Structural Biology

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Lecture - 04

Hi everyone, welcome again to the course of structural biology. We are going through high-resolution structural biology techniques and are currently in the NMR spectroscopy module. We have already discussed basic things about spectroscopy, the basic application of NMR, and the basic principles of NMR. Today, we will start with something I talked about, which is the chemical shift, which I also told you has a very critical role in the technique of NMR spectroscopy. As we know, a given nucleus is surrounded by a cloud of electrons with specific density in any given molecule. These electrons are moving charges that exert a magnetic field. So we said that because of the charge and spin in the nucleon, they are behaving as a magnet. Nucleons are also used as a magnet, exert a magnetic field at the nucleus, which is often opposite in direction compared to the main magnetic field. Thus, the effective field by the nucleus is reduced from B0, which is the external magnetic field, by a factor that depends on the density of the electrons around the nucleus. So if you have more electrons, you have more effect. The precessional frequency is not the same for all the nuclei in the molecule. It depends on the electron density surrounding it, which means it depends on the chemical environment of the nucleus.

What are the factors that affect the chemical shift?

The most common factors affecting the chemical shifts are the hybridization state of carbon, magnetic anisotropy, inductive effect, resonance effect, aromatic ring current effect, steric effect, and hydrogen bonding. If you see, methane CH_4 with 0.23 ppm and ethane CH_3CH_3 0.86 ppm have SP_3 hybridization, and when this SP3 hybridization goes to SP_2 hybridization $CH_2=CH_2$ (ethene), it goes to 5.28. So with the increase of S character SP_3 to SP_2 to SP orbitals, the bonding electrons move closer to carbon and away from the protons. Because of that, it causes deshielding, and because of the deshielding, the chemical shift is enhanced. Magnetic anisotropy arises due to the difference in shielding and deshielding of atoms due to differential electron density in the molecule. The presence of a nearby pi bond or pi system greatly affects the chemical shift. In inductive effect, we are taking a group of compounds with CH_3X where X is replaced by different elements like CH_3F when it is fluorine, CH_3OH when it is oxygen, CH_3Cl when

it is chlorine, CH_3Br when it is bromine, CH_3I when it is iodine, CH_4 when it is hydrogen. The electronegativity of fluorine is 4.0 (the most electronegative), oxygen 3.5, chlorine 3.1, bromine 2.8, iodine 2.5, hydrogen 2.1 and silicon 1.8. The chemical shift for fluorine (highest electronegativity) is 4.26(highest chemical shift), for methanol in the presence of oxygen, it is 3.4, CH_3Cl it is 3.05, CH_3Br it is 2.68, CH_3I it is 2.16, CH_4 0.23 and $(CH_3)_4Si$ it is 0 (that is why it is used as a standard). The effect increases with greater numbers of electronegative atoms and their electronegativity.
If you have chlorine, in the presence of 3 chlorine which is $CHCl_3$, the chemical shift is 7.27, by replacing one chlorine with one hydrogen (CH_2Cl_2)it become 5.3, by replacing another chlorine with another hydrogen it become 3.05. So with decreasing electronegativity containing atom the chemical shift decreases. So the effect decreases with increasing distance, like in CH_3Br if the carbon and bromine is directly attach it is 3.3, CH_2CH_2Br where one carbon further it is 1.69, $CH_2CH_2CH_2Br$ it is 1.25. So with increasing distance from the electronegative atom the chemical shift decreases. In $CH_2=CH_2$ with 5.29 ppm, all the protons are equivalent and resonate at 5.29 ppm. Now if you introduce a carbonyl group $COCH_3$ (keto group), the addition of a ketone group or keto group withdraws electron and decreases the shielding around the proton and enhances the chemical shift. So chemical shift from 5.29 now becomes 6.52. Then you introduce the OCH_3 group. For OCH_3, CH_3 have the positive inductive effect and it puts the effect towards electron. Because of electron pushing the concentration of electron, the electron cloud would be enhanced so there would be more shielding so definitely there would be decreasing chemical shift. So it reduces from 6.52 to 3.93 or you could say from 5.29 to 3.93. So the methoxy group will donate electron to the double bond and increases the electron density around protons which results in enhancement in shielding. Aromatic ring current, external field is applied, and any nucleus above the plane of the ring is shielded. Now there is circulating phi electron, and deshielding. Steric effect, steric crowding around a carbon nucleus increases deshielding and moves resonances down field. Like if you see carbon with 3 hydrogen (methyl), deshielded by one bond, then methylene deshielded by two bonds and methionine deshielded by three bonds. And you see that, here the chemical shift actually change because of the steric effect. Hydrogen bonding, we have taken the example of methyl salicylate. In methyl salicylate which has strong internal hydrogen bonding, the NMR absorption of hydroxyl group is at about 14 ppm way down field. So we have seen the different effects of factors on the chemical shift.

