

Chapter 2

Bonded Bells and Two-Dimensional Spectra

Falling dominos. A bat hitting a baseball out of the stadium. A dog tugging on a leash. Energy transfer is all around us (Fig. 2.1). One of the most elegant demonstrations of energy transfer is “Newton’s cradle” (Fig. 2.2). You’ve probably seen this executive toy: five metal balls are attached to a metal frame by a thin wire, like five pendulums kissing each other. When the first ball is swung (Fig. 2.2a), it hits the neighboring balls, but only the ball at the extreme end reacts and swings out (Fig. 2.2b). The kinetic energy of the first ball is almost perfectly transmitted through the middle balls, causing only the ball at the end to move.

Like the metal balls in “Newton’s cradle,” ringing atoms in molecules also transfer their excess energy to other atoms. NMR spectroscopists call this energy transfer *coupling*. This phenomenon is very useful for characterizing the details of molecular structures because it tells us which atoms are close to one another. We’ll see how this works in the next two chapters.

2.1 Introduction to Coupling

In Chap. 1, we learned that atoms ring like bells when they are in a strong magnetic field. The frequency at which they ring depends on the atom type and its electromagnetic environment (i.e., the types of atoms

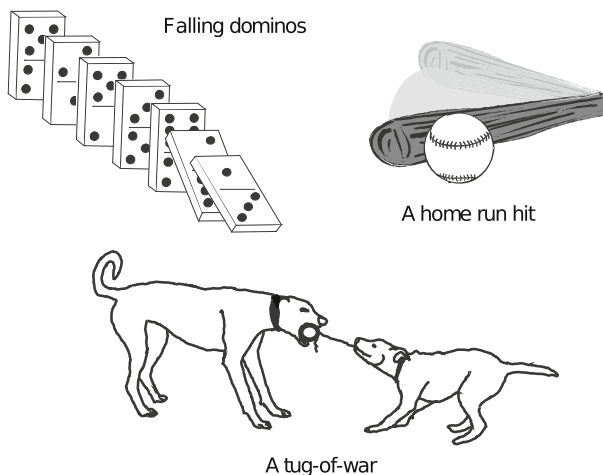


Fig. 2.1 Three common examples of energy transfer: dominos knocking each other down, a bat hitting a baseball, and two dogs playing tug of war with a toy. Can you identify more examples of energy transfer around you right now?

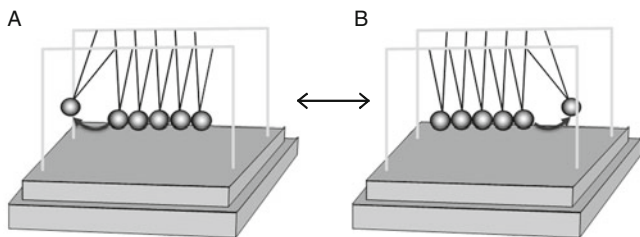


Fig. 2.2 “Newton’s cradle”: As the two metal balls on the outside swing back-and-forth (**a** and **b**), the middle balls transfer the kinetic energy almost perfectly and thus stand completely still

around it). In an NMR experiment, we make atoms ring by applying a strong radiofrequency pulse to the molecule. This pulse hits particular atoms like a big hammer, causing the nuclei to ring and create the NMR signal (Fig. 1.6).

Like a church bell ringing, a ringing atom has excess energy, and it can transfer this surplus energy to neighboring atoms if the two atoms are:

(a) Connected by a covalent bond

or

(b) Close in space, more specifically less than about 5 Å (or 5×10^{-10} m) apart.

In both cases, the type of energy exchanged is nuclear magnetization. You'll hear and read this term often in NMR. For example, "The magnetization is transferred from atom A to atom B." You can think of it like the metal balls in Newton's cradle (Fig. 2.2), moving energy from one nucleus to another. In Newton's cradle, the extra energy is kinetic; in NMR, the extra energy is electromagnetic.

In the language of physical chemistry, transfer of magnetization through bonds is referred to as "J-coupling," while transfer of magnetization through space is known as "dipole-dipole" coupling. Before we take a look at J-coupling (we'll discuss dipole-dipole coupling in the next chapter), let's examine the term *coupling* a bit further.

In the non-NMR world of everyday life, when you hear the word *couple*, you probably think of two people dating or a married couple. Indeed, when two people are a couple, their activities are connected. They eat together, work-out together, and study together. After years of marriage, couples even start dressing the same—a "style coupling"—or eating similar foods—a "diet coupling."

In NMR, *coupling* is quite similar to the more common, everyday usage of the word. When two nuclei in a molecule are coupled, the

activity of one nucleus affects or controls the activity of the other nucleus. When one nucleus starts ringing, it makes the neighboring nucleus join in. Think back to Newton's cradle (Fig. 2.2). When the ball on the right swings, it causes the metal ball on the left to swing as well. Thus, the two balls are coupled. The first ball shared its kinetic energy with the second ball. In NMR, coupled nuclei also share each other's excess energy, just like human couples share so many things in life.

What about when a married or dating couple starts doing the opposite actions of each other. For example, you are so sick of your boyfriend that when he goes to the library to study, you intentionally stay in the dorm and work. You are still *coupled* because his choices strongly affect your choices. However, instead of your actions being *correlated*, they are now *anti-correlated*.

2.2 Bonded Bells: J-Coupling

Two atoms connected by a covalent bond can share or transfer their energy when they ring. This is referred to as J-coupling, and it is extraordinarily useful in biomolecular NMR. It tells us not only which atoms are bonded to each other but can also provide information on angles between neighboring bonds (known as dihedral angles). Let's learn how it works.

