Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules
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Lecture – 20
Protein Backbone resonance assignment and side chain resonance assignment

So, in the last class we looked at the 3D experiment HN CO CA CB towards the end. Let us look at that again this will be the last 3D triple resonance experiment that we will look at in this course. And let us see what information it gave and how it was complementary to HN CACB.

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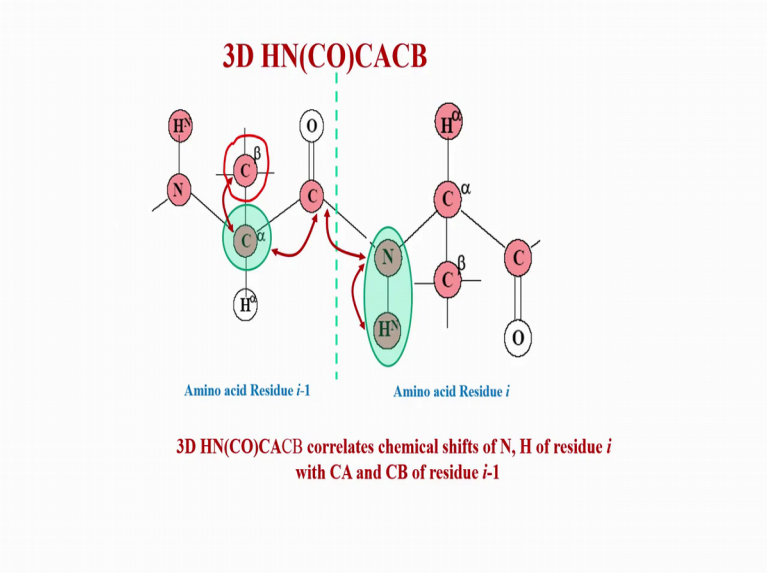

So, this is shown here; you can see in across 2 peptide I mean die peptide amino acid i and i minus 1 H N C O CACB correlates the chemical shift of N N H N of 1 amino acid. We can all it as i to this previous amino acid i minus 1 the C A and C B of those 2 amino acids of that amino acid. So, we saw that it goes through C O, but actually it does not collect the chemical shift of C O. So, it is using it as a relay station it is using it to go from one nucleus to another nucleus, but it is actually not using it for any chemical shift information. So, that is why you put this in the bracket and this is the nomenclature we have been seen and using worth triple resonance experiment.

So, this is what is written here the HN CO CACB experiment correlates the chemical shift of N and H amino acids as atoms of residue i with the C A and C B of i minus 1. So

now, question is how is it complementary to the HN CO CACB which we saw in the previous class.

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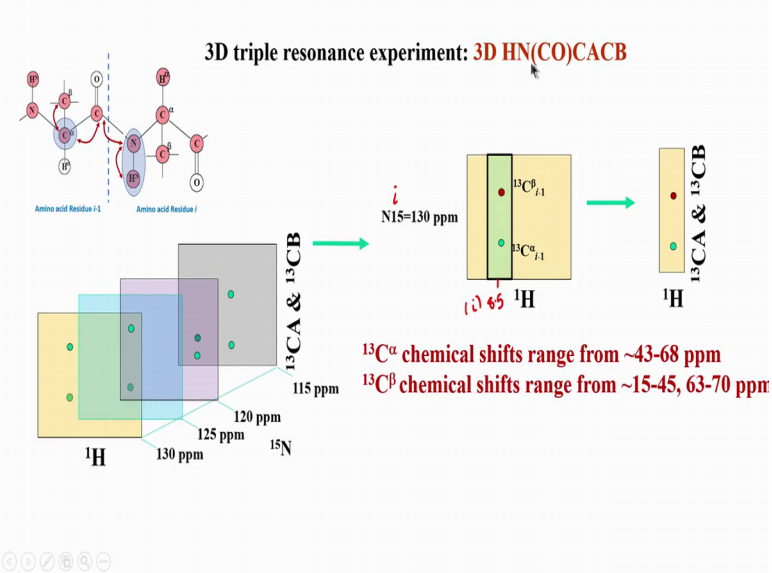

So, HN CO CACB and HN CO CACB their complement in the following manner. So, this is what we saw again in the last class that if you have a 3D HN CO CACB you will get 2 peaks for every proton nitrogen pair. That means, for every amino acid you will get 2 peaks one corresponding to alpha peak alpha carbon of i minus 1 and one will correspond to the beta carbon of i minus 1. And as I said similar to HN CO CACB the phase that is a sign of the peak in HN CO CACB is opposite for alpha and beta peaks. So, this is something very good for us.

