogen bonded to two hydrogens) and amides (nitrogen bonded to a hydrogen and to a carbon that is doubly bonded to an oxygen). The O-H and N-H features vibrate separately from each other and are dependent on the wavelength of the laser. So, what do I mean by they vibrate? If we look at **Figure 8.5**, I am showing how the O-H "vibrates." This motion is the O-H bond stretching and compressing while the rest of the molecule stays unperturbed. With an IR-laser, we typically would see a feature that represents this mode when the laser is placed around 3575 cm^{-1} (wavenumbers). Wavenumbers is defined as the number of waves per distance and is used in IR spectroscopy since

Figure 8.5. The "vibrating" stretching motion of the carboxylic O-H. The O-H bond stretches and compresses. This resonant mode is usually found around 3575 cm^{-1}.

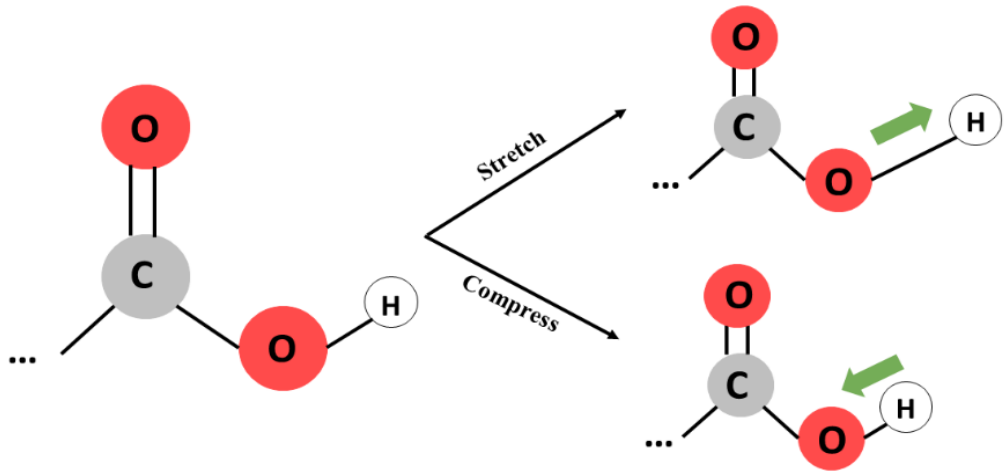
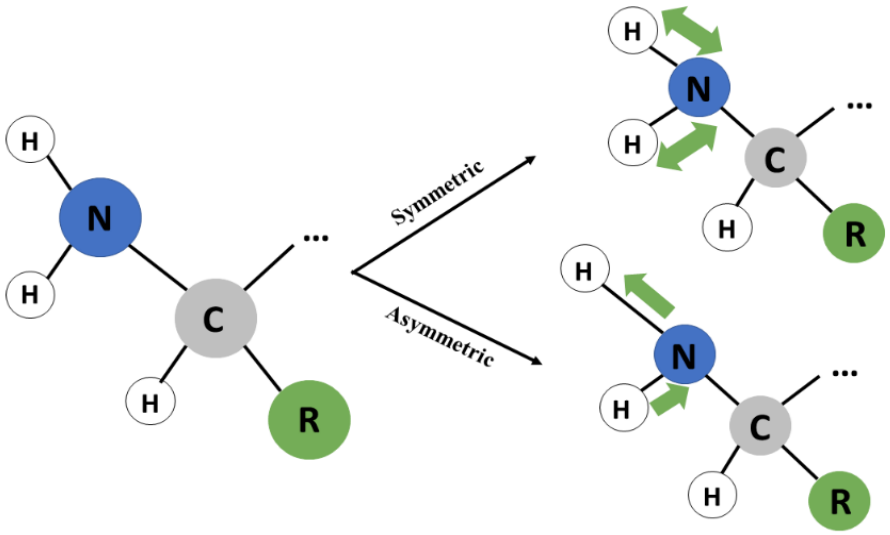


