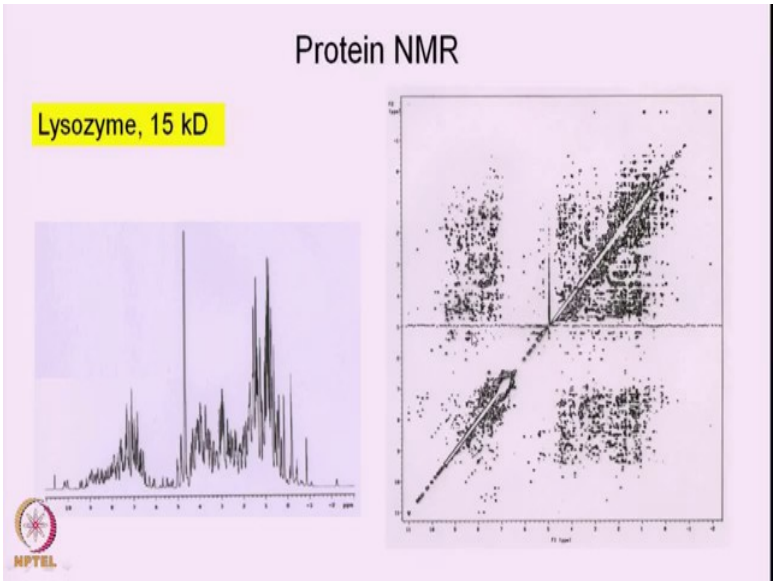
NMR Spectroscopy for Chemists and Biologists
Dr. Ashutosh Kumar
Professor Ramkrishna Hosur
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay
Lecture 55
Multidimensional NMR

During the last several lectures we have discussed in great detail a variety of 2-dimensional NMR experiments, various kinds of correlations have been discussed and we have seen how they will be helpful in determining structures of molecules in solution. One of the major applications of NMR has been to proteins.

So therefore, we will take forward from there to discuss the applications of NMR to proteins and we will see how protein NMR applications have revolutionized NMR itself, it has led to further developments in NMR then what we have discussed so far and that has been a feedback mechanism for NMR spectroscopy developments.

(Refer Slide Time: 1:14)



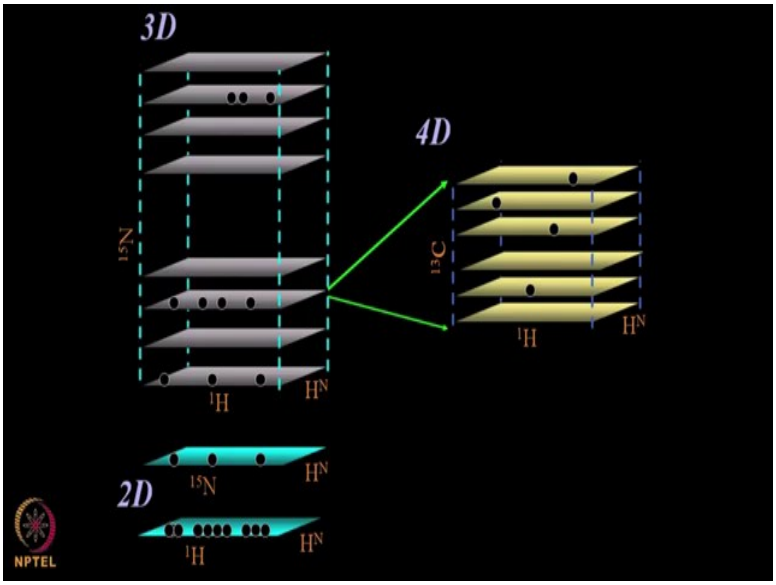
So here I show you first a 1 dimensional NMR spectrum of a protein, this is the protein called lysozyme which is molecular weight of 15 kiloDalton, it is made up of 130 amino acid residues and this is the 1-dimensional say proton spectrum at 500 megahertz. And we can see here this has a very well spread out spectrum, peaks up there all the way from minus 1 ppm to 11 ppm and there are a large number of peaks. This protein has nearly 500 protons.

So therefore and you should expect the complexity of the spectrum to be as seen here, there are a large number of resonances and one can only say from this 1 dimensional spectrum that these belong to the amide protons and here you have the amide protons and the aromatic protons on the basis of the chemical shifts. And these ones belong to the α protons to the β protons and then you have the methyl here and so on.

But more than that you cannot tell anything about what peak belongs to what amino acid residue and which proton in amino acid residue and so on. To circumvent such problems we had to record a 2 dimensional spectrum and this is the so called 2 dimensional NOESY spectrum which we have discussed earlier. But look here this has thousands of peaks here, this actually reflect correlations between the various protons which are close by in space less than 5 ˚A distance and there are some which are 3 ˚A, 4 ˚A or 5 ˚A and things like that and the intensities will depend upon this distance.

But this spectrum is also extremely complicated, we have so much of overlap of the cross peaks here and it is almost impossible to analyse this spectrum in great detail to identify the peaks as belonging to individual protons in the protein. So therefore, this again posed a challenge, so as we went from 1 dimension to 2 dimensions in case of smaller molecules to remove the ambiguities and obtain assignments with the t1 the 2 dimensional spectrum here is not adequate to obtain full assignments of the individual protons.

