Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 16
3D NMR Spectroscopy – part I

We will start with a new topic in this course now which will be 3D NMR Spectroscopy. This is something which will be is which was not covered in the previous course and therefore, this is completely a new topic. I would like you to pay a very careful attention to this set of slides or this set of this topic.

Because, now onwards we will be moving on to essentially all the protein NMR stuff where we look at how 3D NMR is used for protein assignments and how is 3D NMR spectroscopies perform. So, I would really recommend you to be careful attention to each and every slides. So, we will go through this very slowly and build up our knowledge. So, let us start from very basics of 3D NMR spectroscopy. So, the question first is why do we need 3D NMR?

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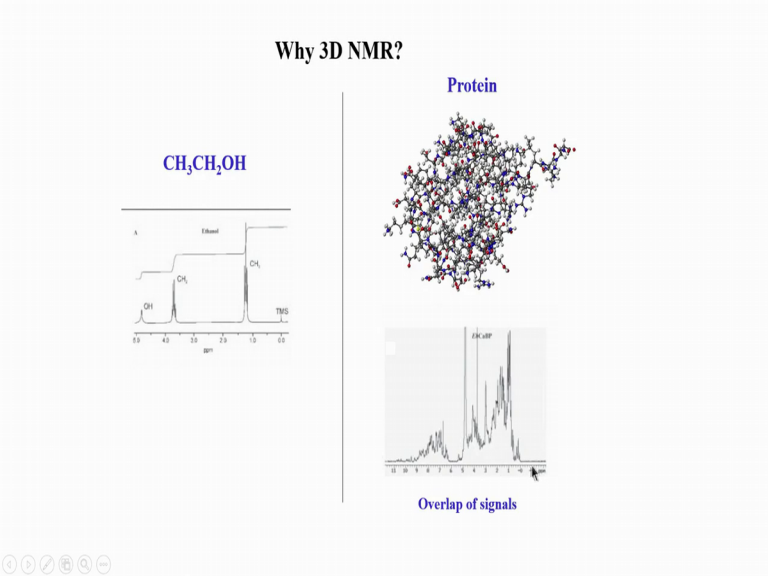

So, this example this is shown here in this case of methanol or ethanol molecule small molecules the number of peaks are very few as you can see here we know for ethanol, it just 3 peaks CH 3 CH 2 and OH. But, when it comes to more complicated and large molecules such as proteins the spectra 1D NMR spectrum becomes really complex. So,

you can see here this is schematic or this is a representation of a protein structure 3-dimensional protein structure and each and every why grey colour sphere here corresponds to a proton.

So, this is a ball and stick model of some protein. Now, if I take a NMR spectrum of a protein like this, each and every proton will give me a peak in the spectrum. Because, each and every proton in this molecule has a unique chemical environment and therefore, it has unique chemical shift value. So therefore, they all will come in this region. But since all of them are basically of the same type of protons like methyl, methylene or methane or anomatic an amide the region chemical shifts range is all the same; this all of them come between around 0 to 11 ppm.

So, you can see minus 1 to 11 ppm. So, you can see that in this range of 11 or 12 ppm values all the proton chemical shifts come. And therefore, if there are about 1000 atoms or more in the case of proteins 1000 hydrogens you will end up with more than 1000 peaks here in the spectrum. And, that is something not easy to resolve because 1000 peaks here compared to 3 definitely the overlap of peaks will be much higher in a 1D spectrum of a protein than a 1D spectrum of a small molecule.

So therefore, 1D NMR spectroscopy is virtually useless to work with for protein NMR except that it gives us definitely some basic information of the protein. For example, when we record a spectrum of a protein like we did in the last part we showed for 2D HSQC how it will helps us to find whether a particular protein is folded or not folded. In the case of 1D that also that information is also somewhat available to us. The chemical shift is spread well in the negative ppm or here we can infer that this protein is well folded.

If the protein were unfolded or denatured they would not be such a spread of chemical shift as shown here, you will see more of lumps of peaks they are all coming at in different groups. So, they would not be spread out or what is called dispersed as in a folded protein, but for any other purpose 1D NMR is no longer useful. So therefore, we need to go to 2D NMR 3D and in fact, we need to go to even 4D NMR spectroscopy.