Figure 8.6. The "vibrating" stretching motion of the amine N-H. The N-H bonds stretch and compress. They can be either a symmetric mode where both N-H bonds stretch and compress in sync or they can be asymmetric mode where one N-H bond stretches while the other compresses and vice versa. These resonant modes are usually found around 3420 and 3470 cm^{-1}, respectively.



wavenumbers is proportional to the energy of the light; the higher the number, the greater the energy of light. Therefore, this mode would have a "resonant frequency" at 3575 cm^{-1}. An amine, which is at the other end of the peptide, has two different stretching motions, shown in **Figure 8.6**. It can have a "symmetric stretch" which is where both of the N-H bonds expand and compress in sync and it can have an "asymmetric stretch" where one N-H bond expands at the same time as the other compresses and then vice versa. These features usually have resonant frequencies around 3420 and 3470 cm^{-1} for the symmetric and asymmetric stretches, respectively.

When it comes to protein folding, there are interactions of the atoms within the proteins that we can probe with our IR laser. Hydrogen bonding is usually the main culprit for protein folding, both within the molecule and outside of the molecule by solvent. Hydrogen bonding is rather simple in definition. It requires there to be a hydrogen to be bonded to another atom (usually oxygen, fluorine, or nitrogen). This bond is weaker than the bonds within the molecule (the single and double bonds) but stronger than other types of interactions the molecule can have. With our IR laser, the interactions probed within (intramolecular) the folded molecule are usually by the N-H features hydrogen bonding to the C=O feature of the carboxylic acid. And once water is involved, water molecules like to hydrogen bond to the N-H and O-H features (intermolecular). When these perturbations happen from hydrogen bonding, we see a "shift" of peaks in our spectrum, usually to the smaller wavenumbers. This is termed "red shifting." By understanding where these features should be unperturbed, and then seeing the features change as a function of different R-groups (an abbreviation for the rest of the molecule) of the various amino acids, we can give information as to how the various R-groups affect the structure of the peptides.

As an example of a real IR spectrum, I am showing protonated Gly-Gly-Gly (GGG) and Ala-Ala-Ala (AAA) in **Figure 8.7**. The structures of linear GGG and AAA are shown in **Figure**

8.8. Glycine and alanine are the two simplest amino acids and only differ by the R-group: the R-group in glycine is a hydrogen and the R-group in alanine is a methyl group. As you can see, in the green region where the O-H stretch should be, there is no difference between the two peptides. However, in the blue and purple regions which are from the amine/amide and hydrogen bonded amine/amide, there are differences between the two, both in peak height and peak placement so we can assume that the methyl R-group that is alanine does something to perturb the folding structure of the backbone.

Figure 8.7. (Top panel) IR spectra of protonated Gly-Gly-Gly (GGG) and (bottom panel) Ala-Ala-Ala (AAA). The various stretching mode regions are highlighted in different colors.