(Refer Slide Time: 3:37)



So, what do we do? So we have to use other strategies as we went from 1D to 2D now we can go to 3D and 4D and so on, how does that help? Let us look at that in a schematic manner

here. This is the proton-proton spectrum schematically and you have the amide protons and then we have the other protons, aliphatic protons, aromatic protons, etc etc here. And at this particular one chemical shift along the proton resonance in the amide region you are seeing 10 peaks here which are obviously belonging to multiple amide protons. So obviously there are more than one amide protons here because one amide proton cannot produce so many cross peaks.

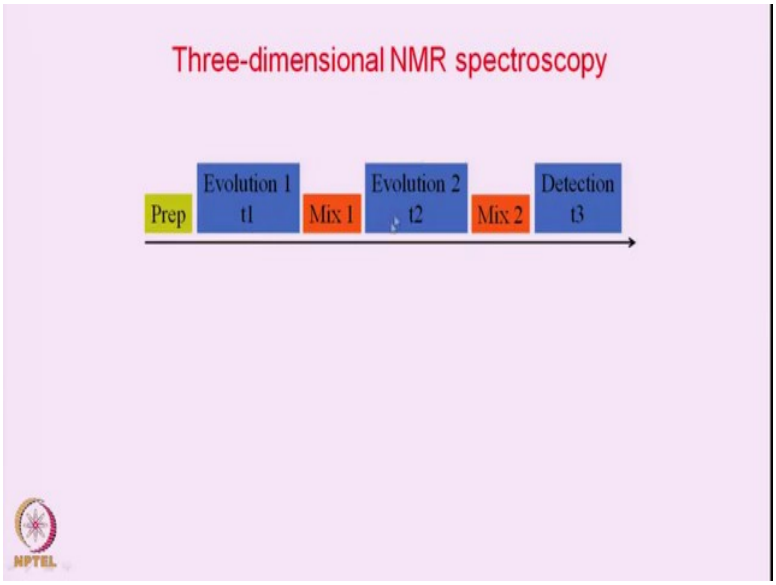
So therefore, we try and see how many are present here then you try and look at the heteronuclear correlation, if they are between amides and the nitrogen, let us do a amide nitrogen correlation here, the amide proton the ^{15}N correlation spectrum which we also discussed earlier. And this is producing at this particular amide proton chemical shift 3 peaks which means there are 3 nitrogen at least at this position, okay.

So now what we do is make use of this ^{15}N chemical shift and disperse these peaks along the third dimension as per the ^{15}N chemical shift. So one of these belongs to this ^{15}N chemical shift, this plane belongs to this ^{15}N chemical shift and this plane belongs to this ^{15}N chemical shift. So the corresponding peaks are now distributed in these respective planes. So there are 3 peaks here at this ^{15}N, 4 peaks here and 3 peaks here, whether there is any degeneracy here can we identify this further as to belonging to the particular protons we can remove this further degeneracy using a carbon13 chemical shift.

Now use carbon13 chemical shift to disperse these peaks for example these 4 peaks on 4 different planes according to their different carbon chemical shifts because they are attached to different carbons but they are producing peaks at the particular ^{15}N and are correlated to the amide proton at that chemical shift. So therefore, you are now able to resolve the various aliphatic protons of the individual amino acid residues at least 3 amino acid residues here and they are distributed like this.

The same will happen for this plane as well and also for this plane as well, so all the 10 peaks will have proton ^{15}N carbon13 and amide proton chemical shifts uniquely identified, so this is a schematic. The idea is you want to get to this. So therefore, how do we do this 3 dimensional experiments? How do we do 4 dimensional experiments? So we will take as an illustration the 3 dimensional experiments, the idea is very simple, it follows the same strategy as we did when we went from 1 dimension to 2 dimensions.

(Refer Slide Time: 6:39)

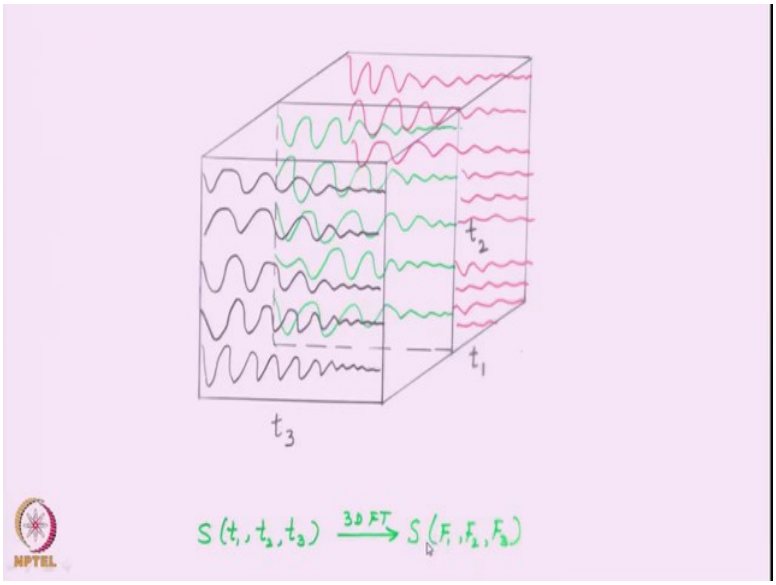


So the schematic of 3 dimensional NMR spectroscopy is indicated here, so here you have a preparation period which is common as in the case of 2D as well. And here you have the evolution time t1 which is an incrementable time which produces one of the time variables and then you have the mixing to transfer magnetization from this one period to the next period.