Two atoms are covalently bonded when their nuclei share electrons. These electrons orbit between both nuclei, keeping the atoms close together (Fig. 2.3). You can think of these shared electrons as springs connecting the nuclei (Fig. 2.4). If we start ringing one nucleus, say the nitrogen atom (Fig. 2.4a), the spring will oscillate and eventually transfer part of the energy to the attached hydrogen, causing it to ring too (Fig. 2.4b). This is the essence of J-coupling.

Although the analogy to a spring is an oversimplification, it does help explain a few important and useful characteristics of the J-coupling phenomenon:

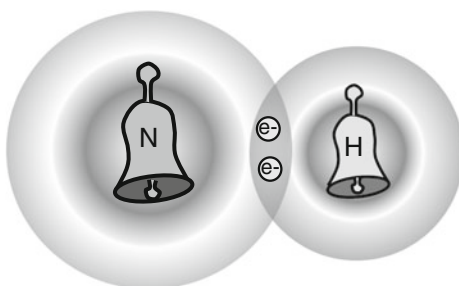
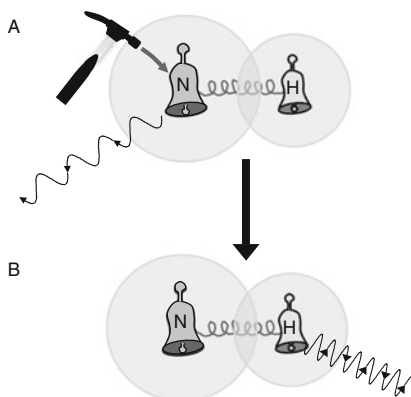


Fig. 2.3 Two atoms are covalently bonded when they share two or more electrons

Fig. 2.4 (a) When we ring the nitrogen's nucleus, (b) the extra energy can transfer through the shared electrons and ring the neighboring hydrogen. Thus, the shared electrons in a covalent bond act like a spring between the nuclei



- (a) As a spring oscillates back-and-forth between two endpoints, energy (or magnetization) oscillates between two bonded nuclei.
- (b) How fast a spring oscillates depends on the material and other properties of the spring. For atoms, the speed of the energy exchange depends on the particular properties of the electrons and the bond between the two atoms.

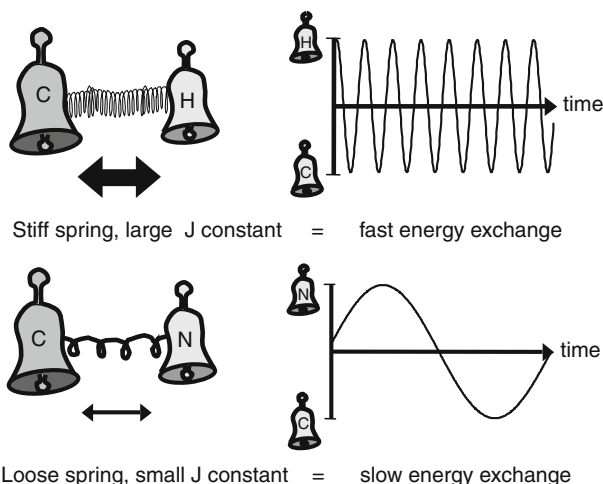
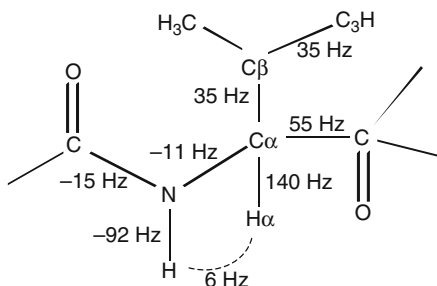


Fig. 2.5 (a) Bonds with large J -coupling constants (stiff springs) transfer excess energy between the two nuclei quickly and (b) bonds with small J -coupling constants (loose springs) transfer the energy more slowly

Stiff springs, like the ones in car shocks, have large “spring constants” (Fig. 2.5a). In this case, when you hit the nitrogen, the spring will oscillate quickly, and the energy moves back-and-forth between the atoms at a high frequency. Loose springs, like a slinky toy, have small “spring constants.” In this case, when you hit the nitrogen atom, the spring will oscillate slowly (Fig. 2.5b), and the energy will move back-and-forth between the two nuclei at a much slower frequency. The “NMR spring constant” for bonded nuclei is called the *J-coupling constant*, and it tells us how fast the shared electrons transfer energy (or magnetization) back-and-forth between two bonded nuclei.

Let’s look again at the valine–threonine peptide as an example. In Fig. 2.6, the values next to each bond show the bond’s J -coupling constant. Notice that the units are frequency—Hertz. Why? Because the J -coupling constant (also known as the scalar coupling constant) tells us how fast energy oscillates between the two connected atoms, just

Fig. 2.6 Typical J-coupling constants for bonds in a protein



like the chemical shift from [Chap. 1](#) tells us how fast a nucleus rings. It's almost like the bond is ringing!

Bonds with large J-coupling constants, such as the one connecting $^{13}\text{C}_\alpha$ and $^1\text{H}_\alpha$ atoms ($^1J_{\text{C}_\alpha\text{H}_\alpha} \sim 140$ Hz), will quickly transfer magnetization between the two bonded atoms (Fig. 2.5a). Thus, the energy state of the $^1\text{H}_\alpha$ nucleus greatly affects the state of the $^{13}\text{C}_\alpha$ nucleus. If the $^1\text{H}_\alpha$ nucleus starts ringing strongly, the $^{13}\text{C}_\alpha$ nucleus will definitely join in and start ringing too. This is analogous to how your mood has a profound effect on your spouse's mood when you are “tightly coupled.”

