Principles and Applications of NMR Spectroscopy
Professor Hanudatta S. Atreya
NMR Research Centre
Indian Institute of Science Bangalore
Module 7
Lecture No 34

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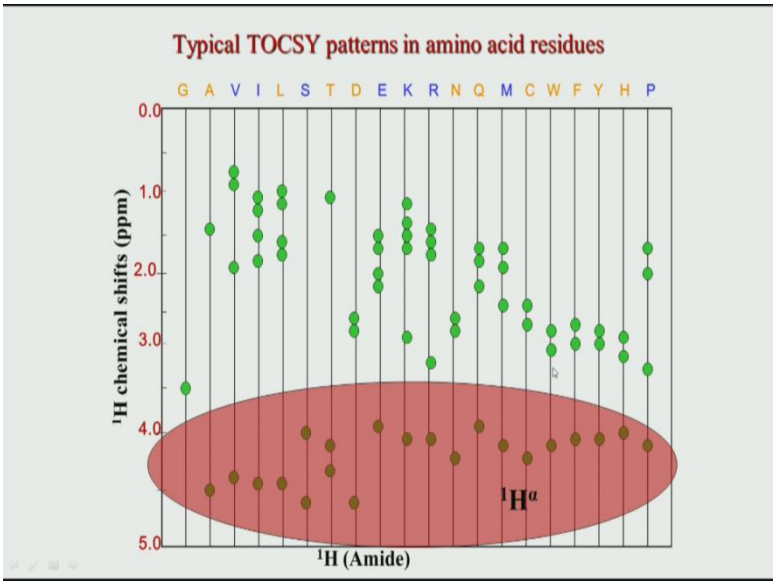

In the last class, we looked at how TOCSY pattern of different amino acids can be used for sequential assignments, so basically the idea is that each of the peaks of these residues such as alpha peak, beta peak and gamma peak, they have very regular pattern for each of the amino acids 20 amino acids, so we call it a fingerprint. So, each amino acid can be identified based on the chemical shifts in the aliphatic portion of the spectrum, so this is shown here, so you can see for all the 20 amino acids you can see that there are very nice different patterns which can be utilized for matching the residues. So, this is what we will use now as we go along and for sequential assignments. So, you can see this is for glycine, there should be as I said there should be 2 peaks; sometimes you may get 1 or sometime may get 2, but they are not chemically equivalent at a times and then you can see here at alanine gives a very clear cut peak at 1.5ppm and the alpha region at, 4.5 sometime even at 4.

So as different amino acids have different characteristic patterns, so basically what we do next is that given the TOCSY spectrum the 2D TOCSY spectrum we have to now identify the different spin systems in the peptide. So remember, peptide is composed of sequence of amino acids and each amino acid as we saw is can be denoted as a spin system. So, that is what we will do we will also look at the H alpha. So you can see, one thing if you notice the H alpha regions is kind of not very distinguishable from each other. There is no particular

pattern as to where the H alpha peaks belong compared to what you see this side. So, here if you notice here on this side that is aliphatic beyond between 1 and 3ppm, there are clear cut number of peaks which are different for each amino acid, okay except here.

So, here you see here these are called AMX spin systems, AMX spin system are basically those which have only the beta carbon and to beta protons for case of phenyl alanine, tyrosine, histidine and tryptophan you have what is called side chain aromatic spin system but we will not look into all those. When we go for sequence specific resonance assignments in which we only look at the backbone and the side chain up to the beta protons or carbon or gamma carbons, okay.

So these are called long side chains amino acid so that glutamic acid, glutamine, lysine, arginine, they belong to what are known as long side chain amino acids. So one thing so coming back to these protons what we see here is that there is no strict pattern but this is the region which we will be using for sequence specific resonance assignment. So, let us go back to this sequential assignment, so this is what we see here, so we saw the COESY pattern we saw for threonine what is that chemical shift type of amino acids and what are the different patterns and we looked at valine.