Coupling in H NMR. So if you look at, we have a compound which is CH_3, you see CH_3CHCl_2; now the compound contains two type of hydrogen it is very easy to understand one type of hydrogen and a second type of hydrogen. So how many peaks you are expecting two type of peaks and you see two types of peaks. So the compound contains two types of hydrogen atoms so a double peak is observed at 2.1 ppm and a quartet in 5.9. So up to now we discuss about peaks, we know two types of hydrogen two peaks, but what are the splitting? Now we will know about the splitting, and why this

splitting is happening?
So in the last class we have studied all the hydrogen NMR spectra and all had different types of protons that are seen as singlets in the spectra. Then we start discussing about the spectra of 1, 1 dichloroethane, spectra of 1, 1 dichloroethane is seen in multiplets. So we are going to more complex situation from singlet spectra of different hydrogen to multiplets. The appearances of multiple peaks are due to coupling with neighboring protons.

What are the rules for spin-spin coupling?

Chemically equivalent protons do not show spin-spin coupling.
Only non-equivalent protons couple.

Protons on adjacent carbon normally used to couple. So if they are one carbon apart they are the best to do spin-spin coupling.

Protons separated by 4 or more bonds will not couple or better they are not going to show the effect.

Spin-Spin coupling

The interactions between the spin of neighboring nuclei in a molecule may cause the splitting of NMR spectrum. This is known as spin-spin coupling or splitting. The splitting pattern is related to the number of equivalent hydrogen atom at the nearby nuclei. So we are taking the case of methyl ethyl acetate $CH_3CH_2OCOCH_3$, the interactions between the spins of neighboring nuclei in a molecule may cause the splitting of NMR spectrum. This is known as spin-spin coupling or splitting. The splitting pattern is related to the number of equivalent hydrogen atom at the nearby nuclei. So if you look at this, this one (H_3) have no nearby proton, so this methyl group will give 1 peak, this (H_2) will give 2 equivalent protons, split 1:3:3:1, they will give quartet, because coupled to the hydrogen of terminal methyl group having 3 identical hydrogen. This (H_3) will give triplets, 3 equivalent protons, with 1:2:1, because coupled to the hydrogen of the CH2 group. So coupling arises because the magnetic field of vicinal or adjacent proton influences the field that the proton experiences. To understand the implication of these we should first consider the effect the CH group has on the adjacent CH_3. The methane CH can adopt 2 alignments with respect to the applied field. As a result the signal of the adjacent methyl CH_3 is split in 2 lines of equal intensity a doublet. Now consider the effect of CH_3 group has on the adjacent CH. So if you see, they are 3, so they have different combinations. The methyl CH_3 proton gives rise to 8 possible combinations with respect to the applied field, there are 4 magnetically different effects, as a result split them to 4 lines with 1:3:3:1.
The proximity of ‘n’ equivalent hydrogen on neighboring carbon atom causes signals to be split into n+ 1 line. This is known as multiplicity or splitting or coupling pattern of

each signal. Different protons (or those with the same chemical shift) do not show coupling to each other very clear. If the neighbors are not all equivalent, more complex pattern arise (this is because of the difference in the values of the coupling constant). When looking at H-NMR, most common coupling that are observed are those between hydrogen atom on neighboring carbon atom (vicinal coupling, H_aCCH_b where 3 bonds are involved). To a first approximation, protons on adjacent sp^3 carbon tend to behave as if they are equivalent (for example the H-NMR of 1 bromopropane). Now we can do more a complete analysis, including the application of the N + 1 rule to 1, 1 dichloroethane. where delta equal to 5.9 ppm, quadrate integration = 1 H deshielded: agrees with the $CHCl_2$ unit next to a CH_3 unit (N = 3, so N + 1 equal to 4 lines).

∂ = 2.1 ppm, doublet, integration = 3H agrees with CH_3 unit, next to a CH.