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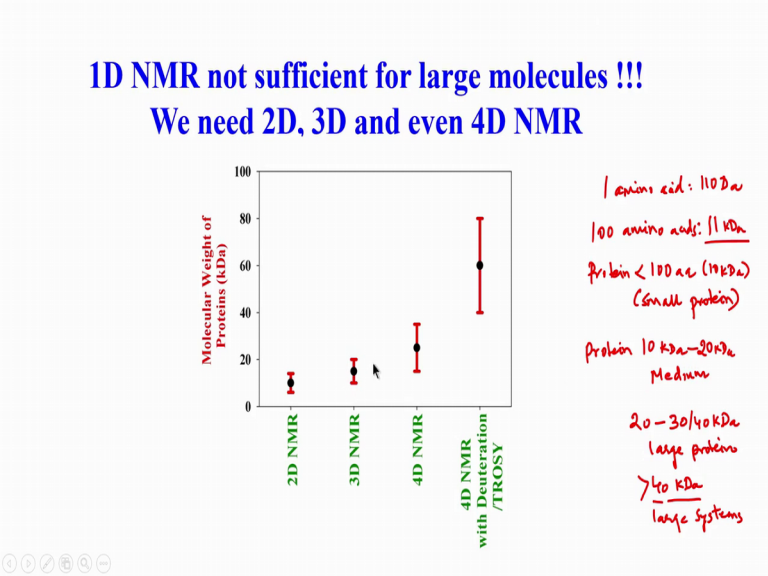

So, this is a picture showing schematically how and why and when we need the spectra higher dimension NMR spectrum for structural studies. So, we can see if you are working with the smaller size protein. So, typically in NMR we can divide qualitatively a protein into 3 different categories one is called as small protein which is somewhere between less than 10 kilodaltons. So, remember in protein when I say 1 amino acid we will see this repeat again several times in the course is about 110 Daltons. So therefore, if I have 100 amino acids it will be approximately 11 kilodaltons ok.

So, any m protein which is roughly a protein which is less than about 100 amino acid; I can call it as a small protein. If the protein molecular weight is somewhere between 10 kilodalton that is 100 amino acid. So, they can say here 10 kD between 10 to 20 amino 20 kD I can say it is a medium size protein for NMR for NMR perspective and if the size becomes 20 to 30 or 40 kDa we can call it as a large protein.

In fact, greater than 40 kilodalton is very rarely in NMR look that and that is really large systems. So, in NMR we a categorise proteins into different categories and based on this particular category we need to adopt different strategies for at looking at the structure of that protein. So, typically when you have a small protein or a peptide we do not need 3D NMR 2D NMR is sufficient. Therefore, when the in the beginning stages of NMR before isotope labelling was invented all the proteins which were solved was about 50, 60, 70

amino acids and they were all less than by 2D homonuclear NMR which we have covered in the previous part.

When you go to slightly bigger like beyond 10 kilodaltons; so, as shown here or somewhere from 8 even 8 or 6 kilodalton onwards you can start doing 3D NMR you can start one can one has to use 3D NMR for structural studies. Because, of the resolution problem which we will deal with or discus again and that is comes in this particular day. But, when you go to larger size proteins, that is 20 to 30 which is roughly sketched here not really in that range, but it is shown in this from 20 to 40; here you need to go beyond 3D NMR. So, you need to do a higher dimensional 4D NMR which we will not do in this course.

Because, that is a little complicated when it comes to analysis, but the basic idea of 4D comes from 3D and basic idea of 3D in fact, comes from 2D. So, we will cover up to 3D NMR in this course, but as we go higher and higher as you can see here in molecular weight when you go to very large systems; you have to actually do 4D NMR with more special techniques called deuteration, TROSY etcetera. Deuteration basically means replacing every hydrogen in your protein every exchangeable non-exchangeable hydrogen with a deuteron or deuterium ok.

So, that is deuteration, that is done with molecular biology approaches as we will see in the case of isotropic labelling. And, there are special NMR experiments which were actually invented to tackle the challenges of large proteins and that is called as TROSY. And, combined with 4D and NMR you can actually attempt to solve look at structures of protein in very large molecular weight which is around 60 70 kilodaltons. But, typically 90 percent of the systems we normally study by NMR falls in this range and most of them actually we use 3D NMR which falls in this 20 25 30 kD. And, we will look at basically how 3D NMR can be used for protein structure and assigned.

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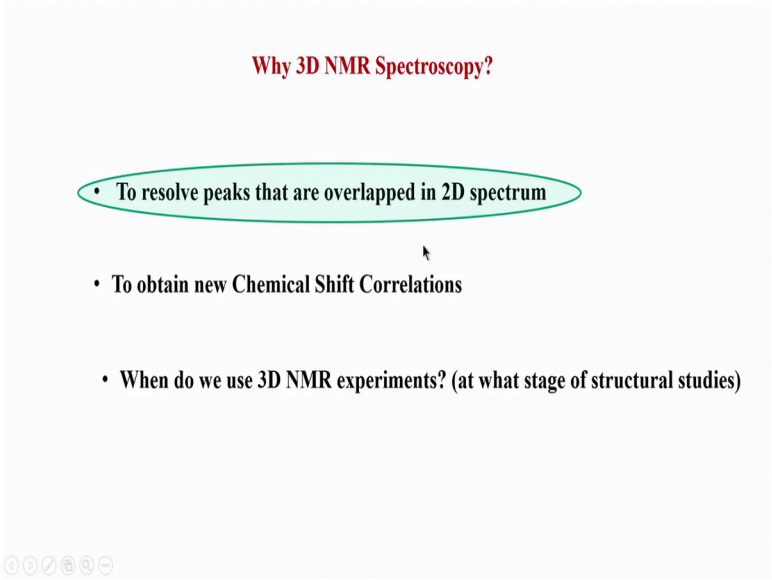