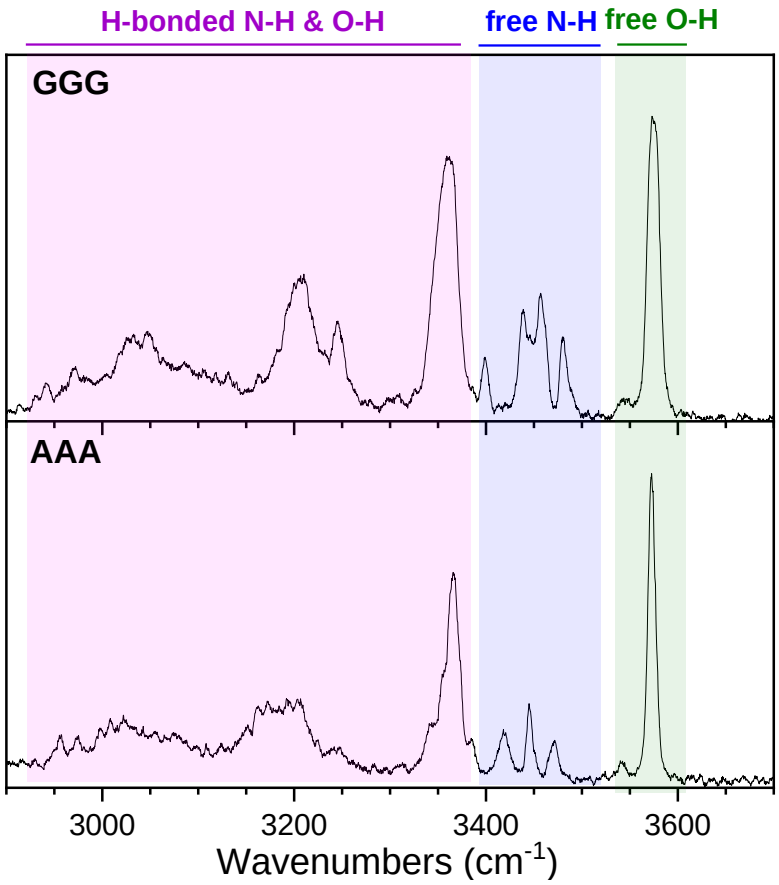
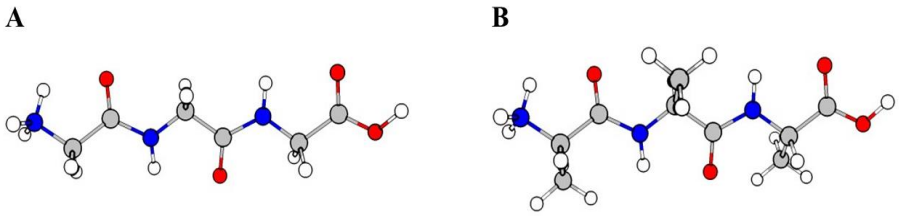


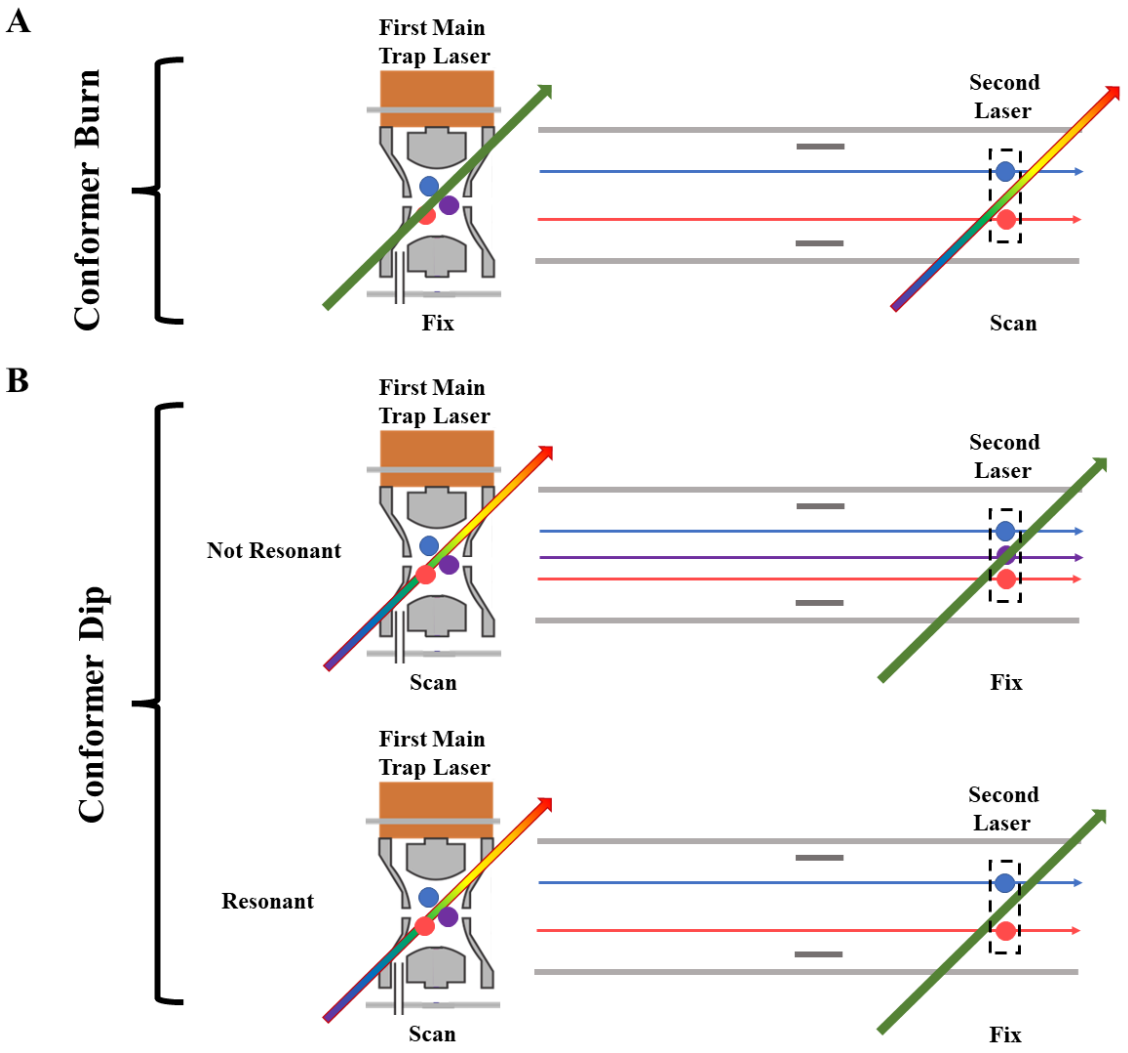
Figure 8.8. A) Protonated Gly-Gly-Gly (GGG). B) Protonated Ala-Ala-Ala (AAA). The difference between Gly and Ala is the R-group replacement of a hydrogen in Gly for a methyl group in Ala.