In contrast, bonds with small J-coupling constants, such as the one connecting $^{13}\text{C}_\alpha$ and ^{15}N atoms ($^1J_{\text{C}_\alpha\text{N}} \sim 11$ Hz), take more time to transfer magnetization (Fig. 2.5b). These atoms are more “weakly coupled,” and their energy states barely affect each other. If one nucleus is ringing strongly, the other nucleus is hardly affected. Perhaps you are “weakly coupled” to your parents right now—if they nag you long enough, you might do what they say, but it takes time and effort.

Notice that $^1\text{H}_\text{N}$ and $^1\text{H}_\alpha$ atoms in Fig. 2.6 also have a J-coupling constant (6 Hz). Although they are not directly bonded, they are connected via three bonds: H_N to N, N to C_α , and final C_α to H_α . This is called a *three-bond coupling* (denoted by a 3J coupling constant). Although 3J coupling constants are usually quite small, as it is here—only 6 Hz, they can provide useful structural information

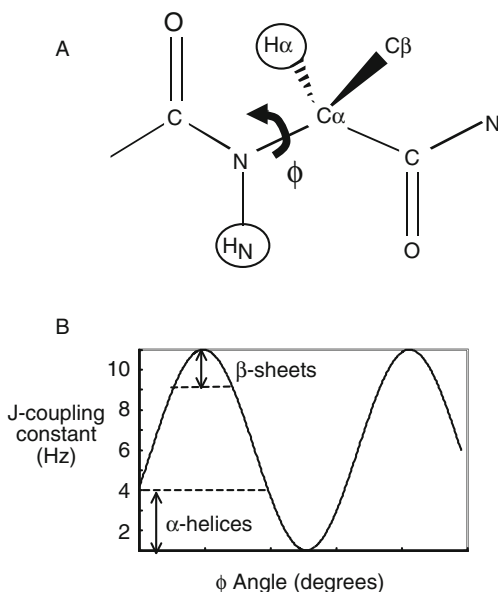


Fig. 2.7 The J-coupling constant between the H_N and the H α atoms in a protein tells us (a) the approximate angle between these two atoms (the torsion angle ϕ about the N-C α bond) and (b) whether this residue of the protein forms an α -helical or β -sheet configuration

for macromolecules. For example, the $^1\text{H}_\text{N}$ - $^1\text{H}_\alpha$ ^3J -coupling constant tells us the approximate value of the ϕ (phi) torsion angle about the N-C α bond (Fig. 2.7a). In α -helices of proteins, ϕ is approximately -60° , and the $^3\text{J}_{\text{HN}\alpha}$ coupling constant is barely detectable at about 4 Hz (Fig. 2.7b). In contrast, extended regions of the protein (called β -sheets) have ϕ values around -135° , and the $^3\text{J}_{\text{HN}\alpha}$ is larger (~ 9 Hz) (Fig. 2.7b).

The relationship between ϕ and the $^3\text{J}_{\text{HN}\alpha}$ coupling constant is described by a geometric relationship known as the Karplus relationship (see Mathematical Sidebar 2.1). It is a great place to start when determining the structure of a protein because with one easy measurement, we find where the protein forms α -helices or β -sheets. Values

less than 4 Hz identify helical regions and those greater than 9 Hz most likely form extended or β -sheets regions (Fig. 2.7b).

Before moving on, we want to emphasize one key point about J-coupling: It involves energy or magnetization transfer between two nuclei via the electrons in a covalent bond(s). Therefore, J-coupling occurs *only* between nuclei that are connected by a small number of covalent bonds. If the connection involves more than three bonds, the J-coupling constant is negligible, and the atoms are, for all practical purposes, not J-coupled.

Mathematical Sidebar 2.1: Karplus Equation

The Karplus equation describes the relationship between the dihedral angle and the vicinal coupling constant. This was first applied to the configurations of ethane derivatives but has been expanded for use in a number of systems, including peptides. The general form of the equation is

$$J(\phi) = A \cos^2 \phi + B \cos \phi + C$$

where J is the three-bond coupling constant between two atoms; ϕ is the dihedral angle involving these two atoms and the two atoms connecting them by bonds; and A , B , and C are empirically derived parameters that depend on the atoms. In proteins, J couplings are largest when the torsion angles are -135° (β -sheet) and smallest when the angle is -60° (α -helix).

2.3 NMR Maps: Two-Dimensional Spectra

Okay, when two atoms are connected by a covalent bond, energy (or more specifically magnetization) transfers between the two nuclei via the bonded electrons. What good is that? For starters, we use this

phenomenon to begin matching up specific atoms in the molecule with the ringing frequencies found in the spectra. Let's see how.

To characterize molecular structures and dynamics by NMR, the first step is to determine the ringing frequency (or chemical shift) for each atom in the protein. This is called *chemical shift assignments*, and it can be quite tricky. Think of a “chicken-and-the-egg” problem combined with a huge “connect-the-dots,” or a Sudoku puzzle with 10,000 squares!

If you are interested only in the biophysical properties of the backbone atoms of a protein, then you need only to determine the chemical shifts for the backbone atoms. But if you want an NMR image of the entire protein, you need to assign the ringing frequency for almost every atom in the protein, which is easily over a 1000 atoms.