The distance between the peaks in a given multiplet is a measure of the splitting effect known as coupling constant. Denoted by symbol J expressed in Hz. Coupling constants are a measure of effectiveness of spin-spin coupling and very useful in analyzing proton NMR of complex structures.
The coupling constant J (usually in frequency unit, Hz) is a measure of the interaction between a pair of protons. In a vicinal system of general type H_aCCH_b then the coupling of H_a with H_b which is J_{ab} must be equal to the coupling of H_b with H_a which is J_{ba}.

So the rule is $J_{ab} = J_{ba}$.

The implications are that the spacing between the lines in the coupling patterns are the same as can be seen in the coupling patterns from H NMR spectra of 1, 1 dichloroethan.

So far we have emphasized vicinal coupling of hydrogen atom on adjacent sp^3 carbon atoms. This coupling constant is typically about 6 to 8 Hz. Coupling is controlled by geometry and the orbitals involved between the coupling nuclei. So other types of system have slightly different coupling constant as compared to the CH_3 carbon, which is 6 to 8. So if you look at as I told, alkane 6 to 8 Hz, in double bond with cis 6 to 15 Hz, trans 11 to 18 Hz, geminal which is in between 0 to 5 Hz, for aromatic rings ortho 7 to 10 Hz, meta 2 to 3 Hz, para 0 to 1 Hz.
Hydrogen atoms on a CH_2 of a alkene do not have to be equivalent (depending on what substituents are on the other hand of the alkene). As a result they can couple to each other. This is a 2 bond coupling. Coupling between hydrogen atoms that are more than 3 bonds is also possible and that is called long range coupling. In general the more bonds involved between the H that are coupling the smaller or weaker the J value. Long range coupling is more common with rigid system including the 5 bond system of alkenes and substituted benzene with delocalized 5 electron. The presence of different coupling (different J values) tends to lead to complex coupling.

The multiplicity of signal is calculated by using N +1 rules. This is one of the rules to predict the splitting of proton signals. This is considered by the number of nearby hydrogen nuclei if N = number of proton in nearby nuclei.

Zero hydrogen atom as neighbor N + 1 = 0 + 1 = 1 which is called singlet. So if the hydrogens are independent have no coupling then you get singlet, one hydrogen atom as neighbor, putting effect is doublet, and two hydrogen atom is triplet in that way.

Pascal's triangle talks about what we talk the N + 1 but it would talk about the intensity. So N = 0 is singlet, N = 1 is doublet where it is 1:1. N = 2, is triplet 1 2 1, N = 3 is quartet, 1 : 3 : 3 : 1 in that way. Individual resonances are split due to coupling with N equivalent adjacent protons. Number of lines in coupling pattern L = N + 1.

The relative intensities of the lines in a coupling pattern is given by a binomial expansion or more conveniently by Pascal's triangle. To derive Pascal's triangle start the apex and generate each lower row by creating each number by adding the two numbers above and to either side in the row above together. So for H-NMR, a proton with 0 neighbors, N = 0, appears as a single line, a proton with 1 neighbor, N = 1 as two lines of equal intensity, a proton with 2 neighbors N = 2, as 3 lines of intensities 1 : 2 : 1.

CH_3CH_2Br, are having 3 independent hydrogen, two lines. These are methyl hydrogen so they would be around in between 1 and 2, having chlorine having deshielding effect, so they are higher. So you know that, there would be 2 lines the first line is for methyl group, the second line is for the CH_2 group. Now this is giving them a quadrate and this is giving them a triplet. So the first one which is of methyl group would be a triplet and this would be a quadrate. In that way, now you could be able to interpret number of signals, indicates how many different kind of protons are present. Position of signal, indicates something about the (chemical shift) magnetic environment. Relative intensity of signal, proportional to number of proton present. Splitting of signals indicates the number of nearby nuclei.

Solvent plays critical role in NMR experiments. The following solvents are normally used in NMR experiment where hydrogen is replaced by deuterium. CCl4 carbon tetrachloride, CS2 carbon disulphide, CdCl3 deuterio chloroform, C_6D_6 hexa deuteriobenzene, D_2O deuterium oxide.

Each different type of hydrogen gives a peak or group of peaks. The chemical shift (the delta and ppm) gives a clue as to the type of hydrogen generating the peak. The integral gives the relative number of each type of hydrogen. Spins spin splitting gives the number of hydrogen on the adjacent carbon. The coupling constant J also gives information about the arrangement of the atoms involved.