Now in the case of 2D here we had the detection period, we collected the data as a function of time and we produced a 2 dimensional spectrum after 2 dimensional Fourier transformation. In this case we will not collect the data here, but we will treat this as another evolution time with the label t_2 then we add another mixing period here called mix 2 and then transfer the magnetization from here to here. And finally we have the detection period which is t_3, we collect the data as a function of time here and therefore this data which is collected will be modulated by what happens here and what happens here in independent manner.

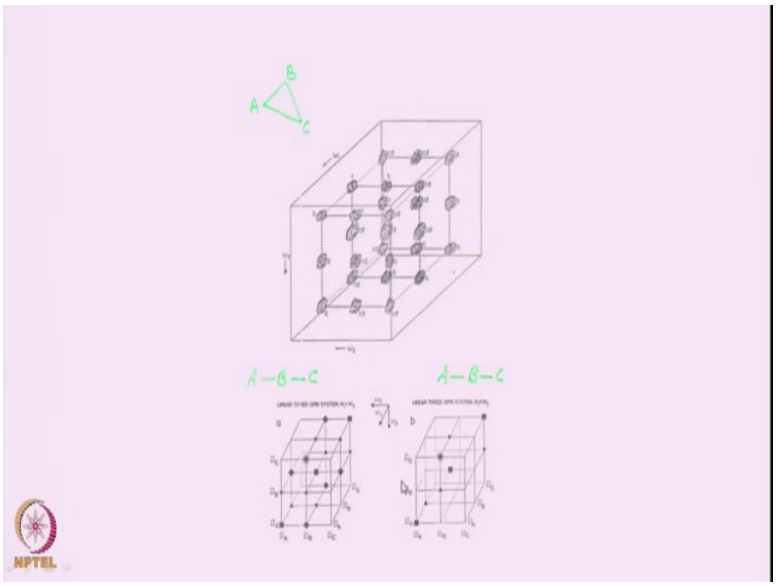
You systematically increment this t_1 period, you also increment this t_2 period and for every one of those you collect the data. So let us say to be more explicit for a particular value of t_2 you collect some let us say 256 FID's and then you increment the t_2 collect another 256 FIDs', increment again collect another 256 FID's.

(Refer Slide Time: 8:09)



Therefore, you will generate a cube of FID here, so t_1 is one variable here, t_2 is another variable here and the t_3 is the third variable here, so you collect FID for independent variation of t_1 and t_2, okay. Therefore, you generate a 3-dimensional time domain data which we write as $S(t_1, t_2, t_3)$. Now, if you do a 3-dimensional Fourier transformation and you will get a 3-dimensional frequency domain spectrum $S(F_1, F_2, F_3)$.

(Refer Slide Time: 8:41)



Okay, so here is the example of a particular 3 spin system. Suppose we have a 3 spin system here A, B and C and those 3 spin systems are here indicated here, their corresponding chemical shifts are at this point. And then you will have correlations between each one of those. So the mixing transfers magnetization between each one of those, so and then if you

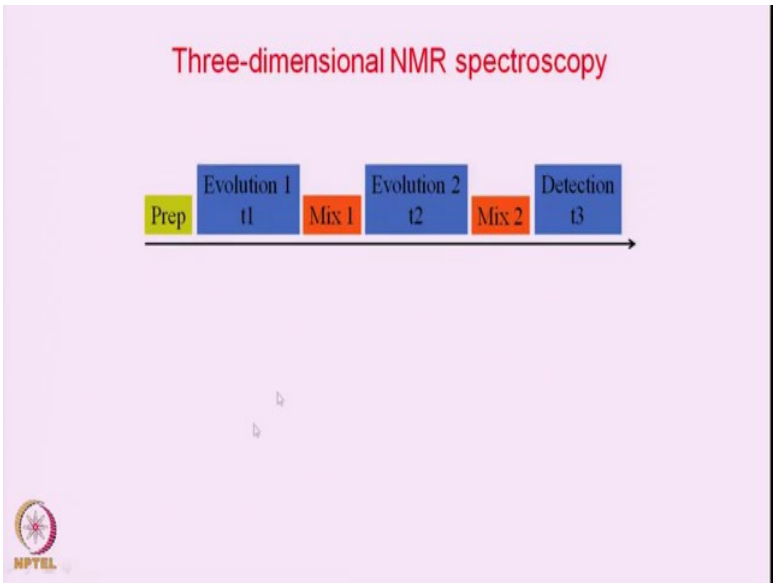
have a 2 dimensional corresponding spectrum for a 3 spin system you will have 9 peaks in the 2D correlation spectrum. right.

So now if you have a the second mixing time then you establish correlations once more with all these 9 peaks in the 2 dimensional spectrum, in the 2D spectrum we had a nine peaks for a 3 spin system of this type. Now when you have another evolution period establish correlations with those once more with those 9 peaks you generate 27 peaks. Therefore, you have in this 27 peaks in this the 3 dimensional spectrum all of them are separated and then by analysing this you can follow the mechanization transfer pathway.