Since proteins and peptides are flexible, we can't expect them to be locked in one conformation, rather there are probably many conformations that exist both in solution and in the gas-phase. One way to know if there are more than one structure that you are seeing in your IR spectrum is to count the peaks. For instance, if we look at GGG we should have one O-H stretch peak, two amide N-H stretch peaks (since there are two amides), and three stretch N-H peaks that correspond to the protonated amine. This then means we should see 6 features in our IR spectrum if there was just one structure present. In just the green and blue highlighted regions there are 5 peaks and in the purple region there are at least 4 more. Since this total is larger than what we predict, then we know that there should be at minimum two different structures within this population. When a species has more than one structure that it can form into, we say that these species have multiple "conformers".

So how do we distinguish the multiple conformers? With our instrument set up, I described the case where we just had one laser at the end. However, we can add an additional laser to the main tagging trap stage and we can do IR-IR double resonance spectroscopy. Basically, this means we can do conformer selective spectroscopy. There are two techniques that we can use: fix the first laser that is in the main tagging trap and scan the second laser or we can flip the two and scan the first laser and fix the second laser. I am going to break down the two different techniques. You can also refer to **Figure 8.9** for a descriptive picture.

Figure 8.9. A) Schematic of conformer burn method. B) Schematic of conformer dip method both when the first main trap laser is not resonant and resonant.



1) Fix the first main trap laser and scan the second laser: This is what we call a conformer "burning" technique. Before describing the technique normally, I want to bring us back to the dog analogy. So, in our population, we can have more than one conformer which is like having more than one type of dog in our household. Each dog has its own quirks as well as its own "sweet spots" when petting it. If we have both dogs on their backs with something weakly attached to their foot, it is possible for me to get the spectrum of one dog without the effects of the other. If I know that one of the dogs is sensitive to scritches under his armpit and the other one isn't, I can scratch them both there at the same time. This leads one of the dogs to kick his back legs like crazy, which dislodges the weakly attached thing on their foot, while the other dog remains still. When I then look at who still has the tie on their foot, I see that the one lying still does, and then I can continue on with petting that dog until I see where their "sweet spot" is.