So, we are basically again coming to this question why do we need 3D NMR. So, there are 3 basic things which we will touch upon; first is to see that how 3D helps to resolve peaks that are actually overlapped in 2D. So, we saw that actually 2D itself helps us to resolve peaks which are overlapped in 1D and we saw some examples of that. But, sometimes even in a 2D a peak can be overlapped with another peak. So, 2 peak 2 or more peaks can overlap with each other in a 2D spectrum

So, in such cases 3D will help us to resolve, we will see also how 3D helps to obtain us to obtain new chemical shift correlations. Again this is similar to 2D, in 2D we saw we got new correlations chemical shifts and that will be also extended to 3D and again we will see how new correlations can be obtained. And, third thing which will 3 with 3D NMR is when do we actually need it, when do we use it, at what stage of our study of structures of proteins do would we need 3D NMR experiments. So, let us start from the first point here that how does 3D help to resolve peaks which are overlapped in 2D spectrum.

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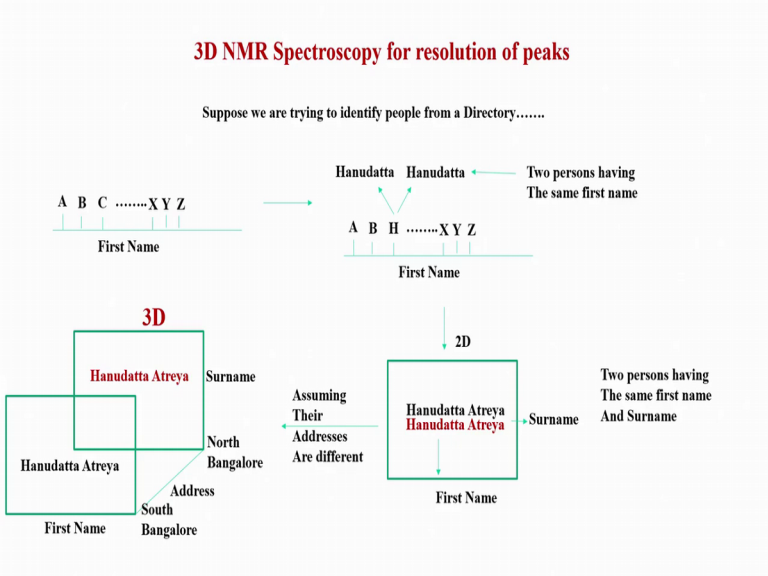

So, this is I am going to give a very qualitative example now; this is a kind of a hand waving example, but this is just to illustrate the point. So, I will give non-real example then we will go to more cases from a real case real system. So, let us say you have a directory with you and you trying to identify people from a directory. So, you are basically given you know the name of a person let us say only the name and you tried to search in the directory.

So, in this case the name I am going to take example of my name that is Hanudatta Attreya. So, let us I will give you have the first name Hanudatta with you then you look at H, but then let us say you end up finding 2 Hanudatta names. So, 2 persons having the same first name then what do you do in such cases, how do you resolve this degeneracy. This is called degeneracy because both are same first name. So; obviously, you have to look for a surname if you want to distinguish this person from this person. So, if user surname and the first name then actually you are gone going into 2D mode.

So, in a 2D your using 1-dimension as surname other dimension as first name and then you will trying to figure out whether these this one this Hanudatta has a different surname compared to this Hanudatta. So, they if they do have a different surname then they are resolved means you are separated them and you can say there are 2 different people. But suppose the names are also having the surname is also same; that means, the 2 persons having the same first name and surname then how do you resolve this problem.

So, this is you see in the 2D; that means, using surname as a independent dimension and a name as another dimension or a variable if you are not able to resolve 2 people who has the same first name and the surname. So; obviously, we have to go beyond 2D now and suppose I use another dimension I add one more dimension here and put that dimension as address of the person because, the directory normally has a name, surname, address, phone number etcetera. So, I expect that maybe the surname of these 2 guys will not be the same sorry the address will not be the same.