So you see here these 3, 9 peaks here and each of these produces a correlation to the other 2, each P produces the correlation to the other 2 and therefore you from each of the 9 peaks you will have a total of 27 peaks in your spectrum, this is in this particular case. And suppose you have a linear system in that case ofcourse you will not have a correlation from *A* to *C* in the normal 2-dimensional spectrum.

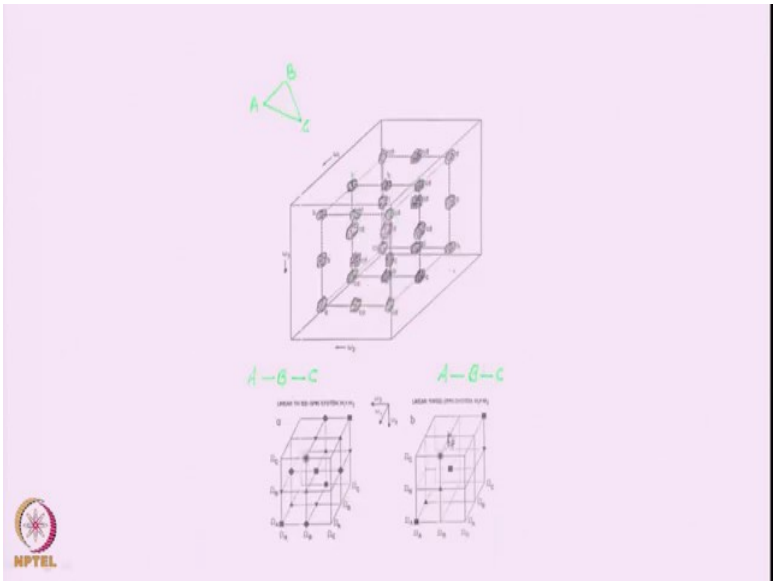
So therefore, the number of peaks will be somewhat less and still it is quite substantial here the 2 mixing periods are assumed to be the same $M_1 = M_2$ the two mixing periods are the same we have written *J* correlation it is a *J* correlation, but in principle we need not have the 2 mixing periods the same, one of them may be *J* correlation.

(Refer Slide Time: 10:35)



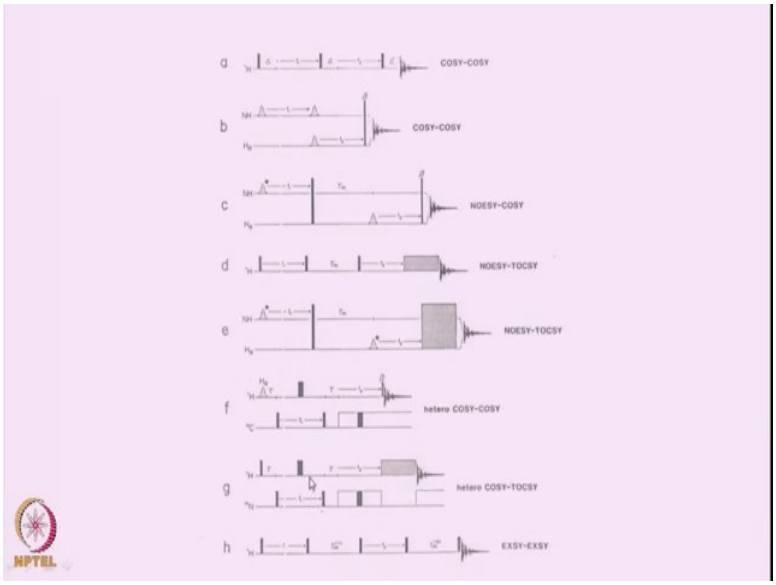
Other one may be I mean in this example this can be a *J* correlation or this can be NOE correlation, this can be *J* correlation, this can also be *J* correlation or this can be NOE correlation, this can also be NOE correlation.

(Refer Slide Time: 10:52)



So like that you can have different combinations of mixing sequences and that will produce different number of peaks in the individual spectrum. So here you have when $M_1 \neq M_2$ you will see much greater reduction in the number of peaks and it will be ofcourse easier to analyse. So this ofcourse becomes quite complicated to analyse but this one will be relatively easier to analyse in the 3 dimensional spectrum.

(Refer Slide Time: 11:13)



Okay, so following this strategy a large number of pulse sequences have been generated. For example here I have a 3-dimensional COSY-COSY pulse sequence that means the first mixing sequence uses a *J* correlation mixing and the second mixing is also a *J* correlation mixing. Therefore you have a 3 pulse experiment here this is a t_1 increment, then you have

the mixing here and they have t_2 increment, another mixing here, then you have a small period for some adjustments and then you acquire the data here at this point.

So this is the COSY-COSY and this is also a COSY-COSY experiment except that here you have a selective pulse applied, here it was a hard pulse, this was a selective pulse applied here for particular group of protons spins. And then you transfer from those particular group of spins to selectively to another group of spins and by *J* correlation and then they evolve here period and then it is transferred back to these protons to acquire the data, this is also a COSY-COSY but then this is ofcourse selective COSY, selectively some peaks are excited here and some peaks are excited here and you observe correlations between only those ones.