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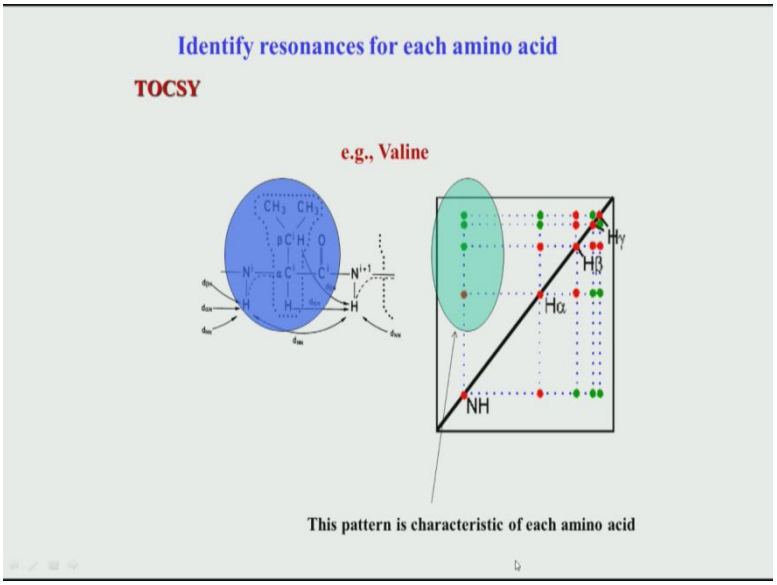

So, in the valine we saw that you have this amide proton and this is alpha, beta, gamma, delta sorry, this is gamma 1, gamma 2 and this is a beta proton, this is a gamma proton and this is 2 gamma protons, so this is a beta proton and this is a alpha proton. So, this is something which

we looked at and as I said this is a peak pattern which is characteristic for each amino acid. So, now let us see how TOCSY can be used for spin system assignment.

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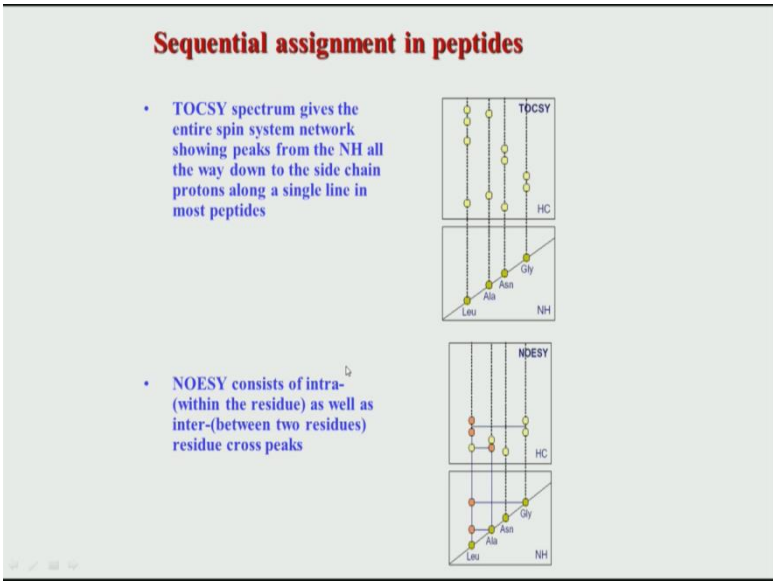

So, this is what is shown here is a TOCSY cut out from a just schematic diagram, this is not from the peptide which we are going to look at. So, what as it says here the TOCSY spectrum gives the entire spin system network showing peaks from the NH all the way up to the side chain protons along with single in along a single line? So you can see if you look at this here if you look at the single line here that means if I look at a TOCSY portion, this is a portion of the TOCSY spectrum, this is not the full TOCSY spectrum. So, for a given for a portion of the TOCSY spectrum you can see from NH this is alpha, this is beta, gamma, delta and so on for each amino acid, and this is what we will be using for a sequential assignments.

So, this is basically in the case of NOESY what happens is, we will see that later but in a NOESY we not only see the spin system along this line, but we will also see the previous the neighboring amino acid peaks also in this. So, right now let us not focus on this we will go ahead and come back to this at the later point.