For chemical shift assignments of proteins, NMR spectroscopists almost invariably start with the backbone amide protons ($^1\text{H}_\text{N}$)—the hydrogens attached to the nitrogens in the peptide bond (Fig. 2.6). Figure 2.8a shows the NMR spectrum for the amide protons in calmodulin, a 148-residue protein found in all eukaryotic organisms. Notice the limits of the x -axis: 5.5–10.5 ppm. This is the frequency range for virtually all amide protons in all proteins. In general, each residue of calmodulin (with the exception of the N-terminal residue and prolines) has one amide proton, so there is approximately one peak for each residue of the protein. For calmodulin, this means we have ~148 peaks between 5.5 and 10.5 ppm.

With so many peaks in such little space, it's tough to pick out specific hydrogens and say, “this peak must belong to the amide proton of residue 29” (arrow in Fig. 2.8a). To do this, we must spread the peaks out. In other words, we need to *increase the resolution* of the spectrum. How could we do this?

1. For starters, we could make the peaks narrower (i.e., sharper). Unfortunately, broad peaks are inherent in protein NMR, and, as we'll learn in Chaps. 4 and 5, they don't slim down easily.
2. Another option is to run the experiment at a stronger magnetic field—say, for example, 900 MHz versus 500 MHz. Why would that

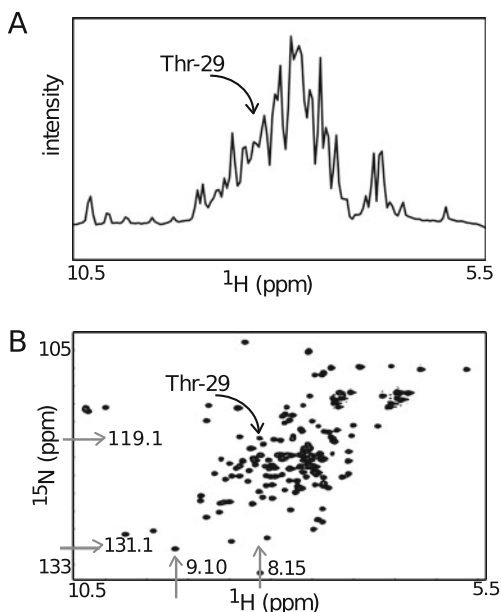


Fig. 2.8 (a) The one-dimensional NMR spectrum of a protein's amide hydrogens (H_N). (b) The two-dimensional version of the same spectrum (an 1H - ^{15}N HSQC spectrum)

help spread the peaks out? (See the end of Chap. 2 for the answer.)

Unfortunately, higher field magnets aren't usually available.

3. In the end, the trick that works best is to *add another dimension*. Why does this increase our resolution?

Think about driving to the mountains. When you are far away on level terrain, all the peaks flatten into one plane like a vertical pancake (Fig. 2.9a). You know that your favorite peak for skiing is straight ahead, but it's difficult to identify the exact one. The problem is that you can see vertically, but not horizontally. Now think about looking at these mountains from an airplane far above: You can easily identify

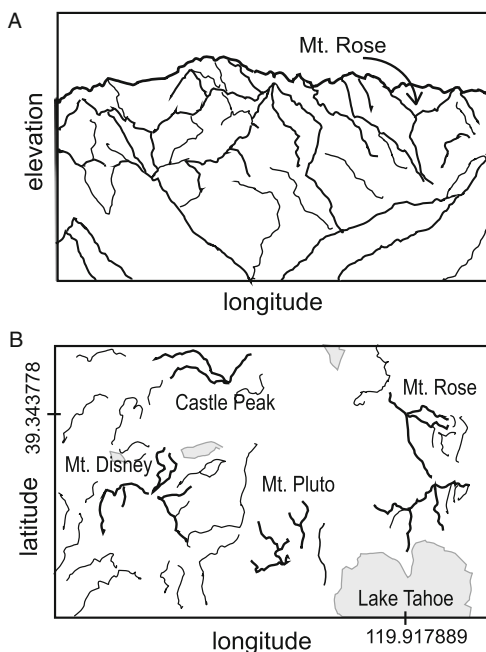


Fig. 2.9 A mountain range (a) from the front and far away or (b) from above

individual peaks because you see in all directions—east to west and north to south (Fig. 2.9b). Adding the extra dimension separates and spreads the peaks out.

In NMR, we add extra dimensions by transferring magnetization between coupled atoms, such as J-coupled nuclei. This is called “two-dimensional” NMR, and it involves four basic steps¹ (Fig. 2.10):

¹This is an oversimplification of the experiment, but it provides the essence of two-dimensional NMR. We’ll describe a bit more of the details below, but if you’re still not satisfied, check out Chap. 7 of “Protein NMR Spectroscopy: Principles and Practice” by Cavanagh et al. (2007).

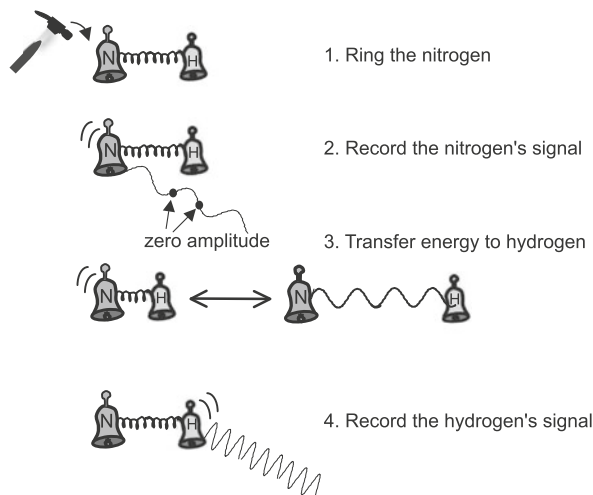


Fig. 2.10 The four basic steps of the ^1H - ^{15}N HSQC experiment for connecting the chemical shift of an amide hydrogen with the chemical shift of its covalently attached nitrogen

1. Ring the nitrogen atom.
2. Record its electromagnetic signal.
3. Let the nitrogen ring the attached hydrogen via J-coupling.
4. Record the electromagnetic signal of the hydrogen.