NMR spectra of ethyl bromide, ethyl bromide has two types of hydrogen, the down filled peak at about 3.5 ppm is for CH_2 deshielded by the bromine and appears as quadrate due to the adjacent from methyl group. The up filled peak at about 1.7 ppm is the CH_3 and appears as triplet due to the adjacent methylene.

Coming to the NMR spectra of propyl bromide. So we have $CH_3CH_2CH_2Br$, three type of hydrogen and three spectra. The middle CH_2 at about 2 ppm seems to appear as a hexet state (6 line) due to the total of 5 neighbors, this is due to an important approximation that helps simplify the spectra. The approximation allows us to assume that the CH_3 which is the methyl group and the other CH_2 are similar neighbors and that the coupling constant involved are the same, the coupling constant of the middle CH_2 to each of the other groups is essentially the same. This is reasonable expectation because both the other groups are sp^3 carbon system and there is free rotation of the chain. If this approximation does not hold or apply then more complex coupling patterns are observed.

Now we will shift to C-NMR spectroscopy. It is useful to compare and contrast H-NMR and C-NMR as there are certain differences and similarities. ^{13}C has only about 1.1 percent natural abundance. ^{12}C is not NMR active, ^{12}C does not exhibit NMR behaviour (I = 0), ^{13}C nucleus is also a spin half nucleus like a proton, ^{13}C nucleus is about 400 times less sensitive than hydrogen or proton nucleus to the NMR phenomena. Due to the low abundance we do not usually see ^{13}C - ^{13}C coupling like 1H, 1H coupling. Chemical shift range is normally 0 to 220 ppm in the higher range.

Chemical shifts are also measured with respect to tetra methylsilane $(CH_3)_4Si$ TMS, which the standard, is also used as standard in proton NMR. Similar factors affect the chemical shifts in ^{13}C as we have already seen and discussed for H NMR. Long relaxation times (excited state to ground state), means no integration. All ^{13}C spectra are broadband proton decoupled so the peaks show as single lines. Number of peaks indicates the number of type of carbon. The general implications of these points are that ^{13}C NMR spectra take longer to acquire than H NMR though they tend to look simpler than the proton NMR. Chemical overlap of peaks is much less common because you know less intensity than for H NMR which makes it easier to determine how much type of carbons are present.

What does broadband proton decoupled mean? The resonances due to 13C nuclei are split by neighboring hydrogen atoms. This splitting would complicate the appearance of the spectra making them harder to interpret. Therefore, in a normal 13C spectrum these couplings are removed by applying a continuous second radio frequency signal of a broad frequency range that excite all the proton nuclei and cancel out the coupling patterns due to the interaction of the proton with the 13C. Now each carbon is seen as a single line, of course information is being lost by doing this, such as how many hydrogens are attached to each carbon and that is why we need 2D NMR. In off resonance decoupling the one

bond C-H couplings are written so the signal for a particular carbon is given by the number of attached hydrogen in accord with N + 1 rule. For example, a CH_3 shows as a quartet and a CH_2 as a triplet.

^{13}C chemical shifts, we discussed about 1H, the most significant factor affecting the chemical shifts are electronegativity of the groups attached to the carbon, hybridization of carbon, a simple correlation table of 13C chemical shift which you could see here, you will see that when you have carbon-carbon saturated single bond these have lower values, carbon is attached with electronegativity, then it enhance, carbon is triple bond it enhance, then carbon double bond more enhance, aromatic more enhance, carbonyls have the highest. So, single saturated bonds have values in lower side carbonyl and modified carbonyls have values in higher side.

Interpreting carbon NMR spectra: The following information is to be gained from a typical broadband decoupled ^{13}C NMR spectrum. How many types of carbon? Indicated by how many signals there in the spectra, so how many lines that many carbon. What type of carbon? Indicated by the chemical shift of each signal.

Carbon NMR spectra of ethyl ethanoate $CH_3COOCH_2CH_3$:

How many carbons? 4 carbons. How many peak? 4 peaks.
Ethyl ethanoate have 4 type, so 4 peaks are observed, ester carbonyl peak is at 171 because it is carbonyl carbon, deshielded CH_2 group at 60 ppm and 2 methyl carbons are at 16 and 20.