So, you the name and surname are same maybe the address will separate them or differentiate them. So, if I use a 3D approach where I look at now address as a criteria for separation you can see here that this person may live in North Bangalore whereas, another person maybe in South Bangalore. So, I can now I have separated them by using address as another dimension. So, you can see from this example what essentially we are doing here is, we are adding independent dimensions to resolve the peak.

So, you can think of these as peaks, you are trying to separate peaks which have the same chemical shift. So, if you look think of this as a chemical shift now, first name as a chemical shift a proton chemical shift, this H had this peak, peak at this at the same proton chemical shift. So, instead of 1D I then added a carbon chemical shift to it directly attached carbon. So, that for example, becomes an HSQC there again probably the both proton and carbon has same that is both name surname in this case are same. So, again there was an overlap in a 2D.

So, I added one more dimension or one more chemical shift; let us say nitrogen and that molecule if it has nitrogen of course, it has to have nitrogen otherwise we cannot add a dimension. But, let us see those 2 molecules had different nitrogen chemical shifts then they can be resolved. So, we are essentially relying or connecting more and more chemical shifts so, that peaks can be resolved. So, this is one of the aims of 3D NMR extra spectroscopy is to resolve what could not be resolved in a 2D spectrum. So, let us now look at actual molecules.

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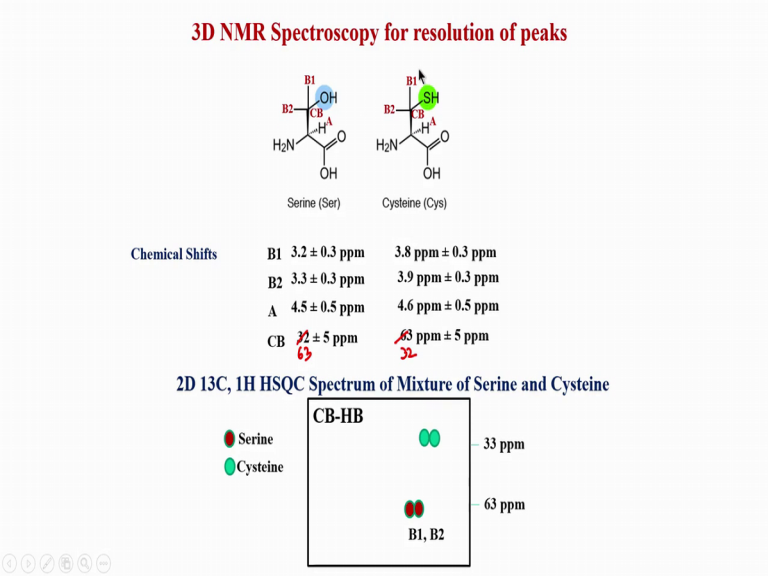

So, let us say we have these 2 molecules serine and cysteine. So, serine and cysteine are actually 2 amino acids in a protein. So, many proteins have both of this sum this is also a will rare although in molecules proteins, but let us say we have this both present in a molecules. So, if you look at the structure they are nearly the same, they look the same except there is sulfhydryl group here and there is a hydroxyl group here. So, there is a slight difference in that sense, but if you look at the chemical shifts the chemical shifts of A is alpha hydrogen here and here are almost the same within the deviation expected from molecule to molecule or structure to structure. B that is this proton B 1 B 2 here this 2 methyl protons methylene protons and these 2 methylene protons are slightly different.

So, if I recorded 2D TOCSY spectrum of a mixture of serine and cysteine. So, I have mixed this two, they can be the same concentration or different. So, for example, in a protein I may have 3 or 4 cysteines I may have 2 or 3 sernies. So, they are actually like a mixture now. So, if I mix the 2 and add they come very close to each other you can see here although there is some over differences because of this chemical shift values, but the 2D TOCSY spectrum is able to slightly differentiate, but still there is some overlap.

So now, the question is can I use a 3D NMR experiment to separate or further resolve or make it better separated for these two molecules. So, I can do that with the 3D. So, you can see a 3D how do I do that? I need one more chemical shift. So, this is something I mentioned in the previous slide that we had name and surname were same, but we use a

third possibility that is address in to resolve. Similarly, here I can go to I have these 3 which are almost similar I do although these are two are different, but let us assume these are all very similar.

But, this look at this C beta chemical shift of these 2 amino acids, there is a huge difference. So, this is slightly mistake here this should be actually 63 ppm and this is 32 per say. So, you can see here that a cysteine comes around 32 ppm that is its when it is a SH, in SH state. And, serine beta 1 that is the protons at the carbon for beta that is this carbon; now remember we are looking at this C beta here comes around 63 ppm.