The result is a two-dimensional map of the NMR spectrum (Fig. 2.8b), similar to the aerial view of the mountains (Fig. 2.9). The ringing frequencies (or chemical shifts) of the amide hydrogens are along the x -axis and the ringing frequencies for the attached nitrogens are along the y -axis (Fig. 2.8b). With the extra elbow-room of an additional dimension, many peaks are now completely separated and distinct, making the spectrum easy to analyze and significantly more useful (compare Fig. 2.8a to 2.8b).

In this spectrum, there is a peak for each $^1\text{H}_\text{N}$ atom bonded to a ^{15}N atom, or each " $^1\text{H}_\text{N}$ - ^{15}N pair." (To learn why we study ^{15}N atoms

with NMR instead of the more common ^{14}N atoms, see Mathematical Sidebar 2.2.) The location of the peak is at the point $x =$ hydrogen chemical shift, $y =$ nitrogen chemical shift. You can read this two-dimensional spectrum in the same manner that you read a map on a longitude–latitude grid. Say your favorite place to ski is Mount Rose. How would you find Mount Rose on a map if you know it is located at 39.343778° latitude (x -axis) and 119.917889° longitude (y -axis)? In Fig. 2.9b, you could draw a horizontal line passing through the y -axis at 119.917889° and a vertical line passing through the x -axis at 39.343778° . Mount Rose is where the two lines intersect.

Analogously, you can find the cross-peak for threonine-29 on the two-dimensional NMR map in Fig. 2.8b if you know the chemical shifts (or ringing frequencies) for its amide hydrogen and nitrogen. Say the amide hydrogen for threonine rings at 8.15 ppm, and the amide nitrogen rings at 119.1. To find the cross-peak of threonine-29 in the two-dimensional spectrum, simply draw a vertical line through 8.15 ppm on the x -axis and a horizontal line through 119.1 ppm on the y -axis—voila! Like Mount Rose, threonine-29 is where the two lines intersect. Since this peak is now distinct from all the others, we can use it as a probe to characterize the structure and dynamics of residue 29 in calmodulin. We'll see how this works in later chapters.

You can probably see now how this two-dimensional spectrum (Fig. 2.8b) helps us start matching up atoms with their specific ringing frequencies. Say you know that residue 137's amide proton rings at 9.10 ppm, but you don't know the chemical shift for its attached nitrogen. To make this chemical shift assignment, simply draw a vertical line through $x = 9.10$ ppm in the two-dimensional spectrum. This line cuts straight through a peak at $y = 131.1$, telling us that the amide nitrogen rings with a frequency of 131.1 ppm.

Obviously this procedure wouldn't work in the center of the spectrum where multiple peaks overlap and pile on top of each other (Fig. 2.9b). In this case, we would need to add another dimension to spread these peaks out further. We'll talk more about these "three-dimensional" spectra in the next chapter, but for now, let's focus on the most important spectrum in biomolecular NMR: the HSQC.

Mathematical Sidebar 2.2: Why ^{12}C and ^{14}N Atoms Are So Shy?

When studying a protein by NMR, one of the first steps is to create a “ ^{15}N -labeled protein” in which all the ^{14}N atoms are replaced with ^{15}N atoms. Then to solve the structure of the protein NMR, we also need to replace the ^{12}C atoms in the protein with ^{13}C atoms to create “ ^{13}C and ^{15}N -labeled protein.” But why? Why can’t we study ^{12}C and ^{14}N versions of proteins? To answer this question, we need to learn more about why atoms ring in the first place.

Atoms carry a few intrinsic properties that dictate how they respond to forces in nature. First, atoms possess mass. The more mass an atom carries, the more it responds to gravitational fields. Atoms also have a charge: positive, negative, or neutral. The charge on the atom tells us how the atom responds in an electric field. Although we don’t usually “see” the charge, we can observe it (e.g., lightning bolts). Sometimes we can even feel the charge of atoms. Simply rub your feet across a carpet and then touch a friend, and you’ll definitely “feel” charge.

The third property of atoms is *spin*. The spin tells us how an atom responds to a magnetic field. Like charge, spin comes in multiple flavors:

- Nuclei with even mass numbers (i.e., the total number of protons and neutrons) and even numbers of protons have spins of zero. For example, ^{12}C has a spin of zero.
- Nuclei with even mass numbers and odd number of protons have integral spin numbers. For example, ^{14}N has a spin of one.
- Nuclei with odd mass numbers have half-integral spins: $1/2$, $3/2$, $5/2$, etc. For example, ^1H , ^{13}C , or ^{15}N each have a spin of $1/2$.

Each flavor of spin acts differently in a magnetic field. Those with a spin of zero, like ^{12}C , are very shy and don't ring at all in the magnet. Thus, all those ^{12}C atoms in proteins are not very useful for structure determination by NMR. Nuclei with spins greater than $\frac{1}{2}$ (i.e., spins of 1 and larger) do ring in the magnetic field, but, unfortunately, their ringing is very short. These atoms possess what is called an “electric quadrupolar moment,” which dampens their ringing so much that detecting their signals becomes extremely difficult. Thus, ^{14}N atoms in proteins are also not useful for NMR studies.