So, given 2 peaks you will immediately know which is beta and which is alpha based on the colour of the peak, the colour will be opposite in phase. Of course, we can also use our chemical shift judgment to also figure out because, remember I mentioned that C alpha comes somewhere between 43 or 44 to 63 ppm 65 ppm. Whereas, C beta comes from 13 15 ppm to 45 and then again starts from 63 or 60 ppm and goes up to 70 ppm

So, this is what is written here. So, there is as distinct range of C beta and there is slight overlap, but not much. So, we can also use chemical shift based information to distinguish a C alpha from C beta, but colour of the peak definitely helps makes it more easier. So now, combining this experiment with HN CO CACB; what kind of information we get?

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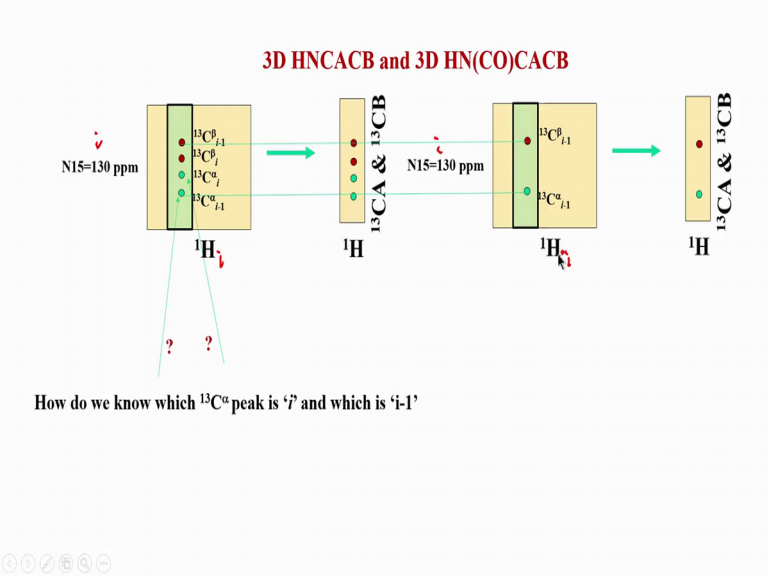

So, this is shown here. So, we had we pose this question in HN CO CACB part that if you have 4 peaks in HN CO CACB remember we get 4 peaks per amino acid and each amino acid H i N i pair. For that we will see this i minus 1 alpha C i alpha similarly i beta and i minus 1 beta. But, between i and i minus 1 alpha or between i and i minus 1 beta there is no way to tell which peak corresponds to i residue and which peak corresponds to the i minus 1th residue. So, remember i we call it as a self-amino acid self-residue and i minus 1 is called as sequential. So, we will use this nomenclature in the second part of the course where we talk about protein assignments.

So, one should bear this in mind that self and sequential basically referred to i and i minus 1. So now, if I compare this with i H N C O CACB here, I will be able to distinguish which is i and i minus 1. Because, HN CO CACB will exclusively give me peaks to i minus 1 of alpha as well as i minus 1 of beta. So, this is what we saw in the last part in the last lecture and this is how we are use these two experiments to help in our assignment process which we will come in the subsequently in this course. So, this basically kind of brings us to the end of HN CO CACB.