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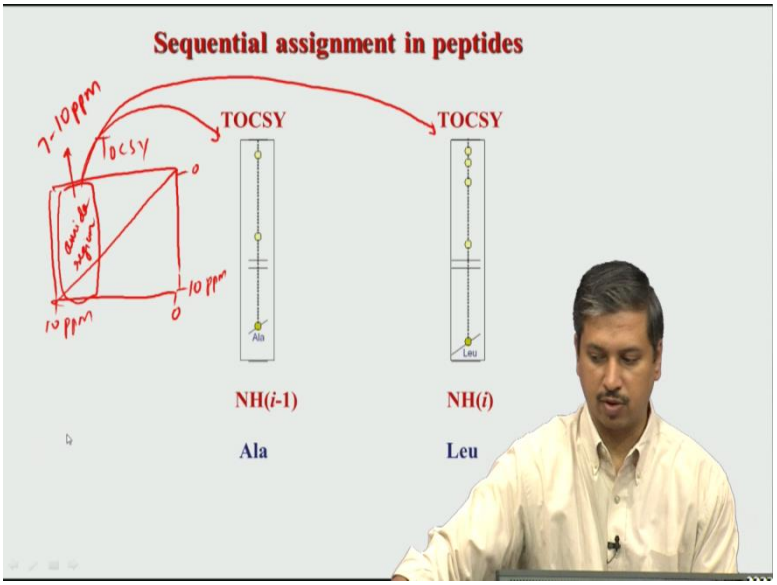

So, this is basically what how do we assign the sequential peaks. So, we can see here, for example, you have a TOCSY peak which is showing for alanine you have alpha and beta this is a protons and let us say you have a leucine, so now I am looking at a dipeptide okay. So, when you look at a dipeptide region of a amino acid what we are going to see? So, let us say that this is a NH of residue i and this is a NH of residue i - 1. So, let me again illustrative where we are actually focusing on here. So if you look, this is a TOCSY spectrum. Okay. So, this is diagonal. So, we are looking a TOCSY and this is between 0 to 10ppm and this is between 0 to 10ppm, okay. So, the amide region comes in this portion this is 7 to 10ppm so this is a amide region okay. So, what we are looking at in all these cases is basically this region, so we are looking at a particular strip.

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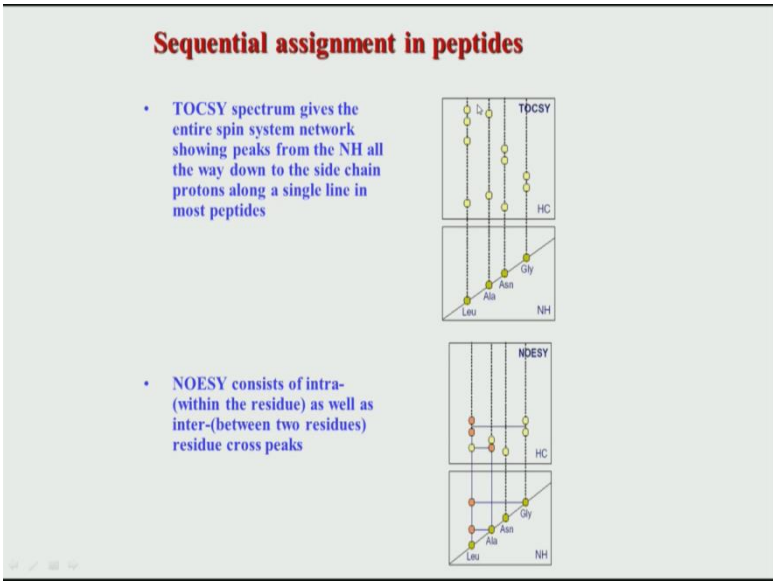

So, we are looking at particulars residue, so if we go back to the previous slide. So, if you see that in this previous is this slide. We see here we see this strip. Here we can see line of peaks belong into one amino acid and this is what we can analyst (())(06:49:01) strip, this strip because I can cut out a strip like this and that will correspond to one amino acid. So, in that particular region this is the TOCSY region. But again remember we are looking only at the amide region 7 to 10ppm and this is the region where all the peaks are coming in the amide portion.

So, if you look from amide region that is a NH the amide means NH. So, for a given residue let us say we have identified suppose a spin system and we know it is leucine's spin system okay. So, right now in this class we are going hypothetically to through schematic drawings we are not looking at real peptide. We will see the real peptide in next few slides and in the data from that actual peptide, but so right now it is all in the schematic mode. So, if you see if you look at an amide NH region of amino acid which is somewhere here in this region of the spectrum. So, this is a total TOCSY spectrum and this is amide region here and link particular one let us say one particular spin system we identify as leucine okay. And there is another spin system where we identify as alanine okay, so these are 2 different spin systems both of which are present in the TOCSY region okay.