So now I am going to describe the technique without the analogy. Once we have the normal one laser spectrum, which is what I showed before and described in the instrumental set up, we can pick a feature that we believe might belong to one conformer (just like me scratching under the dog's armpit). From there, we fix the first laser on that wavelength. Since the laser is on, anything that is resonant with that wavelength will vibrate and pop off the D_2 tag in the main trap (as the dog did who had a sweet spot in the armpit). When the whole population leaves the main trap (me looking at which dog still has their tag on their foot), whatever does not have a tag gets kicked out of the system and what you are left with is anything that was not resonant with that wavelength, i.e. does not have that peak in the spectrum (just the dog lying still). With this technique you are getting rid of part of the population early on in the instrument and observing the spectrum of what is left through the second laser.

2) Scan the first main trap laser and fix the second laser: This is what we call a conformer "dip" technique. Before describing it normally, I want to bring back my dog analogy. So, let's say we have our two different dogs in the household, but I just want to understand where the Golden's "sweet spot" is without the effects of the other dog. I know that the golden loves getting petted on his ribcage and the other one is neutral about it. So, I am going to focus my pets there. First, I will start petting both, moving from the top of their belly to their legs, and at each step I am going to check who still has the tie around their leg. Then, I will pet the ribcage of whoever still has the tie around their leg. I am expecting my Golden to always shake the tie off when I pet his ribcage (therefore have a constant "signal"). However, if he shakes off the tie earlier when petting his belly, when I move to the second step of petting the ribcage, I won't see anyone shake their tie off because my Golden has already done it earlier! This is then a "dip" of signal in each place this happens.

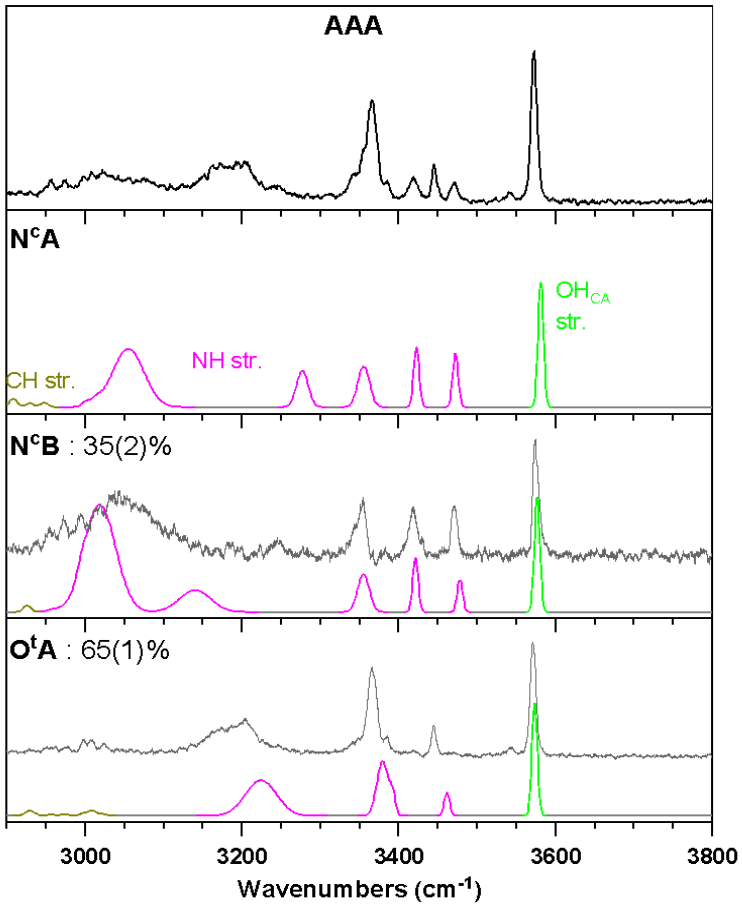
Now I will describe this technique plainly. Similar to the burn method, once we have the normal one laser spectrum, we pick a feature that we believe might belong to one conformer and we set our second laser at the end of the instrument to it (petting the ribcage of the Golden). This means that no matter what, we should see a signal from this conformer. When we scan the first main trap laser, if it is not resonant in the main trap, it passes through the rest of the instrument and to the second laser, so we see its constant signal. In the main trap, if the conformer that we are probing is resonant, the molecule vibrates and loses its D_2 tag and is kicked out of the system there (if the Golden loses its tie earlier during the pets). So, when we are probing with our second laser, we actually lose the signal that we have. The resulting spectrum is basically the dip of a signal and if we invert it, it is the spectrum of just the conformer that has this feature that we were probing.