So, you see there is a very big difference 30 ppm difference between the two, but all other values seem to be similar. So therefore, if I use the C beta as a discriminator or a third dimension means I have 2 protons in a TOCSY, but I can add one carbon dimension and make it a 3D HSQC TOCSY. Then in such a spectrum I should be able to completely separate this spin system this family from this family right.

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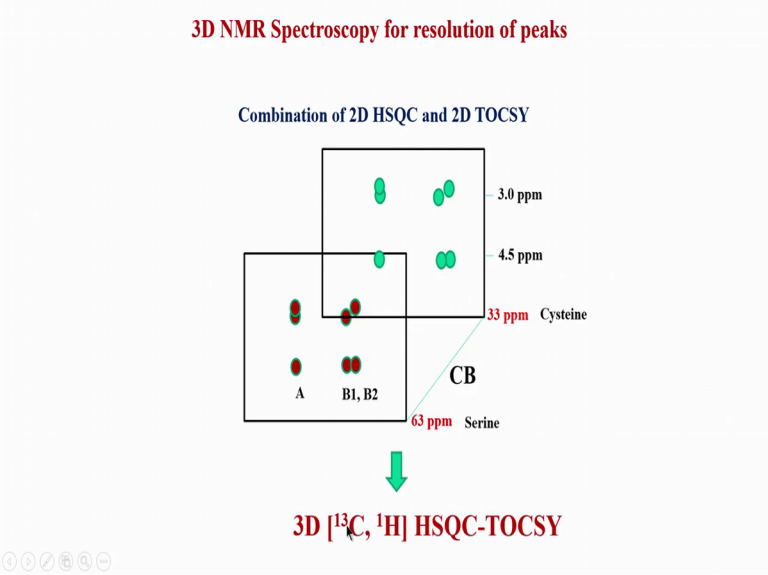

So, that is something what we can be done using a 3D HSQC TOCSY which is done by combining a 2D HSQC with a 2D TOCSY. So, like this by combining 2 2-dimensional NMR I can construct or I can create a single 3 dimensional NMR spectrum experiment where in, now I am using carbon is a third dimension. So, if you remember this plane here this what is shown here this green peaks and red peaks they were actually the TOCSY planes ok. See if I merge these two, if I join these two it becomes the 2D

TOCSY of the mixture. But, now I have separated them by using C beta as a separator or a discriminator or a third dimension and by virtue of that I am able to separate the cysteine family of spin system from the serine family.

So, this is one other this is one of the examples how we are able to separate 2 molecules 2 amino acids just because, we had a very good possibility in a carbon dimension to distinguish them. So, like that we can construct a 3D experiment. So, not only serine cysteine it would be possible to also separate some other 2 amino acids which are having closed chemical shift in protons. But, which have very good C beta or C alpha or C gamma some carbon differences and in HSQC TOCSY we should be able to separate them.

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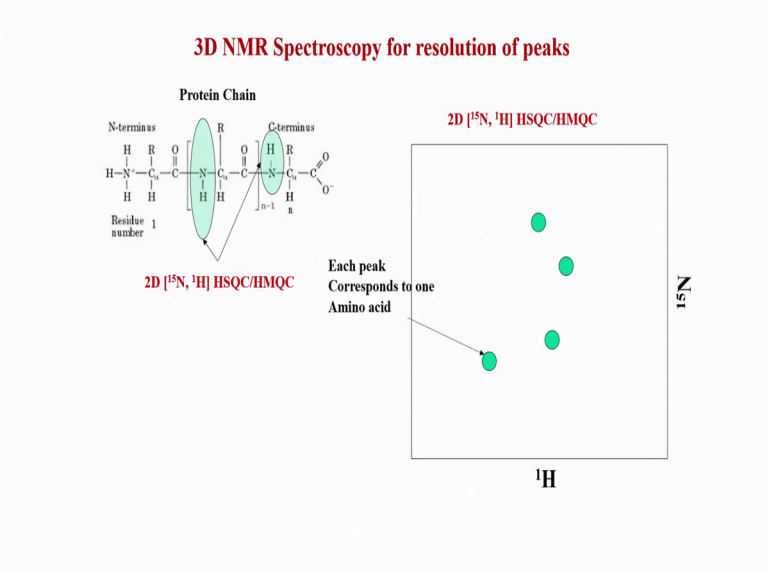

So, this is an example of how resolution can be obtained. Now, let us come to the protein NMR experiment which we normally use which is called 15 HSQC. We have looked at this in detail in the previous classes. Now, we will again look at say let us say in a 2D as I mentioned we each peak here corresponds to 1 amino acid and that is this shown here.

So, this is the peptide bond. So, we have the each amino acid has an amide except the N terminals and proline, but this amide proton and amide nitrogen come in a as a peak.