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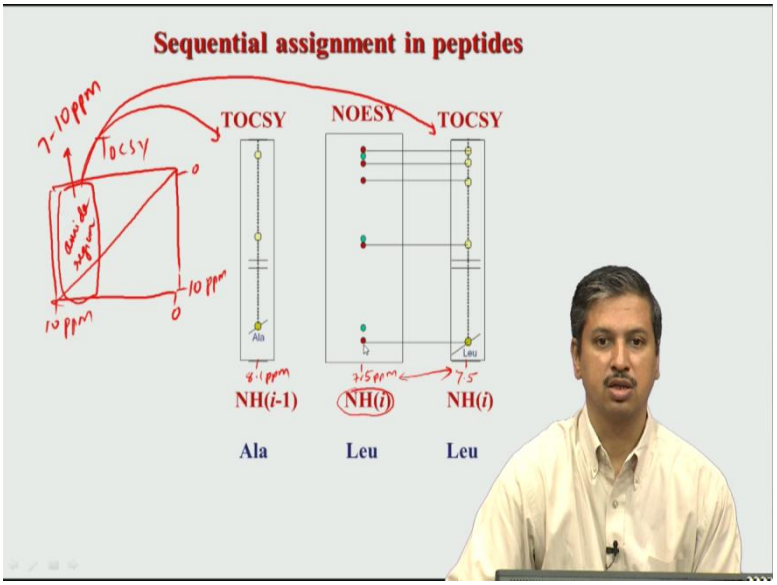

So, now we want to figure out which is coming after this we can see as per the as per the latter's written down text here NH is residue i and NH is residues i - 1that means alanine comes is coming before leucine okay, but how did we find that out? We can find it out from the NOESY. So, what will happen in a NOESY is that the 2 different spin systems are which are there in TOCSY that will be combine you will see in a NOESY okay. So, if you look at the NOSEY NH region, so if you look at the NH chemical shift of the leucine, the same chemical shifts you will see this is the leucine's spin system okay and there is this residue i which is NH value is somewhere let us say 7.1. So let me again write down so that is clear. So let us say that this one is 7.5 let us say (())(09:12) so let us say that this is 7.5ppm and let us say this is 8.1ppm okay, so this is the amide chemical shift.

Now, here this particular region is going to be we are looking at 7.5 okay, so this is same as the leucine okay. So, now in this particular region here when it is 7.5 that is the same chemical shift but this is coming from a TOCSY spectrum and this is coming from a NOESY spectrum. So in the NOESY, what you will notice is that you will notice the leucine chemical shift which are shown in the red color okay. So the leucine chemical shifts are coming in the red color in the same ppm value as in a TOCSY, and this is not surprising because this is nothing but the same amide chemical shift as the TOCSY, so TOCSY and NOESY are giving the same correlations. They always give the same correlations but additionally now if you see so this is what is shown by this line.

What you will see this green peaks here? They correspond to alanine chemical shifts. So this is the beta proton of alanine and this is alpha proton of alanine and also we are getting it

correlation to amide. So, what is happening here is that in a NOESY spectrum you not only see correlations of the TOCSY from the same residue where you are looking at. So for example, I am looking at this line which is 7.5ppm and the same line 7.5 ppm in TOCSY it will give me a spin system and therefore, that spin system is completely observe observed here means it is present in this line also, but additionally in this line we will also get the correlations from i - 1 okay. So, this is where the most important aspect of NOESY and TOCSY combination comes into play the idea here is that in TOCSY you see individual spin system that is individually amino acids, but in a NOESY you get a combination you get a dipeptide you get 2 amino acids together and why is this happening?

This is happening because the neighboring amino acid is not very far away from the amino acid which we are looking at. So, we are looking at residue i and i - 1 is not far away, it is within 5 armstrong. So if you draw this picture again here, so we are looking at dipeptide like this so we are looking at dipeptide like this, this is some side chain, this is amide, this is another side chain. So, what we are seeing is we are seeing a correlation from here to this residue in a NOESY and correlation from here to this residue in a NOESY. So, the NOESY gives both this correlations okay, this is what happens in a NOESY because this is what I written as 7.5ppm in this picture. So, I am getting correlations that is connectivity information from not only this amide this portion here to the self, self means to the residue i. So, I will call this i, I am also getting correlation to i - 1 in a NOESY spectrum, but not so in the TOCSY.