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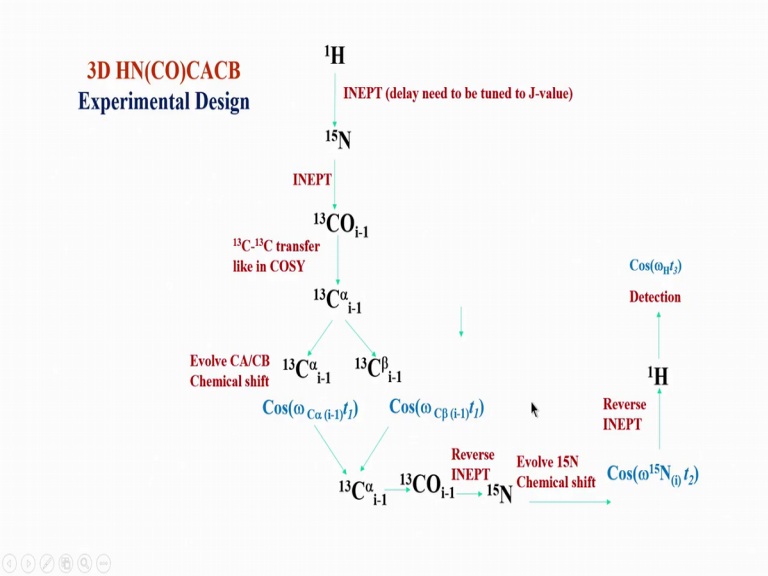

So, let us look at the experimental design before we move on to the side chain assignments. So, this is how it starts you start from amide proton, you go to N 15 by INEPT again based which has to be delay INEPT delay which is based on the this coupling. In you not only that this INEPT in general is to be tuned everywhere according to the coupling involved. So, here also you will need to worry about the coupling constant J coupling between 15 and i minus 1 which is typically about 15 hertz this coupling. So, your INEPT delay will be 1 over 2 times 15 which we saw comes to about 25 to 30 milliseconds. From i minus 1 you go to next step the C alpha i minus 1 which is simple C 13 C 13 transfer because, remember both are carbon 13.

So, there is a like COSY transfer between the two. Once you come to this nucleus you excide this nucleus and then further transfer it into two parts and this is the called the branching out. The flow of branching the flow of magnetization which kind of resembles river which I showed you in one of the previous classes. So, here when you branch out you use a CC transfer again and part of the magnetization is retained on itself and part of heat is transferred to C beta of the same amino acids. So, we are on i minus 1 now; once you transfer to this you can then collect their chemical shift by evolving them ok.

So, that period evolution is called of elaboration is called t 1. Once you have evolve the chemical shift you need to go back again to this original C alpha from where you started. From here to C alpha C O minus i minus 1 which we have we came here and from there

to nitrogen back and all the way back to amide protons. So, nitrogen chemical shift is evolved during the back transfer during the transfer when it is going back you do that and after that you go back to amide proton and then detect that amide proton which is t 3.

So, that is how we correlate or connect the chemical shift of amide proton and nitrogen and C alpha C beta of i minus 1 on in this case. So, this is how basically all the triple resonance experiment work essentially you are looking at starting from an amide proton going to N 15 going to C O minus 1 and so on. And what is advantage of this kind of experiment? Advantages are that you actually start from a proton and you again go back to proton. So, if you recall the discussion on detection sensitivity; we discuss that is most sensitive part experiment would be where you start from proton and you detect proton.

So, this satisfies that criteria. The second helpful the reason for this is that suppose you deutrate your protein. So, this is something which we will see in the in the next part, but I can show you this picture here. See if I deuterated this protein, what does deuterated means; deuterated means you take this proton here this every all the non-exchangeable proton means not this proton and not the amide proton. Take all the non-amide protons H alpha, H beta here, H gamma so on. If you take all those protons and replace with deuteron; how do we do that is a different story we will look at it when we come to isotope labeling. But, let us say we have deuterated then we can still do this experiment because we still have this proton left.

So, how is this left out because this is called an exchangeable proton and it does not go to deuterium as soon as you put it in water, the deuterium which is present here will get exchange to water depending on the excessive water is in excess it will remain as H 2 O sorry H; if it is deuterium is in excess in the solvent it will become D. So, depend on that this population of this will depend on the population of protons and that will become protonated. And once it is protonated you can do HN CA HN CO CA and all the experiments which we discussed here; they would not be affected by whether the protein is deuterated or not. In fact, it is good to have deuteration for larger protein this experiment become even more sensitive.