So we can make all such combinations here, so therefore you generate a whole lot of experiments, here is an experiment called as NOESY-COSY and you selectively excite a certain number of protons here and you transfer the magnetization to the *Z*-axis here and do a NOESY mixing this is the NOESY mixing. So after the NOESY mixing you apply selective pulse once more, transfer the magnetization to some other protons and then you do a t_2 evolution use frequency label those ones and then you transfer the protons once more do a second mixing and then this is the *J* correlation mixing.

So therefore, this becomes a NOESY-COSY experiment, so you have here the NOESY mixing period, then this pulse acts as the *J* correlation mixing or the COSY mixing therefore you will have the NOESY-COSY 3 dimensional experiment. So likewise we can also have the NOESY-TOCSY here homonuclear experiment, this is the t1 evolution for the particular group of protons which are excited and then you have the NOESY experiment here you do the same thing as before.

Magnetization is kept on the *Z* axis and then you do a NOESY mixing and after that this is converted into the transverse magnetization and then the frequency label those after the NOESY mixing you do a frequency labelling and then you do TOCSY mixing okay, so this is the second mixing period you have the frequency labelling here, then you have the second mixing period here and therefore and then you collect the data. So this produces a NOESY-TOCSY correlation.

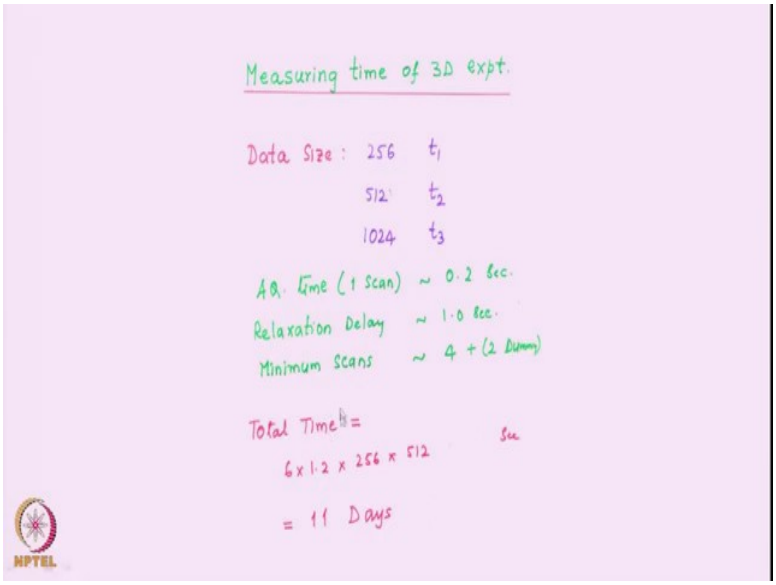
So correlation between F_1 and F_2 will be NOESY and F_2 and F_3 will be TOCSY. So therefore, this will go in that manner. So you can do such kind of experiments various combinations are possible so you have NOESY-TOCSY, here you have the hetero COSY-

COSY invoke the hetero nucleus here transfer to the hetero nucleus and then you do a kind of HSQC type of experiment here t1 evolution here and transfer back to this is the proton and then you carry through some mixing period here and then you do a decoupling and this is the COSY mixing here.

Okay so this is homonuclear COSY experiment that will here it is a hetero experiment, this is a heteronuclear correlation after that it is a homonuclear correlation during this t2 evolution there is a homonuclear transfer from one proton spin to another proton spin. And this tau periods are required to convert anti-phase into in-phase and things like that, so all of those is thing which you have already discussed and we will not go into the further detail theoretical calculations here, but the principles are the same.

So it is easy to follow by actually working out the product operators at the individual steps, what are the product operators at various places, why do you need the delays at various certain points and how one can proceed with this? So a large number of experiments have thus been developed and one can use it as per the need.

(Refer Slide Time: 15:24)



A rough calculation here of the experimental time, you have the measuring time of a 3D experiment this is not true for ofcourse all the experiments this is typical numbers which you have put here depending upon what numbers you puts your data the time factor will be different. Suppose you have 256 t_1 experiments and you do 512 t_2 increments and you collect 1024 data points along the t_3 for each one of those.

And assume that the acquisition time the FID length is 0.2 seconds and relaxation delay which is 1 second, then suppose you do a minimum of 4 scans, typically one needs this to do phase cycling as I indicated to you earlier, if you want to filter out certain pathways and select certain pathways some number of scans will be required minimum and then ofcourse you will have that sort of a minimum number of scans and you also use some dummy scans for the spin system to reach a steady state initially.

Okay so therefore, if you calculate this time this works out to be 11 days and this is a huge amount of time. Therefore, for the information you gain by dispersing the peaks in the 3 dimensions you certainly pay a price in terms of the machine time here, if you do experiment in this manner. So but generally one is not doing this strategies, one is adopting certain kind of a combinations of nuclei so that you do not need to do these many increments in each of these dimensions and then you will be able to save time.

This is a particular advantage of heteronuclear experiments, where we can actually choose a small section of the spectrum and collect the data in a rather less time.