So, we have seen this in the previous class that in a TOCSY you get correlation only from the a self-amino acid that is only from it is own amino acid this is i, but you do not get i - 1 correlation which is present only in the NOESY spectrum okay. So, now we can see what is the advantage is that I am able to get a correlation between i and i - 1 between 2 amino acids, so this actually can be used for what is called as sequential assignment, so what is sequential assignment?

Sequential assignment is the process in which we establish a link between 2 neighboring amino acids. So, if I go from i I establish a link correlation to i - 1, now if I go to i - 1that is this NH here 8.1ppm and if I go to the NOESY of that so that is not shown here, but if I go to the NOESY of that residue then I will get correlation to i - 2. Similarly, if I go to i - 2 I will get correlation to i -3 and so on. Similarly, if I go to i + 1 which I do not know which is i +

1, any residue suppose there is residue i + 1 that should show a correlation to the residues of the spin system i so in the NOESY okay.

So, this is how we formed what is called a sequential link. So, we are essentially establishing connectivity from i to i - 1 to i - 2 and so on. Similarly, we are going in this direction to i + 1 will give in to i, i + 1 will give into i + 2. So, we start from an arbitrary i arbitrary which we do not know what residue it is, we will start from arbitrary i and then we move in both directions and basically that will give me the sequential assignment. So that is the goal of this whole exercise is that I am trying to establish sequential resonance assignments and that is done by which starting from an arbitrary spin system i which we do not know what residue it is, we can identify it based on the TOCSY that it could be a particular amino acid type and starting from I, then look for a correlation to i - 1 in the NOESY then I will also see in NOESY, which other spin system is giving correlation to i that should be then i + 1 because i + 1 will give it to i.

So, one thing you will see in this case in this in this discussion is that we are only unidirectional it is a one way path meaning I am only is looking at correlation from i to i - 1 that means in a NOESY spin system in a NOESY peak in a NOESY spectrum, for a given residue I, I will get correlation only to i - 1 and why is that so that we will see that later you can also get a feel from this picture here that this amide residue is closed within 5 armstrong for the NOESY to be observed in to their sequential i - 1, but if it has to see correlation to i + 1 that is little far away, it goes beyond this here because there is a amide bond here and then comes a side chain.

So typically, the i - 1 is the closest for the amide proton of a residue i but i + 1 is not so close so therefore, any spin system i in a NOESY will show correlation to mainly i - 1 spin system okay. So, if you want to see i you have go to i + 1 and i + 1 will give correlation to i and i + 2 will give to i + 1. So, you see we are going in the direction from i to i - 1. So, this is basically the point in the entire exercise when we use NOESY for sequence specific resonance assignment. So, let us see now what we will do is, we will only focus on this portion in the next few in the next slide and from there we will see how we can do the sequential assignment from by going from i to i - 1 to i - 2 and so on or i to i + 1, i + 2 and so on. So, that is basically our goal, our goal is to establish a link between the spin system i and other spin system in the molecule in a peptide.

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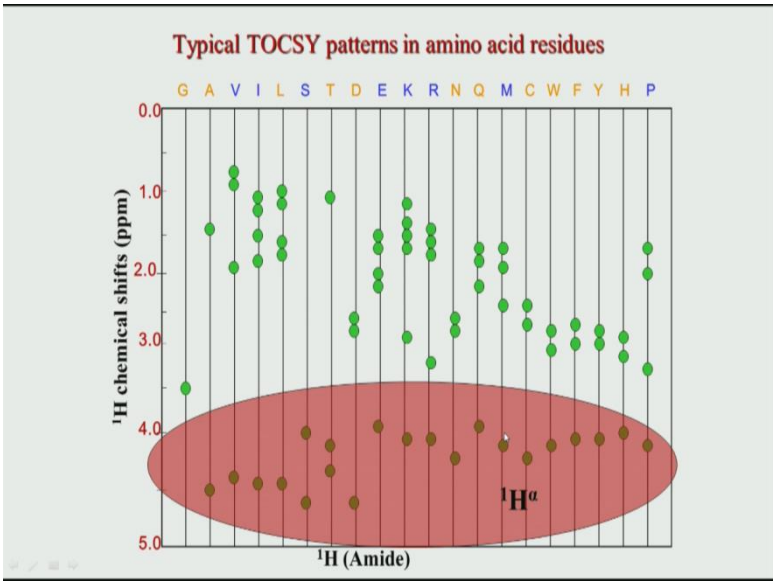

So, this is what I was showing that we are going to look at only the alpha region okay. The idea is that this alpha region will be used for establishing the connectivity, this means i and i + 1 or i and i - 1 at any point and then once you identify the connectivity, then we will go to the side chain here to identify in amino acid it is, so let us do this exercise.