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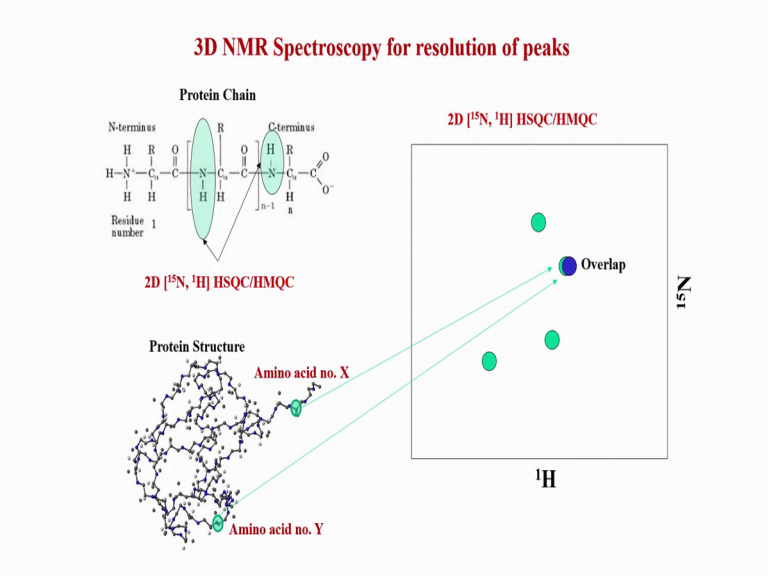

But suppose I have a case like in a protein, we have very complicated structure is always possible that 2 amino acids have accidentally the same chemical shift value; that is there the same amide, proton and amide nitrogen chemical shift. That means, this amide, proton and nitrogen for both of them are same. So, therefore, these 2 peaks are now sitting on top of each other. So, normally we will not be able to distinguish these 2 peaks because they are exactly or very close to each other so, they will come as a single peak appear as a single peak.

I have shown it different here different colour just to illustrate, but in a real spectrum you will not be able to separate the two they will look be overlapped. So, in such cases how do I know that there is there are 2 peaks here? I will not be able to figure out looking at the peak because the peak will look like a single peak. So, I have to do something more to achieve that separation or resolution and say that yes there are 2 peaks here not one. So, for that I need to go to 3D NMR now. Now what do we do here, what 3 what kind of chemical shifts another dimension we need here I write like we showed in the previous slide.

So, we need to add a third dimension another chemical shift. So, there are varieties of possible options. I can use C alpha as a discriminator; that means, it is it may be possible that the C alpha chemical shift of this 2 amino acids are not the same. This has a different C alpha and it could be having different. So, this is a guess I do not know it may be or

may not be or I can I could use C O as a discriminator; you see this has a C O be decided beside this N. And, there is a C O beside this N and every N H pair in the amino acid in the protein will have a C O of the previous amino acid.

So, I can use C O as a discriminator and it probably possible that amino acid X has a different C O compared to amino acid Y as a in chemical shift terms; that means, the C O chemical shift of these two are not the same. So, that is a possibility I can use that as a third dimension. So, that these are different experiments for example, if I use C O to distinguish the 2 amino acids; that means, I am adding C O dimension I am going to add C O dimension to this spectrum. So, it will become H N C O because C O will be another dimension. So, it becomes a 3D experiment wherein I have 3 chemical shifts H N and C O correlated with each other ok.

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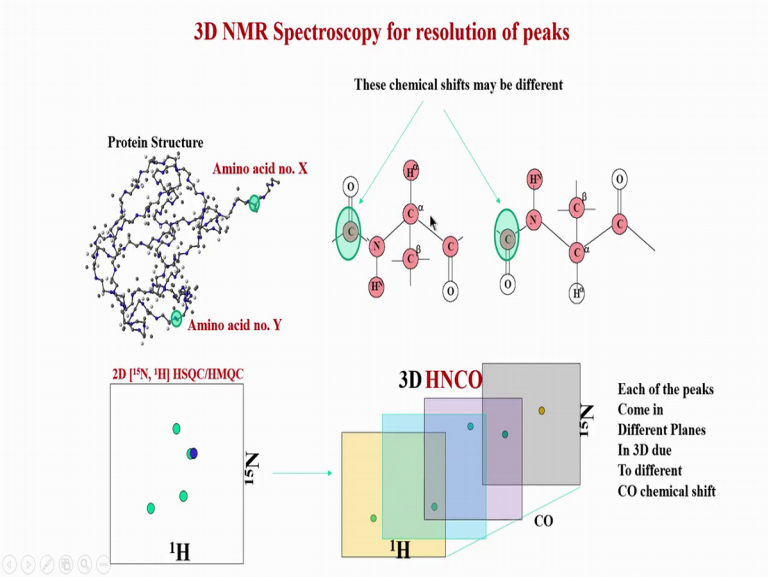

So, let us see how that can be done. So, this is exactly what is shown here, that they have 2 amino acids and somewhere in the sequence and accidentally remember they are just by chance they had the same chemical shift values. So therefore, we need to separate them or distinguish them to know that there are 2 peaks. So, what I can do? I can probably use C O which is close to the nitrogen you see there are close to it, say every amino acid has the C O attached to nitrogen and that can be used, because they may be different.