So, sensitivity of the experiment now increases if you do deuteration of the remaining proton the background protons; background meaning the side chain protons and this proton. So therefore, this kind of experiments triple resonance amide detected, it is

proton detected experiments amide detected are very useful, because, we start from amide and go back to amide. We also use the word out and back something which I have not mentioned in this 3D part out meaning you go out from proton and back meaning you come back to proton. So, this kind of experiments are also called out and back triple resonance and NMR not only this, I mentioned it referring to all the triple resonance 3D experiments.

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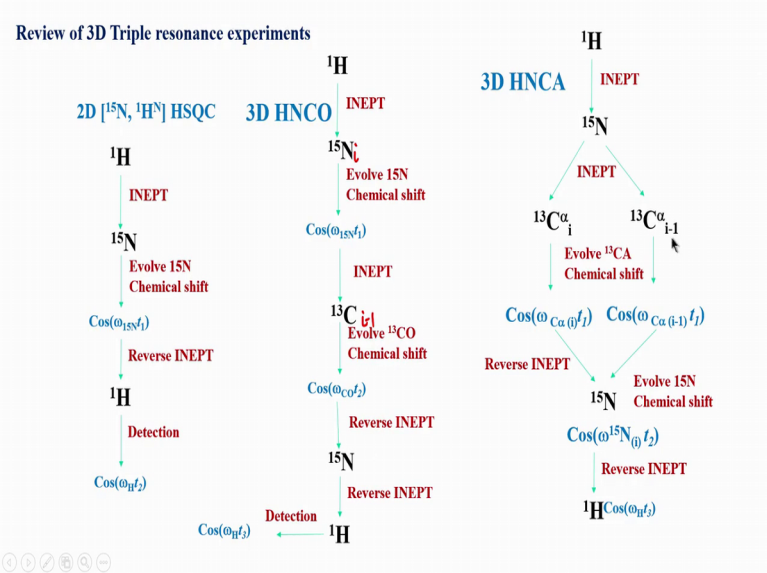

So, let us now review the experiments that we have seen up to now; we have seen quite a few experiments the let us go with them through one by one again because, this will be the end of this part of the course. And so, we should just look at it again then so, that we refreshing for us. So, let us start from the most basic experiment which is not a 3D it is a 2D experiment, but if you recollect or if you see now we are basically what we are doing is we are going to take this basic experiment N 15 HSQC and keep adding to this for 3D. So, 3D comes basically from here only; you take this basic setup and keep expanding this setup to generate different 3D.

So, we will see now how that comes about. So, in a HSQC N 15 this is something we have seen several times, you start from a amide proton. This is in protein, you apply INEPT, go to nitrogen, evolve the nitrogen chemical shift, come back to the proton, INEPT again that is reverse INEPT and detect that proton chemical shift direct detection

and that becomes a t 2. So, t 1 t 2 or f 1 f 2 or omega 1 omega 2 for the two dimensions along which we got the peak or correlations.

So, this is a basic 2D setup this is how we do; now you will see how 3D comes actually from here. So, if you look at the next basic the most basic 3D H N C O experiment; we started like the same as HSQC you start from proton go to nitrogen, you can evolve if you can evolve here or down it does not matter. So, we evolved similar to HSQC then we added 1 INEPT step which was not here, but are simply adding a INEPT step I could go to the next nucleus which is C O; C O of the i minus 1 remember.

So, this is residue i with this is i minus 1 that is how we did and we got now an option or opportunity to collect the chemical shift of carbonyl and then we go back to the proton and detect this is similar to here. So, you can see that all we have done compared to HSQC is we have added an extra element or transfer from nitrogen to carbonyl evolution of carbonyl back to nitrogen. So, this was the extra part which was not there in this part so, but by simple addition of these extra part we could now generate a 3D H N C O.