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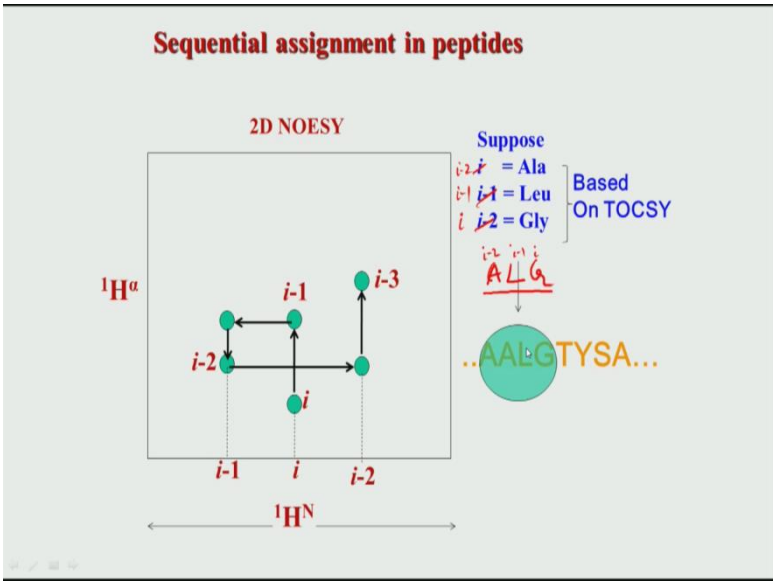

So, this is what we shown again as a schematic, the real example as I said we will take it up subsequently. So, this is a 2D NOESY spectrum and if you again you should keep careful attention to the point that we are looking at very small region of the whole spectrum, this is not the full spectrum, the full spectrum will go from 0 to 10ppm on both the axis, but here we have only looking at amide region which is 7 to 10ppm and we are looking only at the alpha

region H alpha and if you recollect from the previous slide it comes somewhere between 3.5 to 5ppm, so this region what you are seeing on the y-axis is actually only from 3.5 to 5ppm.

So, now what we will do in a ROESY taking a NOESY or it can be a ROESY, it does not matter both experiment give remember the same correlations. So I will take a ROESY or NOESY spectrum and then I will zoom into a particular spin system I, and remember again this is an arbitrary and this looking at some peak in the ROESY spectrum and I will call that as i and this is in the H alpha region. Now from the TOCSY, I will know the spin system which is corresponding to i but in the NOESY I will only look at the correlation it is giving to another peak.

So, in a ROESY spectrum it will give me a correlation to i - 1, then i - 1 if I go more further, it should now come correspond to some this is H alpha of i - 1. So, when I actually look at self-chemical shift of i - 1 which is here, then in that place not only this i - 1 will be there this peak, but it will now show correlation to i - 2 because I started from a residue i which is arbitrary and I found it is i - 1 because in a NOSEY I get both the correlations.

Now, I searched along this line horizontal line and so where this comes and I found that it comes here and that vertical line now corresponds to i - 1 because it is the self means its own and this was it was sequential. So, when the self of i - 1 is found, there if I look I may get another peak which will now belong to i - 2. So, remember this peak can come either down or up that is not that is not important, what the important see important here is that this along the horizontal line you go, you find the peak corresponding to i - 1, then we go along the vertical line and you get i - 2.

Now, similar X if I continue this I should go along this line then I will get somewhere where this two peaks coincide and that means that peak here this line vertical line should belong to i - 2 because i - 2 will give correlation to i - 2 self that is called it is own and it will give correlation to sequential, which is now i - 3. So, we can see here what we are doing essentially you are staring from it a self means some residue i which is it a self-chemical shift it is own chemical shift moving along the vertical line finding in this line within this regions.