Nuclei with spins of $\frac{1}{2}$ are perfect! They ring in a magnetic field, and they don't possess “electric quadrupolar moment.” So their signals stay strong and loud for a long enough time that we can easily detect them. Thus, for biomolecular NMR spectroscopy, the most important nuclei are those with spins equal to $\frac{1}{2}$: ^1H , ^{13}C , and ^{15}N . *Can you name another nuclei often found in biological molecules that could have a spin of $\frac{1}{2}$?*

2.4 The ^1H – ^{15}N HSQC: Our Bread and Butter

The spectrum we've been analyzing in Fig. 2.8b is called a ^1H – ^{15}N *Heteronuclear Single-Quantum Coherence* correlation spectrum, or HSQC for short, and we create this spectrum using the steps outlined in Fig. 2.10. You need to understand this spectrum well because it's the starting point for almost all other biomolecular NMR experiments. In other words, it's our “bread and butter” experiment.

The ^1H – ^{15}N HSQC spectrum shows us which protons and nitrogen atoms are connected by a single covalent bond. There are also ^1H – ^{13}C HSQC spectra that tell us what protons are attached to what carbons, but we'll focus on the ^1H – ^{15}N correlation experiment since it is the spectrum most often seen in protein NMR publications. (To learn why

we study ^{13}C atoms with NMR instead of the more common ^{12}C atoms, see Mathematical Sidebar 2.2.)

Here are the five most important attributes of the ^1H - ^{15}N HSQC for proteins. Read them, understand them, remember them:

- (a) **The Axes:** The x -axis gives the ringing frequency or chemical shift of each protons attached to a nitrogen atom. The y -axis gives the ringing frequency of each nitrogen atom attached to a proton.
- (b) **The Cross-peaks:** A cross-peak appears in the center of the spectrum wherever a hydrogen is bonded to the nitrogen.
- (c) **Total Number of Peaks:** The total number of peaks is approximately equal to the total number of residues in the protein:²
 - If the spectrum has more peaks than this, the protein is probably adopting multiple conformations or forming heterogenous oligomers (i.e., heterodimer, heterotrimer, etc.).
 - If the spectrum has less peaks than predicted, regions of the protein are probably dynamic, exist in multiple confirmations, or partially unfolded.
- (d) **Peak Intensity and Shape:** For “well-behaved” proteins, all peaks should have approximately the same linewidth and therefore peak height, like the spectrum in Fig. 2.11a. If a significant number of peaks are weak and broad (or completely missing), regions of the protein are probably dynamic and/or exist in multiple conformations.
- (e) **The Peak Pattern:** Peaks should be relatively well separated with minimal overlap (Fig. 2.11a); this is a good sign that the protein is well folded and not aggregating. Otherwise

²Specifically, the number of peaks in a ^1H - ^{15}N HSQC spectrum of a protein is given by the number of residues plus two times the number of glutamines and asparagines minus the number of prolines minus one (for the N-terminal residue, which has a rapidly exchanging NH_3 group instead of an NH group).

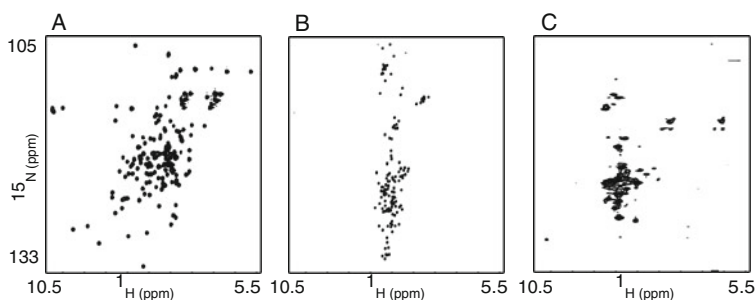


Fig. 2.11 Examples of ^1H - ^{15}N HSQC spectra for (a) a well-behaved protein folded into one configuration, (b) a completely denatured, unfolded protein, and (c) an aggregated protein in multiple configurations

- if peaks are sharp but largely grouped together between $\delta = 7.5$ and 8.5 ppm (Fig. 2.11b), the protein is probably unfolded in a random coil-like configuration—think of a string of spaghetti in boiling water;
- if the peaks coalesce into one big blob near the center of the spectrum, the protein is probably aggregating or forming high-order oligomers (Fig. 2.11c).

As this list demonstrates, the ^1H - ^{15}N HSQC provides substantial information about the state of the protein even before we assign the chemical shifts to specific atoms. In fact, crystallographers and other biophysicists often use the ^1H - ^{15}N HSQC to check if their proteins are folded and well-behaved before they set up crystallization screens or perform other time-consuming experiments.

Before we go on, test your understanding of the ^1H - ^{15}N HSQC spectrum with the following question:

The sidechains of glutamine and asparagine residues have amide groups with two hydrogens attached to one nitrogen. With this knowledge, which peaks in Fig. 2.8b belong to glutamine and asparagine sidechains? Explain why you have selected these peaks.

2.5 Hidden Notes: Creating Two-Dimensional Spectra

For the last part of Chap. 2, let's look closer at how two-dimensional spectra, such as the ^1H - ^{15}N HSQC in Fig. 2.8a, are created. We'll use an analogy to clarify the confusing world of "indirect" dimensions.

Do you ever listen to talk radio or sport broadcasts on AM radio? Although its super old school, AM radio has an endearing quality that takes you back in time. Its characteristic raspy, tinny sound is a direct result of AM's broadcasting style, which shares surprising similarities with two-dimensional NMR.