(Refer Slide Time: 17:04)

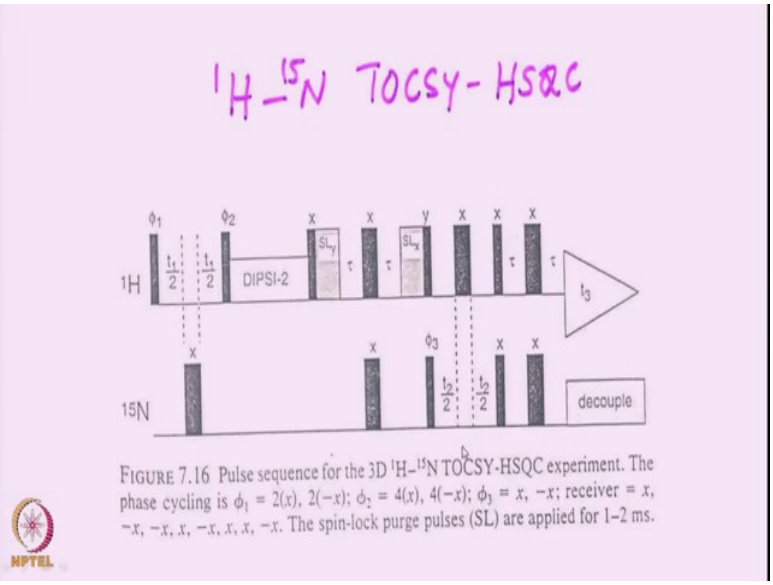


FIGURE 7.16 Pulse sequence for the 3D ^1H–^{15}N TOCSY-HSQC experiment. The phase cycling is $\phi_1 = 2(x), 2(-x)$; $\phi_2 = 4(x), 4(-x)$; $\phi_3 = x, -x$; receiver $= x, -x, -x, x, -x, x, x, -x$. The spin-lock purge pulses (SL) are applied for 1–2 ms.

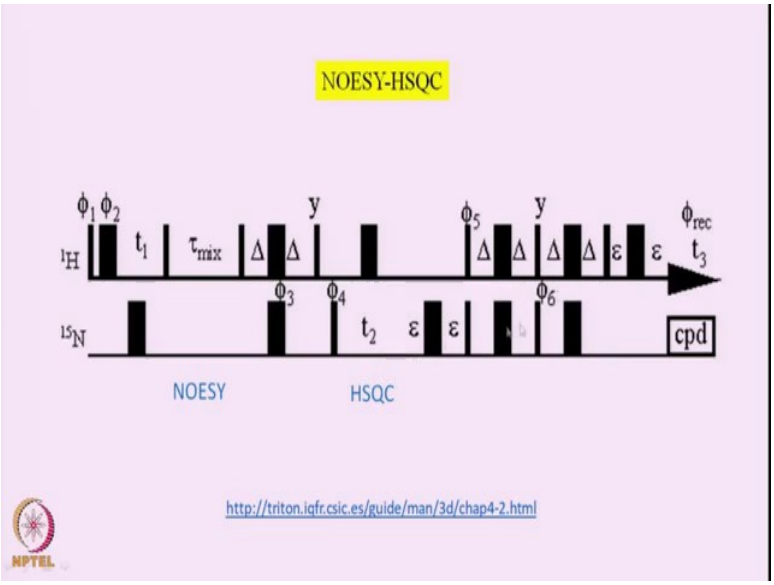
So here is one of the heteronuclear 3-dimensional experiment which is called proton-^{15}N TOCSY-HSQC. So the first mixing here is a TOCSY mixing and the second mixing is the HSQC mixing. So this is the heteronuclear transfer, the TOCSY means it is a proton-proton homonuclear transfer that is indicated here, the experiment goes like this you have the first 90˚ pulse on the proton and you evolve it during the period t_1 here.

But you apply a 180˚ pulse in the middle of the t_1 period on the ^{15}N channel so as a result of this there will be no proton ^{15}N coupling evolution okay this will decouple this 180 pulse decouples proton from ^{15}N and you will have only proton-proton coupling evolution going on. Therefore, you do a TOCSY mixing of the proton-protons here from here to here. The DIPSI-2 is a TOCSY mixing sequence, these pulses are applied to flank this DISPI, this is the whole thing is a part of the DIPSI-2 mixing.

Okay, so then after that you have transferred the proton magnetization from one spin to another spin among the proton coupled network and now you do a HSQC experiment from here to here it is a INEPT transfer from proton to the nitrogen15 here, then ofcourse you need 180˚ pulses on both the channels at this point and this magnetization is transferred to the ^{15}N. Now here the magnetization is on ^{15}N and this will evolve with the its characteristic frequencies and this time label is now t_2.

At the middle of this you have 180˚ pulse as in the case of normal HSQC, so we have only frequency labelling of the ^{15}N nuclei here and then from here you transfer the magnetization back to the proton at this point. And therefore, now it is anti-phase magnetization of proton which is anti-phase with respect to the nitrogen and then during this τ-180-τ period you allow this anti-phase magnetization to refocus and you can collect the proton data here. You collect proton FID and when you are collecting this you can decouple the nitrogen15. So this is the TOCSY-HSQC experiment.