That means, C O may be different from for this amino acid from this amino acid. So, that is again an assumption we do not know that will have be true or not, but we can take a guess and then design a 3D experiment H N C O which was which will now help us to resolve those 2 peaks. So, such an experiment will look like this. So, that is spectrum of 2D as a 2D spectrum which I showed you within overlap here. Now, if I record a 3D now 3D will look like this because, it has 3 dimensions H N and C O and the C O is now acting like a separator like a discriminator.

So, you can see each of the peaks of here shown here will come in different planes because, there C O values may not be the same ok. So, they are able to we are able to separate those peaks for example, though these 2 peaks here which were actually overlapped in the 3D spectrum in the 2D spectrum. We are able to separate them because, of this nature of C O which help us to distinguish the peaks. This is an experiment we can name it as H N C O because we are correlating H and N C O. So, simplest 3D experiment we can think of iron and in fact, this is a most basic 3D NMR experiment known and very sensitive in nature.

But one thing you have to keep in mind is that we have nitrogen which is N 15 and carbon also has to be labelled C 30. So, basically we need to label isotropically make this molecule enriched rich with C 13 and N 15 then only we can perform this experiment. Because, next carbon is natural abundance C 12 is only 1 percent and natural abundance of nitrogen is 0.3 percent. So, if I want to do an experiment like this on an unlabelled means natural abundance on any molecule it will be virtually impossible because, I will have very low abundance of this isotope which are required for NMR.

So therefore, I need to label the molecule or protein with C 13 and N 15 and there for all this 3D experiments which you will be seen hence forth, are actually carried out on labelled protein samples. So, that is something which we will come across, we will see that as we go on because without labelling is not possible to do this experiment.

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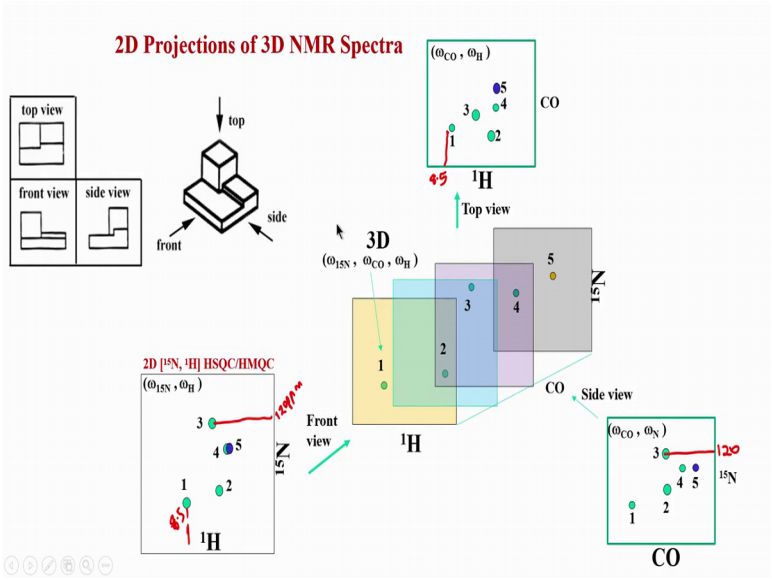

Now, let us see what are the different things in 3D NMR one should understand. So, as I showed you in the previous slide a 3D experiment, but we let us look at the different projections. Now, this is something which one has to understand very carefully. So, let us say this is taken from Google. You can see suppose you have some drawing or some 3-dimensional object like this and suppose I want a view a different views of this from different sides. So, typically in engineering we talk about front view side view and top view; see that is shown on this side here.

Your top view may look like this, front view if you are looking from here it may look like this, side view may be look like this. So, similar to this kind of a picture we can actually have also for NMR spectrum 3D NMR spectrum. So, this is a 3D spectra which I showed in the previous slide. So, you can see this is 3-dimensions. Now, if I want to look at different projections of this similar to that how will they look. So, let us start from the very basic front view. So, if I am looking for this angle basically what will happen is, this CO will dimension will disappear like here. When you are looking from the front side you do not look the side dimension disappears ok.