How does AM radio work? In a nutshell, the AM radio in your car converts an electromagnetic wave into sound waves through your speakers (Fig. 2.12). Each AM radio station in an area is assigned a specific frequency at which to broadcast its signal. For example, in Washington, DC, WCYB, which plays gospel music, broadcasts its signal on an electromagnetic wave with a frequency of 1340 kHz (Fig. 2.13b). On the other hand, WTEM, which plays local news and talk shows, broadcasts its signal on an electromagnetic wave with a frequency of 630 kHz (Fig. 2.13a). When you dial in "AM630" on your radio, you're telling the radio "pick up only waves with a frequency of 630 kHz and ignore all other frequencies."

For simplicity, let's say that both AM630 and AM1340 are broadcasting one note each (remember, a note is merely a sound wave at a particular frequency): AM630 is playing the note

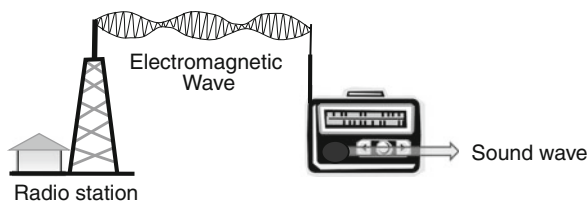


Fig. 2.12 AM radio stations broadcast a signal with oscillating amplitude. Your radio converts it into a sound wave by analyzing the amplitude changes in the electromagnetic wave

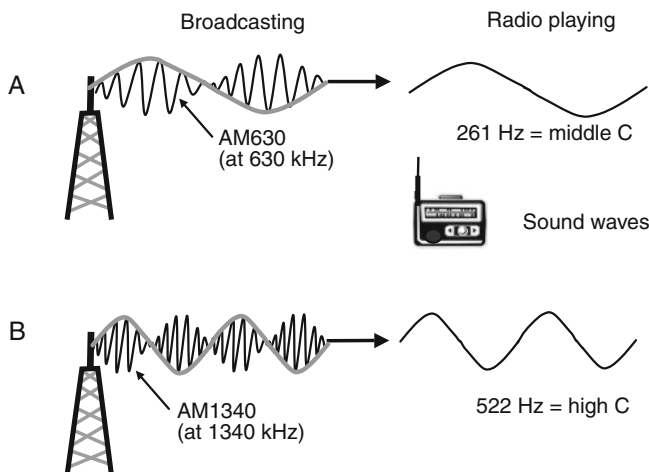


Fig. 2.13 (a) AM630 broadcasts its electromagnetic signal at 630 kHz, but the wave's amplitude oscillates at 261 Hz, the frequency of the note middle-C. (b) AM1340 broadcasts its signal at 1340 kHz, but the wave's amplitude oscillates at 522 Hz, which the radio plays high-C

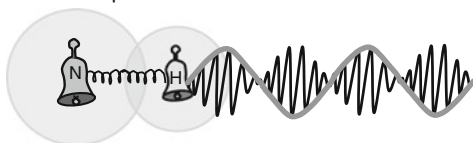
“middle-C” = 261 Hz (Fig. 2.13a), while AM1340 is playing a C note one octave higher = 522 Hz (Fig. 2.13b). How does each station tell your car radio to play the right note? The radio station can't actually send out a wave at the note's frequency because the station can broadcast only at its assigned frequency. So the radio station needs to hide or imbed the note's frequency inside its electromagnetic wave. AM radio accomplishes this by varying the wave's amplitude (i.e., its maximum height) at the frequency of the specific note (Fig. 2.8a). Thus, your radio knows what sound wave to produce in your speakers by following the fluctuations in the electromagnetic wave's amplitude. This type of broadcasting is called “Amplitude Modulation,” or “AM” because the signal is created by modulating the electromagnetic wave's amplitude.

The note on AM1340 is twice the frequency of AM630's note, so AM1340 changes its amplitude twice as fast as AM630 (Fig. 2.13a

versus 2.13b). You can easily see these hidden notes in Fig. 2.13 by drawing a line along the edge or amplitude of each station's radio wave (gray lines). The resulting sine curves have the exact frequencies of the sound waves played by the radio stations. If we plot the radio stations' frequencies, 630 and 1340 kHz, on the x -axis and the frequency of the note they broadcast on the y -axis, 261 and 522 Hz, respectively, the resulting graph looks quite similar to the two-dimensional ^1H - ^{15}N HSQC spectra in Fig. 2.11a. This is no coincidence! Like AM broadcasting, the peaks in a two-dimensional NMR spectrum are also created by modulating the amplitude of the NMR signal.

For the ^1H - ^{15}N HSQC in Fig. 2.11a, each amide hydrogen in the protein is a tiny radio station broadcasting at its ringing frequency or chemical shift, which is approximately 500 MHz (Fig. 2.14). Similar to how AM630 plays the note middle-C by modulating its amplitude, the hydrogen nucleus "plays" the note of its attached nitrogen by modulating its amplitude at the nitrogen's frequency (i.e., ~50 MHz, Fig. 2.14). The result: a two-dimensional spectrum that shows which hydrogens are connected to which nitrogens (Fig. 2.11a).

A HSQC experiment



B Hydrogen
500 MHz



C Nitrogen
50 MHz



Fig. 2.14 (a) In a two-dimensional ^1H - ^{15}N HSQC experiment, the hydrogen broadcasts its signal at (b) 500 MHz, but the wave's amplitude oscillates at the ringing frequency of the attached nitrogen, approximately (c) 50 MHz

How do you get an amide hydrogen to “play” the attached nitrogen’s note? We use J-coupling! And, we run the experiment multiple times. Here’s how it works.