So, you can see these experiments are basically expansions or basically building on the existing experiments ok. So, they are not completely different from what you can say, if you understand HSQC you can very easily understand H N C O. Now, one more the next experiment we saw was HNCA there was a slight complication again similar to HSQC; we start from proton go to nitrogen there we branched out into two parts. Because, J coupling from here to here is same as a J coupling it is not same similar as J coupling from here to here.

So, we were able to branch out into two parts again INEPT was used for this transfer then we evolve the chemical shift of C A. Now, both chemical shifts got evolved that is alpha of i and i minus 1, then we came back to nitrogen evolve the nitrogen shift and we did a reverse INEPT and back to amide proton. So, you can see again this is an expansion of this portion. So, from N 15 we branched out to 2 and came back to nitrogen, so, this part is extra compared to what you see in HSQC.

So, the can see again is very simple compared to H N C HSQC we have essentially just added an extra element. And so, by adding these kind of modules these are called transfer modules an INEPT CC transfer so, on we can build complicated 3D experiments. So, this

was 3D HNCA then we saw that you get 2 chemical shifts here. So, we are not able to distinguish which peak corresponds to i and which peak corresponds to i minus 1.

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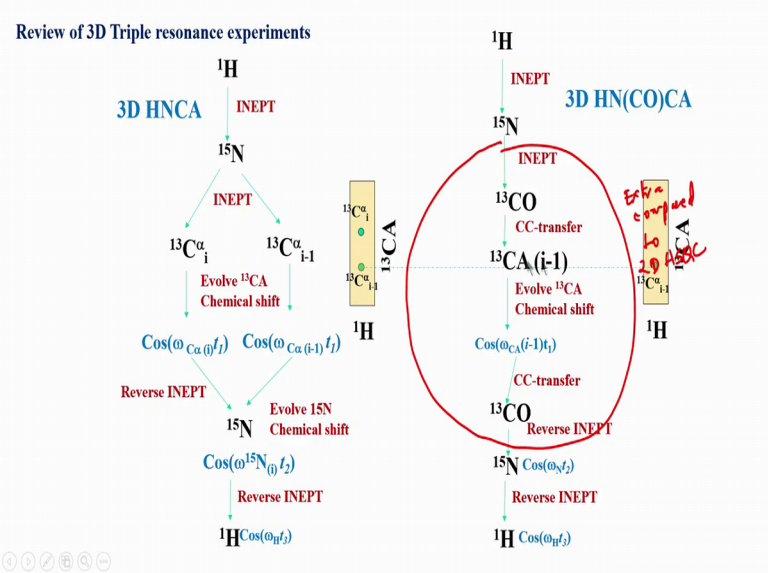

Therefore, we came up with another experiment which is 3D HN CO CA. So, HN CO CA if you compare let us compare these two now. So, if you look at this experiment we start from amide proton, we are going to nitrogen and from nitrogen we go to C O; now this is the step which is not here. So, here it directly went to C alpha, but here I am going through C O to C alpha. So, this was a relay concept that is a train going from one station to another without stopping in somewhere. So, that thoroughness is involved here; we are going through the carbonyl and this helps us to go exclusively to i minus 1. We do not go to i whereas, here there is no exclusivity we cannot go only here or here.

Of course, there are special experiments published in literature; if you scan through the papers you will see there are experiment which can selectively get this side or can give you this side. This side is of course, H N C O C A there are also experiments you can only give this information this pathway. But, for a regular HNCA we do not do that in regular HNCA we get both the peaks and those peaks here we are able to now get exclusively only i minus 1. And then we go back to C O then nitrogen and so, again compared to HSQC you can see what you have added extra is this part. So, this is extra compared to HSQC.

So, you can see that 2D experiment again can be converted into any 3D by adding extra steps in the sequence. So, this gave us exclusively this peak and because, of that I can use this spectrum and this spectrum together and distinguish the correlation for this and from this. So, therefore, we need these two together; you cannot record only HNCA and expect to analyze the data without recording this. So, these two experiments have to be recorded together in NMR. So, that is one thing and as I said the peak pattern is like this you get basically 2 peaks here and you can see nicely this i minus 1 matches with this i minus 1. And therefore, you can assign from here which 2 is i minus 1 which is i. So, that is distinction is basically coming from these two combination. So, these are the two experiments we saw.