So, like that you will get with only HSQC spectrum back. So, actually in a 3D the HSQC spectrum is one of the projection of a 3D and that is something which we should know because, we took an HSQC and we use carbonyl or C O as a discriminator as a separator. So therefore, if I did not have this dimension I would have got a standard HSQC and that

is what is one of the projections of a 3D spectra ok. So, this has this overlap which we could resolve, but if we do not have this dimension it does not get resolved.

Now, let us see how see how the top view will look like, top view basically means you are looking from the top here similar to this kind of a view, perspective. And, here the peaks are now coming in different positions they are no longer same as this. Why? Because, if you look from the top this now we are looking at the edge C O axis. So, it is not nitrogen here it is not HSQC, it is a proton carbonyl projection. There it depends on the carbonyl chemical shift where the peaks will come. The proton will be the same as this proton for example this one here let us say has a proton peak here which corresponds to 6.5.

So, let me show write down to make it more clear. If this proton chemical shift is let us say 8.5, this proton here also will be 8.5. So, that will not change because we are looking at the same proton axis in the both cases, but that carbonyl will depend on what is a carbonyl value, current C O value in this dimension. So therefore, the peaks looks now re-arrange in a different order compared to this because of this C O dimension. Now, you can I can look from the front side view, if I look from the side view I will get C O nitrogen correlation ok.

So, this is again a different something completely different from these two because, now I am getting C O and N value. So, if you look at N now this N will remain the same. So for example, let us say this 3 if this had 1 20 ppm, this also will be 120; so, that will not change. So, this is just an example to show you that this chemical shift of 3 which is nitrogen part is same as this nitrogen here because, this axis and this axis are the same. So, we can see in NMR 3D NMR experiments we therefore, have to focus on projections of NMR spectra to understand what is happening and this is how NMR spectra 3D analysed.

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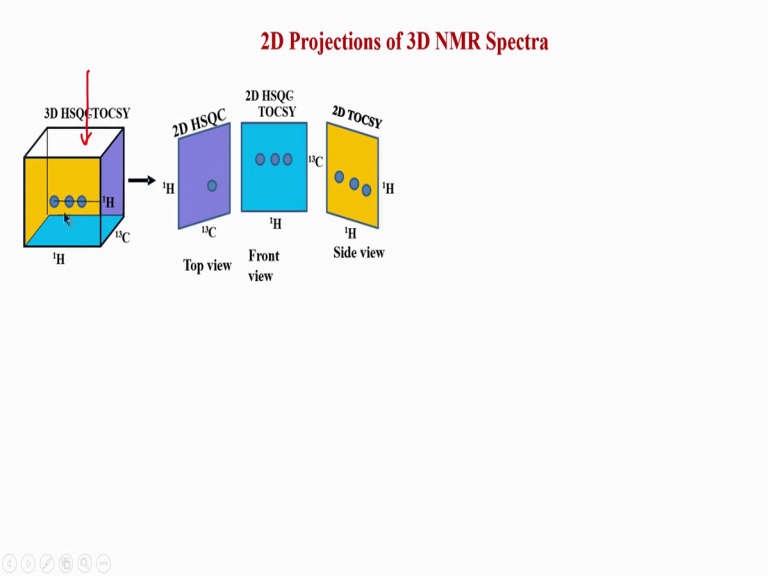

We will look it again a few more examples later, we will quickly now just look at a projection example for a 3D HSQC TOCSY. So, this is a 3D HSQC TOCSY which I showed you just few slides ago; how that helped us to resolve cysteine and serine, but it will have a peak pattern like this. So, if you look at this box 3D box, if I look at different view; the top view will look like this because I am looking from the top from here.

So, the top view is basically coming from here, this is the top view and you can see that is basically a carbon proton plane which is shown here ok. And, then if you look at front view that is proton-proton TOCSY is shown here and then side view; you need to look from this side here you will see a proton regular proton-proton TOCSY and this was basically carbon proton TOCSY.

So, will have looking from this angle. So, you can see this blue plane here, this blue plane is basically this blue plane here proton carbon HSQC TOCSY. So, this 3D HSQC TOCSY we will look at the different projections they are actually 3Different experiments.

But, they all got combined into a single experiment and I got a 3D spectrum out of it. So, basically a 3D experiment if you look at the different projections of the 3D are actually 3 different spectra which normally would be collected with different x pulse sequences; like this has a separate pulse sequence we saw in the previous part. This has a separate pulse sequence this has a different pulse sequence. We have seen actually the pulse

sequences of all these 3, but these three are not separately collected and joined and made like this. What is done is this 3D HSQC TOCSY is a single experiment which is recorded in a single data set.

But, you look at its projections they will end up separating into 3 different spectra. So, we normally do not analyse projections. So, like this, but it is useful to know how I truly projection of a 3D look like. So, we will continue this aspect in the next class, look at few more examples and then we will see how actually a 3D spectrum is analysed and interpreted.