In a two-dimensional NMR experiment, we start the nitrogen atom ringing; listen to its signal for a brief period; and then let it transfer magnetization to the bonded hydrogen via J-coupling (Fig. 2.10). We record the hydrogen’s ringing as the NMR signal and then perform a Fourier Transform to determine the hydrogen’s chemical shift.

Notice in step 2 of the HSQC experiment (Fig. 2.10) that the nitrogen’s signal is a sine curve oscillating between a positive maximum value and negative minimum value. At some time points, the nitrogen’s signal is even zero (arrows, Fig. 2.10).

To get the hydrogen’s NMR signal to oscillate at the nitrogen’s frequency, we merely run this experiment multiple times and vary the length of time the nitrogen is allowed to ring (Fig. 2.15). For example, during the first experiment, we let the nitrogen ring for the exact time that it takes for its electromagnetic wave to make one full cycle. At this point in time, the nitrogen’s signal is close to its maximum value, so the amount of energy transferred to the hydrogen is maximal and the amplitude of the hydrogen’s signal is large (experiment 1 in Fig. 2.15). In the second experiment, we let the nitrogen ring for a shorter amount of time, so that its signal is closer to zero. This time the amount of energy transferred to the hydrogen is significantly less than in the first experiment (experiment 2 in Fig. 2.15). This causes the hydrogen’s signal to be smaller than in the first experiment. In the third experiment, we let the nitrogen ring so that its signal is almost zero when it transfers energy to the hydrogen. Then the amplitude of the hydrogen’s signal is almost zero too (experiment 3 in Fig. 2.15). If we reduce the time period that the nitrogen rings even further (experiment 4 in Fig. 2.15), more energy is transferred to the hydrogen than in the previous experiment. Thus, the hydrogen’s amplitude is larger in experiment 4 than in experiment 3.

The length of time that the nitrogen rings is called the t_1 *evolution period*. As we gradually shorten the t_1 period, you can see on the right-hand side of Fig. 2.15 that the amplitude of the hydrogen signal will

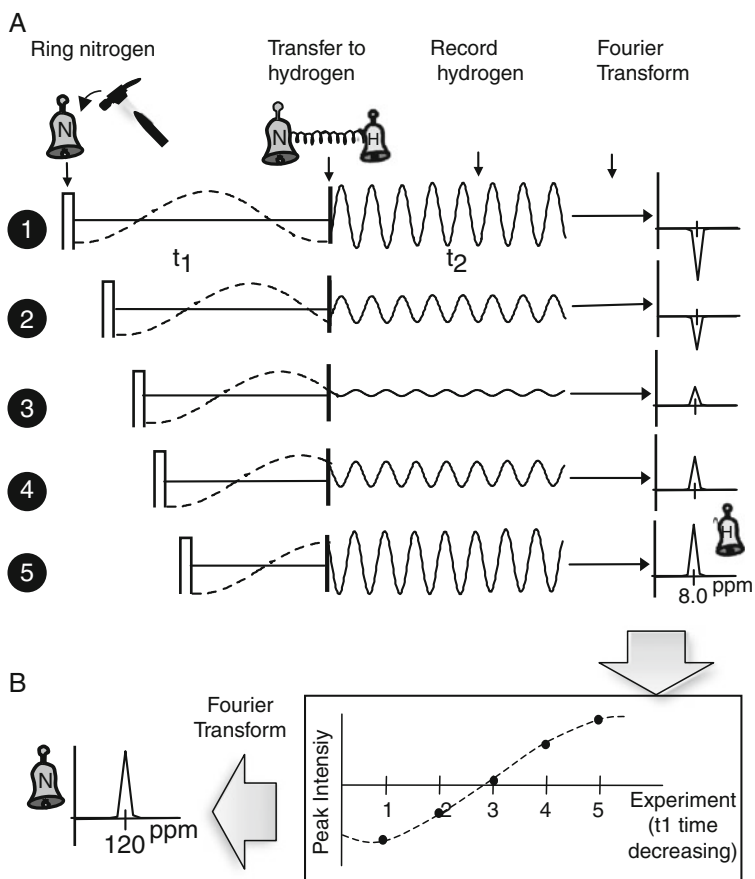


Fig. 2.15 Details of a two-dimensional ^1H - ^{15}N HSQC. (a) We run the one-dimensional experiment multiple times, like the five experiments shown, varying the time (t_1) that the nitrogen atom is allowed to ring. This causes the intensity of the hydrogen peak to oscillate up and down at the nitrogen's frequency (graph inset). (b) If we apply a Fourier Transform to the plot of peak height versus t_1 time, we retrieve the ringing frequency of the nitrogen atom

oscillate up and down at the frequency of the nitrogen's signal—voilà! The hydrogen's amplitude is changing at the frequency of its neighboring nitrogen (Fig. 2.15). In other words, the hydrogen is broadcasting the chemical shift of its neighboring nitrogen by amplitude modulation, similar to an AM radio station.

All that's left is to convert the two time signals into frequency signals, with the Fourier Transform. In this case, it's a double Fourier Transform. First, we perform a Fourier Transform on the hydrogen signal in each experiment. Notice that the resulting peak is at the same frequency in all experiments (the hydrogen frequency) but the height of the peaks varies (Fig. 2.15, far right). The curve created by these oscillating peaks encodes the frequency of the directly bonded nitrogen. To get this frequency, we perform the second Fourier Transform on the curve created by the oscillating hydrogen peak (Fig. 2.15b). That's it!

References and Further Reading

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