So, remember we are only focusing on this region and this region is called the H alpha region. So, within the H alpha region I go up I find i - 1 which is the sequential, then I go along the horizontal then I see where this i - 1 comes in this spectrum and I notice that it

come somewhere here in this vertical in line and that vertical line now becomes i - 1 NH means the amide of i - 1, so again remember we are looking at the amide portion.

Once I find that i - 1 NH, again I search either up or down to see if I see another peak and if I get another peak that peak should not belong to i - 2 because in a NOESY i gives only to i - 1, i - 1 gives you only to i - 2 and so on. So, now once I got i - 2, I will go a horizontal along this line find an another spin system where this is present and that spin system now should belong to i - 2 and it is sequential will come as i - 3.

So, this is the manner in which we carry out sequential assignment, we are essentially linking again remember the word link where linking or forming a sequential correlation between a residue i and its sequential i - 1. So, now let us come to this place here so suppose when I started arbitrarily from the residue i or peak I, from the TOCSY as I said you will know the spin system and suppose that spin system belongs to a residue alanine, it means suppose it is an alanine spin system and let us say i - 1 which is along this line vertical line is let us say suppose it is another spin system leucine and i - 2 let us say is glycine.

So, what we have done now based on this TOCSY we have identified the spin system corresponding to i, i - 1, i - 2 and we have also formed a link between them using the NOESY spectrum. So, what we have to do now is search in our sequence of amino acid, if there is such a system if there is such string of amino acid; this is our peptide sequence which is shown here. So, suppose this is our peptide sequence, now I will search for a residue which is i should be Ala, L should be i - 1 hence that means it should be GLA spin system.

So there is a typo here, so let me correct this so, this is basically this will be so I will call other way round. So let me say that this i is glycine, i - 1 is leucine and i - 2 is alanine. See it does not matter because this just schematic right now so we are only looking for a tripeptide because we have formed the tripeptide connection here. So, that means I am looking at a system i is glycine, so alanine, leucine, glycine, so this is i - 2, this is i - 1 and this is i. So what I have done here is based on the TOCSY I have identified a tripeptide TOCSY and NOESY, Identified a tripeptide which is ALG and now I am trying to look for this tripeptide in my peptide sequence okay. So, the peptide sequence you can see I can see that there is ALG here and which is I got from my linking of the residues. So, this is how we say that we have assigned the spin system. So, we can say now that we have assigned these tripeptides ALG okay.

So, remember one thing is that yes ALG each of them is unique amino acid. So, you may have to still establish a link between them, only then you can say that I have completed the assignments without establishing a link between amino acids like we do based on NOESY and TOCSY, we cannot say that we have than the sequence specific resonance assignment.

So, sequence specific resonance assignment now if you recollect it is based, there are 2 things we have to achieve in that, number one we have to do spin system identification means from the TOCSY we have to identify all the amino acids, what amino acid is belong to and from the NOESY we have to establish a sequential link. So let us take an example, real example of a peptide and with data and then go through this exercise again with real experimental spectrum.

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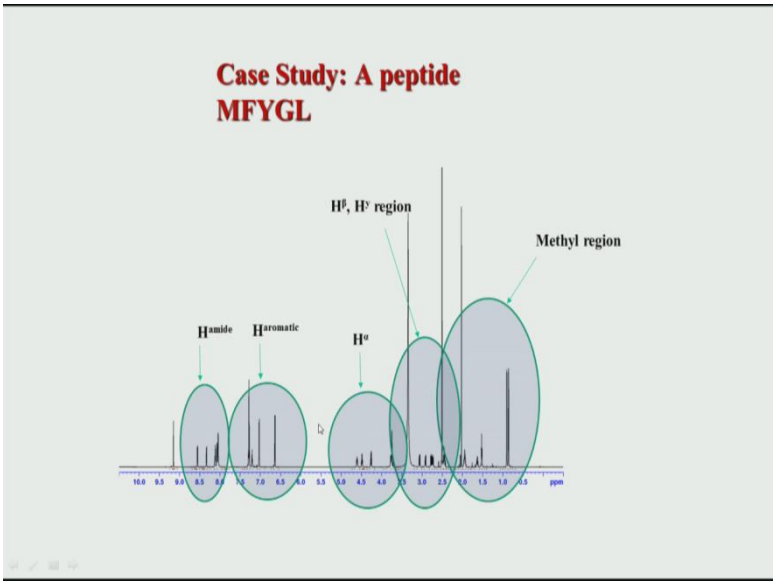