(Refer Slide Time: 19:19)



Okay similarly, you can also do a NOESY-HSQC experiment, the NOESY-HSQC goes in the same manner here, you have the proton magnetization excited here and then you have the evolution for labelling the proton frequencies and this is done once again to remove the proton nitrogen coupling, so this is only proton frequencies here and this now whole block from here to here will be the NOESY part, so you have the NOESY mixing.

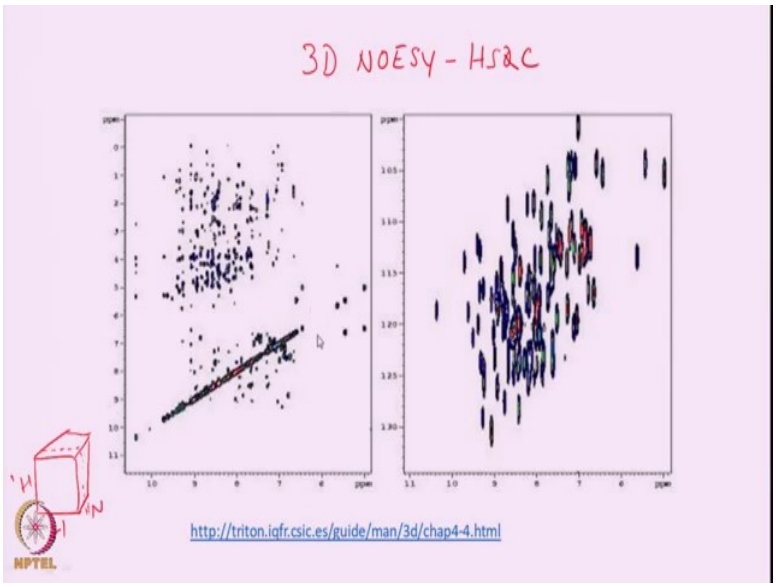
Therefore, the transfer of magnetization happens from one proton to another proton because of NOESY mixing and then you start your INEPT sequence from here onwards you actually transfer the magnetization to the nitrogen15. So from this the INEPT sequence you transfer it from here to here and now the magnetization here is nitrogen15 and to a HSQC type of experiment.

These pulses which are present here are some improvements, so we will not go into those details, I mean of course compared to the standard HSQC what I have given you earlier there are some differences here, but these ones are to improves the performances, remove some artefacts and things like that. So but let us not go into those details. Similarly, of course this one pulse also which is applied for some removing some artefacts and imperfections.

And then you transfer the magnetization back to the proton at this point and then now you have to do a refocusing and there is also some other additional which is added here which we have not discussed this is to increase the sensitivity of the experiment and finally you collect the data here and do decouple the nitrogen frequencies. Okay, so therefore this is something which is additionally put for improving the sensitivity of the experiment.

So this is called NOESY-HSQC basically you have in the first mixing it is NOESY mixing period and in the second mixing you have the HSQC this is heteronuclear correlation that is the *J* correlation. And then you collect the data as a function of proton frequencies.

(Refer Slide Time: 21:24)



Okay, now let us look at some experimental data, so here is an illustration application to a protein. This is a 3D NOESY-HSQC spectrum, this is the 3D HSQC spectrum. Now on this side a 2 dimensional HSQC spectrum is shown for ease of understanding, so on this axis in the HSQC of the protein you have the proton dimension here and on this axis you have the nitrogen15. So you have the nitrogen15 chemical shifts here and the proton chemical shifts along this axis okay, so these are amide proton chemical shifts, okay.

So as we said earlier this is actually fingerprint of your protein, you will have one peak per residue and by actually counting the number of peaks here you can actually figure out how many amino acids are there in your protein. Now a 3-dimensional spectrum and NOESY-HSQC will look like this, so on one of the axis you have the nitrogen15 here as I indicated you in schematic and then you have other two axis have the proton here or here and here, okay.

But this portion what is chosen here is the amide proton chemical shift because it is only the amide protons which will show correlation to nitrogen15 okay, no other proton shows the correlation to the nitrogen15 through coupling. Therefore, if you are actually passing the magnetization from the proton to the nitrogen it will only be from the amide proton to the

nitrogen. Therefore, I will only have in this case amide proton to nitrogen correlations here okay.

Now after the nitrogen15 it is transferred they are doing an HSQC experiment, you label the nitrogen15 and transfer it back to the amide proton and do the detection. So before you actually transfer you have done the proton-proton NOESY and then you are transferred to the nitrogen 15 and you have done the HSQC there and you generate a 3-dimensional spectrum. So therefore, this is the 2 dimensional HSQC spectrum amide proton N15 correlation here.

Now, if I take a particular cross section here at the particular N15 chemical shift here and I plot that here in this and this is what we have got. So you see even though this is also complex, but still it is much less than what you would have in a simple 2-dimensional spectrum all of them the peaks appearing at one place.

So, if you take a particular cross section means supposing I am taking a cross section at this point you can see here how many amide protons are there, so you can gives at least 10, so you can count more. So there are so much of amide proton degeneracy at this ^{15}N chemical shift okay. So now all these amide protons will show NOE correlations to the respective aliphatic protons. So this is for example from here you show the correlation to the α proton to the β proton, the Υ proton, the δ proton and so on.