Acetophenone has 6 type of carbons, carbonyl carbon, methylene methyl carbons, aromatic carbon directly link with acetyl group, aromatic carbons ortho meta and para. So 6 peaks are observed, 4 types of aromatic carbons having peaks between 125 to 140, keto carbonyl at 198 ppm and methyl carbon at 27 ppm.
An 1D NMR spectrum contains the following information for analysis: Chemical shifts, spin-spin coupling, intensity, all this information are used to deduce the structure of the molecule. Manytime it is not possible to assign the entire chemical shift if the spectrum is complex. Hence only the region of interest is analyzed for example if a substitution reaction on a benzene ring is to be checked on the aromatic region need to be analyzed, also that leads to develop multidimensional spectrum.

So far the NMR spectral methods we have discussed have been one dimensional since they have a single chemical shift into the coordinate axis. With the development of more advanced spectroscopic methods as computational power has increased it has become possible to obtain two dimensional spectra. In two dimensional experiments both the x and the y axis of chemical shift scales and the 2D spectra are plotted as a grid like a map. Information is obtained from the spectra by looking at the peaks in the grid and matching them to the x and y axis.

The basis of 2D NMR is interaction of nucleus spins 1H with 1H, 1H with 13C etc. General spectra 1D NMR are plots of intensity versus frequency. In 2D NMR intensity is plotted as a function of two frequencies called F1 and F2. In general 2D NMR is divided into two types homonuclear, heteronuclear. Each type could provide through bond (COSY)type, through space (NOESY type) coupling information.

Four periods in 2D experiment: First, the preparation, then evolution, then mixing and then detection. Preparation which means spin system relaxes and then excited by the radio frequency. Then T1 chemical shift and spin-spin coupling evolved. This is the time domain which is incremented during the 2D experiment. Mixing radio frequency pulses are applied and create observable transverse magnetization and detection T2 observable transverse magnetization is recorded it is usually labeled with T2.
In 2D NMR spectra we have two frequency axes often symmetrical about the diagonal correlates peaks in 1D NMR spectra plotted on the sides, the diagonal peaks they are same. Cross peaks: connect different peaks in 1D NMR spectrum that are interesting arise from scalar coupling or other magnetization transfer mechanism.
These will give us more information in the 2D spectra. So, correlation between two chemical shifts. So, the compound is $CH_3CH_3COCH_3$, there are three types of hydrogen because they are equivalent, they are connected to one carbon, three peaks in proton spectrum, and four peaks in ^{13}C spectrum. 12 combination of pairing proton with its C ^{13} peak. Based on the peak position and coupling yes get correct pairing, but is there a direct way to get the correct 1H to 13C interaction pairing. So this is an example of correlating to heteronuclear because we are combining 1H with 13C. The 2D spectrum is said to correlate the frequency. Routine 2D NMR experiments: There are majorly three type of NMR experiments homonuclear through bond correlation method (homonuclear through bond correlation method is as we have seen the bonded interactions between one proton with another proton), homonuclear through bond correlation method carbon and hydrogen, heteronuclear and through space.

When you look at homonuclear through bond correlation method there is correlation spectroscopy or COSY, exclusive correlation spectroscopy or ECOSY, total correlation spectroscopy or TOCSY, incredible natural abundance double quantum transfer experiment or (INADEQUATE). Now heteronuclear through bond correlation methods are heteronuclear single quantum correlation spectroscopy or HSQC, heteronuclear multiple quantum correlation spectroscopy or HMQC, heteronuclear multiple bond correlation spectroscopy or HMBC. Through space correlation methods, nuclear overhausser effect spectroscopy or NOESY, rotating frame nuclear overhausser effect spectroscopy or ROESY heteronuclear overhausser effect spectroscopy or HOESY.
So in the next class we will discuss about that all the routine 2D NMR experiments how they converted into 3D NMR experiments and most importantly how 3D NMR experiment help us studying protein. So in this class today we had gone through several

topics starting from the factor affecting chemical shift to spin coupling, spin coupling constants, the NMR spectra of one proton and its effect of all of these things. Carbon spectra how carbon 113C carbon is affected, how they are coming together, what are the factor they are affected with and how they look like then we come to concept of 2D NMR how or why 2D NMR spectra is required, how they are interacting, what type of 2D NMR spectra are there. In the next class we will continue with the details of at least brief details of all those methods. Thank you very much.