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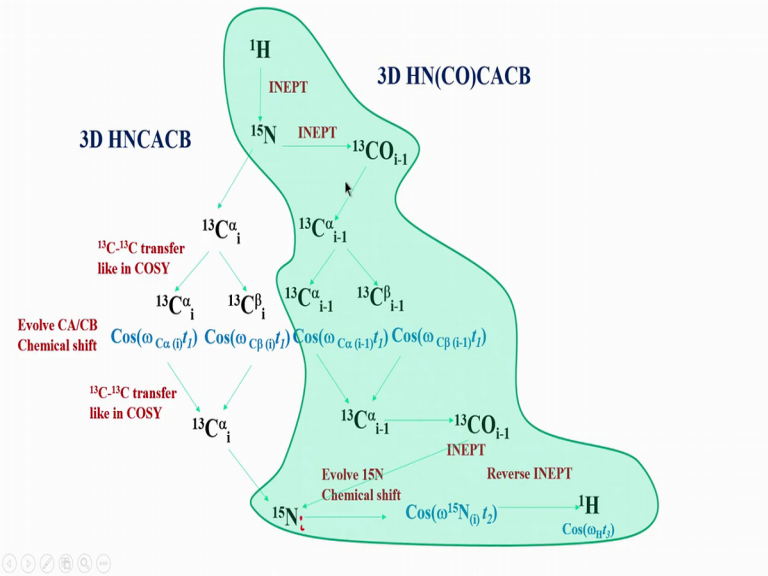

Then the next two experiments were HN CO CACB and HN CO CACB. So, let us review that now. So, you can see in HN CO CACB now was little more complicated in HNCA. In H N CA we went up to here and then we came back to nitrogen here, but now here what we do in HN CO CACB we branch out again one more time. So, here there was already have a branch out, but we further branch out here into i C alpha and C beta C alpha and C beta, but one is i minus 1 other is i.

So now, we get 4 chemical shift correlations in 3D HN CO CACB for a given amide pair. So, remember we always start from 1 amide. So, this is an amide for 1 amino acid and

for that amino acid you are now getting 4 peaks because, this amide again is finally what we detect. So, this are HN CO CACB concept.

Now, again because we have 4 peaks we do not know which peak is which, but fortunately the C beta is opposite in phase compared to C alpha. So, there is one way to distinguish C beta from looking at just the phase of the peak or color of the peak. But, this and this you cannot distinguish you would not know which is i; which is i minus 1; similarly you would not know which is beta i and i minus 1.

So, then we need to therefore, take this other experiment HN CO CACB. So, if you look at HN CO CACB it is basically this pathway the one of the pathways. But, except that there is a difference here we go from N 15 not directly here like we did in HNCA part HN CO CACB part, but you have to go via through C C O i minus 1. And this is something which is extra step which is introduced so, that we get exclusively i minus 1. Similarly, you go here then again you have to come back; you have to come back from i minus 1 through C O you cannot directly go here.

Because, if you directly try to come here you will also end up going to the nitrogen of i minus 1 remember this is i here. So, i let me write down this is i ok. So, if you are going if you cannot directly go from here to here that is from C alpha i minus 1 to i because that will make it difficult because, there is a coupling also to N i minus 1. So therefore, you have to go out only through C O to get this exclusiveness and that is done through CC transfer here and INEPT; similarly here there is INEPT and CC. So, it is a reverse of what we did here and you come back to the amide proton and detect. So, HN CO CACB now gives us exclusively the HN and C alpha C beta correlations of i minus 1 only.