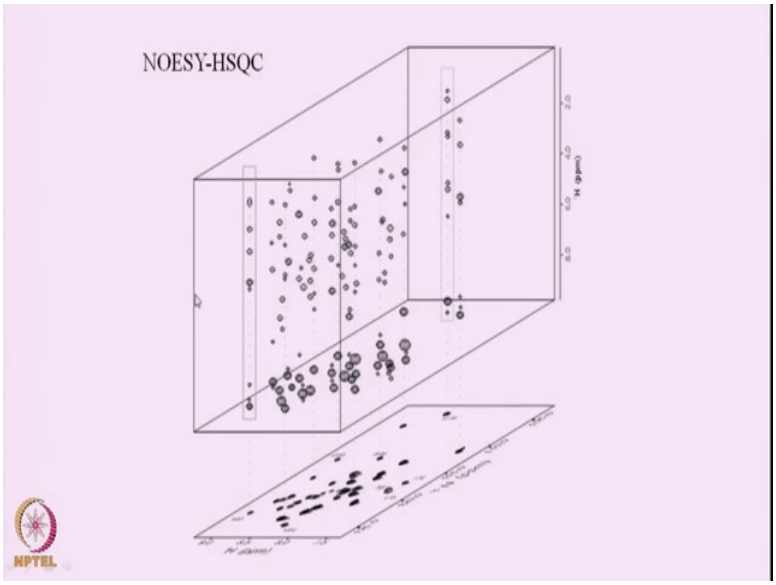
So likewise for this one you see this amide proton is showing a correlation to these alpha protons here and then the beta proton and perhaps the methyl protons here. So likewise all of these amide protons which are present here they are on this axis and they show correlations to various the other aliphatic protons and some of them show correlations to the aromatic protons as well. So all these which are present here these are amide-amide correlations and amide-aromatic correlations.

Aromatic protons also appear in this region right, so they appear between 6 to 7 ppm, 7.58 and all the amides are present in this area. Therefore amide-amide correlations appear in this area and this area belongs to the amide aliphatic correlations. So therefore, since the particular ^{15}N chemical shift there are so many amide protons here you will have so many peaks present in this and this is analysable because we can go plane by plane and look at the correlations between the various groups of protons, okay.

So and often one does to simplify the procedure one takes an area where there is not too much of degeneracy. For example here, and typically these ones belong to the glycine's okay

and then you will have a good starting point, once you start from such unambiguous positions you will be able to establish correlations between the amino acid residues and then we will be able to walk along the polypeptide chain. So that shows a combination of a lot of these experiments are used TOCSY-HSQC and NOESY-HSQC are used to identify resonances as belonging to the individual protons, okay.

(Refer Slide Time: 26:23)



So let me show you here how does the actually the experiment in the spectrum look a 3 dimensional spectrum the same NOESY-HSQC here. Now you see the 3-dimensional cube so you have here the peaks on the various planes. So in principle you can take cross sections at various chemical shifts and analyse the individual peaks, you can see, now there are only a small number of peaks here and this plane also has a certain number of peaks, this is behind that.

So if we come forward then you will have some peaks here which belong to this particular proton. So you now you take a projection down this axis you produce a 2 dimensional spectrum here. So therefore, essentially as I indicated to you in the schematic this 2 dimensional spectrum is now pulled apart along this, but with the NOE correlations, in this case the NOE correlations are not there, this is the say HSQC spectrum.

Now NOE correlations are displayed along the third dimension here. So therefore, this is the proton-proton axis, so you will have correlations from this particular amide proton at this particular N15 chemical shift to these many aliphatic protons here and likewise this one will

also show these many aliphatic correlations and therefore it becomes much easier to analyse a 3 dimensional spectrum.

And this allowed to determine the structures of reasonably sized protein molecules something like 150 and sometimes even 200 amino acid residues have been analysed. And of course it all depends upon what is the quality of your spectrum, how well resolved or the peaks in the HSQC spectra, if they are well resolved then the protein is well folded, it indicates the protein is well folded.

So once you have well folded proteins the peaks will be better resolved then you can go to larger and larger systems. So this has been a success story and for all of these developments you know this Professor Wuthrich was awarded the Nobel Prize for protein structure determination using NMR.

The methodology of 2 dimensional NMR and 3 dimensional was developed by Professor Richard Ernst who also invented the Fourier transform NMR technique and he was awarded the Nobel Prize in 1991 for this technique development and the applications were elegantly done by Kurt Wuthrich's group in Zurich incidentally both of them were in Zurich in the same institute ETH.

And Wuthrich won the Nobel Prize for these applications in 2002. And this is of course this was the beginning and now we have gone much further using more of ^{15}N and heteronuclear experiments, carbon13 experiments which was, which I mentioned to you and we do not have time to do all of that in this course, probably one may have to do another course.

And then you have to develop triple resonance experiments where you use not only over 2 nuclei at a time, but 3 nuclei at a time you can use and disperse the peaks and these are called triple resonance experiments and hundreds of such experiments have been developed and this has allowed us to push the limits of molecular weights to several-several tons of kilodaltons. The ambition is to go as large as possible, study large assemblies, study intrinsically disordered proteins and so on so forth. I think that is well beyond the scope of this course and maybe one has to do this at a later stage, with that I will stop here and I hope you enjoyed the course.