8.6 Matching IR Data to Real Conformers

So now that we have seen some data and can physically see changes in peptide folding due to different R-groups, we want to be able to know how these peptides actually look. For this we use computations that are done on the computer. We are able to build our molecules with Gaussian 16 software[2] and utilize other programs given to us from collaborators in the Hopkins group at the University of Waterloo[3-6] to help us search ways in which the peptides can fold. From there we can optimize geometries and get vibrational frequencies of the molecules. These vibrational frequencies are calculated frequencies that predict where the molecules' various vibrations should appear. We can compare these to our spectrum and get determinations of what structures we are seeing in the gas-phase. An example of this is shown in **Figure 8.10**. Here I am showing the IR spectrum of AAA which you saw in the bottom panel of **Figure 8.7**, however, I am overlaying the conformer specific spectrum to the calculated computation. As you see, I am able to highlight what features are due to what stretches as predicted with the calculations and there is really good agreement.

Now knowing the specific conformations, we are able to make informed opinions on what we believe is occurring. We can use other sets of computations to help guide our reasoning until we get to the bottom of what is going on. And many times, it is not just one factor. There may be a combination of things occurring which leads us to the result that we obtained such as sterics of the R-group on the amino acid (which is if the group is bulky and obstructs the peptide's/protein's ability to fold), electron donating/withdrawing effects from the side chain, proton affinities, etc.

Figure 8.10. (Top panel) The IR spectrum of AAA compared to the (Bottom panels) lowest energy calculated spectra and the conformer specific ion dip spectra for each structure. Each structure's population percentage and respective errors, in parenthesis, are denoted next to their names.



8.7 Impact

This research has not just helped put another piece together of the larger puzzle that is research, but it has also taught me a lot. I did not come with this fundamental research background, and I also had to learn all these techniques from the ground up. And though the techniques might be very specialized, more importantly I learned how to think and problem solve like a scientist.

No matter what endeavors I take in the future, the skills I have learned through this research (critical thinking, working independently and in a group, perseverance, and many more skills) will be extremely beneficial and I can use them all to my advantage.

I hope as I have written this chapter you have begun to grasp some of these techniques that I use on my instrument to answer these fundamental questions. My particular research has focused on these small peptides and understanding how their R-groups and solvation affect their folding (as I introduced and discussed above). I have been able to progress the gas-phase spectroscopy community by showing and understanding that gas-phase structures do make sense and give us insight into what might be happening in solution phase. Though these questions may be fundamental in nature, they help us understand the facts that we have been told many times over without justification. They also help other scientists make informed decisions on peptides to make if they want a particular structure and therefore a particular function. This fundamental knowledge is the basis of all science.

And where do I go from here? I have learned so many valuable techniques from my work in my PhD. Not only that, I have also learned how to critically think about a problem and try to use the fundamentals to give an answer. I will be taking my knowledge and skills that I have learned and used in the Garand Group and will be going on and doing a postdoc in the Remucal Group at UW Madison. Though the science is very different as I am moving from a fundamental physical chemistry field to an environmental chemistry field, I will be able to use everything I have learned and hopefully bring new insights to this different research. In particular, I will be able to bring my expertise about how small changes in the structure of a chemical's backbone can lead to different effects in a different class of compounds, Per- and Polyfluoroalkyl Substances (PFAS), also known as the "forever chemicals."

8.8 References

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