So this is a peptide, which we are going to look at and how we can assign using the approach which we have learnt just now. So, this is the 1D spectrum of this MFYGL peptides M means methionine, phenyl alanine, tyrosine, glycine and leucine. So, now looking at these 1D spectrum it is not possible to assign because there are lot of peaks and which are overlapped and the multiply structure is not clearly visible so we will basically have to use the 2D approaches that we have learnt in this class. So, let us look at the different regions of this 1D spectrum. So, this is called the methyl region because it is all the peaks belonging to the methyl group will come between 0 to 1ppm, 1.5 to 2ppm. So, what are the methyl amino acids here?

It is basically leucine and there is a methyl group in methionine so those are the peaks which will appear here. Then the next region is called as H alpha, H beta, H gamma, this is the methylene region, in the methylene region you get CH2 peaks and that comes typically between 2.2 to 3.5. Then comes the H alpha that is the backbone H alpha region of all the amino acids and they are invariably always between 3.5 to 5ppm depending on in the secondary structure, so this is going to be very important region which we will focus for sequential assignments which we saw. And this is the aromatic region which comes between 7 to 7.5 and this is similar to what you see in the organic compounds. We saw that we also get aromatic peaks in this region and then comes the amide region.

So, amide region is basically the backbone CONH amide and as well as side chain amide. Side chain amides are present in some amino acid such as asparagine, glutamine they are amides and side chain amins or also there in amino acids such as Glycine. So, we are now going to focus on how we can use these two regions. So, essentially the H amide and H alpha region will be useful for us. So, let us go through the different 2D spectrum for this peptide.

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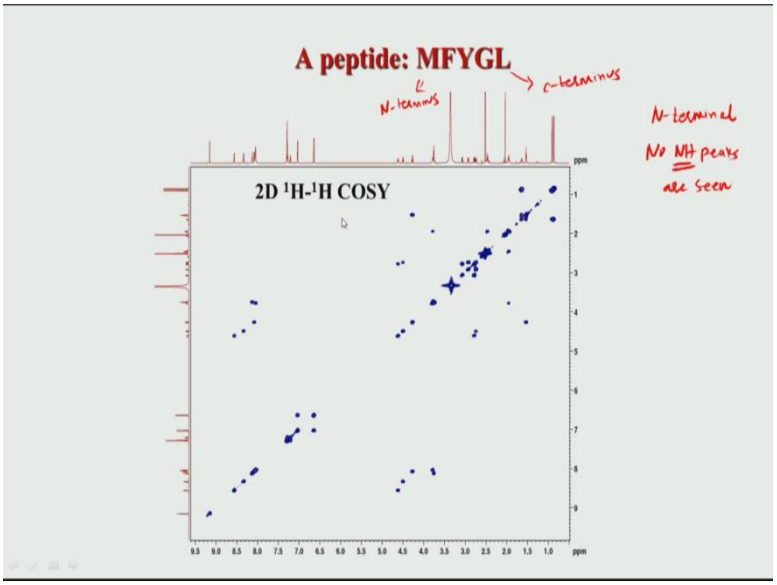

So, the first experiment you would record is the 2D proton- proton COESY and as we have seen this experiment basically correlates two protons which are close to each other. So, in this peptide we will call this side as N ter- and this side as C ter-, this is typically the nomenclature or notation which is used in peptide assignment or protein assignments.

Now, one thing one should keep in mind is that when we have peptide dissolve in water, in this particular example we have taken it in DMSO, but if you have water sample dissolved in

water or D2O what happens is the N terminal the NH3 +, so this residue will not have a peptide bond. It is peptide bonded to this through the carboxylate group but the N-terminal NH of this is free and that is NH2 or NH3 + and that exchanges very rapidly with the solvent and because of that we will not get any peak for NH peak for this amino acid. This is exchange part we will see that in the next part of the course, when we go to the advance topics but this is typically one has to keep in mind, but when it comes to the DMSO solvent, DMSO is A-protic and there we do not need to worry about this problem.

So, now we will in the next class we will basically see how these correlations that is NH alpha correlations can be used for sequential assignments as we have seen in case of a schematic drawing.