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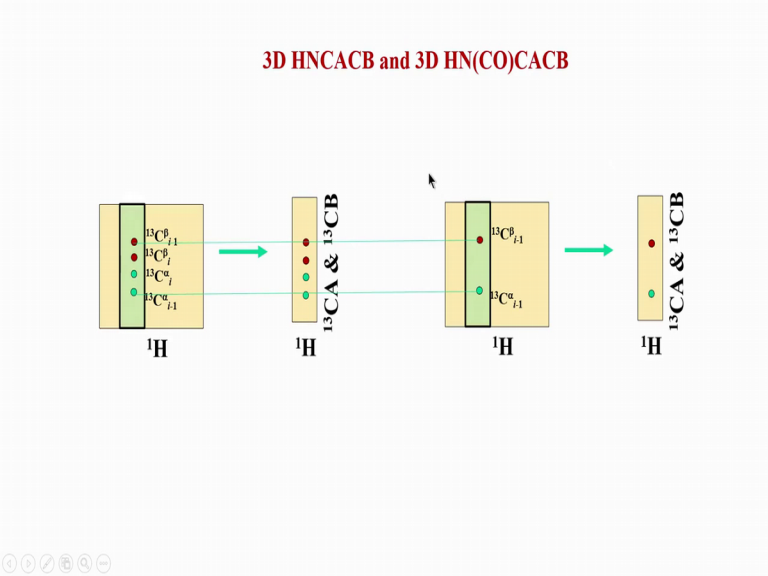

So, combining these two we are able to look the get the full information that is what is shown here. That you have 4 peaks for a given amide proton in HN CO CACB, but by matching it with a HN CO CACB for the same amide chemical shift; we can distinguish which is i and which is i minus 1. So, this is very useful so therefore, these two experiments also have to be recorded together. You cannot record this alone and this alone separately, you have to record these together for any assignment process.

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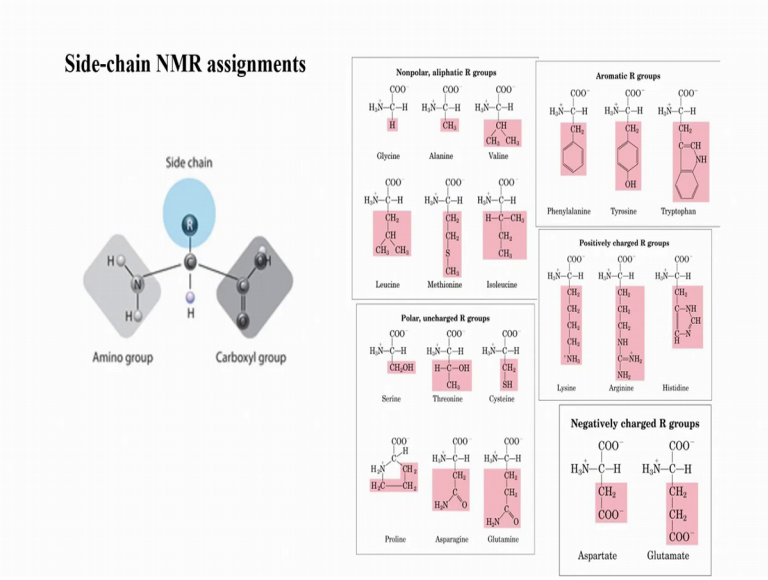

So, basically this brings us to the end of the backbone triple resonance NMR experiment. So, all these experiment HNCA HN CO CA etcetera work for backbone assignments. But, what is left now is how do we do experiments 3D experiments for side chain NMR assignments. So, we will very briefly look at one experiment and then we will come back to that in the second half of the course where we look at how to assign the side chains.

So, what is the side chain in amino acid all of us know this is the backbone structure C alpha is this amide proton. This will be involved in a peptide bond for with amino acid this side, this will be involved in a peptide bond amino acid this side. So, this is carbonyl and C beta and C gamma etcetera come under this side chain category. So, here you can see here this is standard taken from a website textbook, you can see the different side chains of different amino acids.

So, you can see they are really different each one has a very different structure one typically in NMR, when you are looking at biomolecules it is necessary to memorize the structure of the 20 amino acids. Because, if you memorize then it is very easy to understand what is alpha, beta, gamma and so on. So, if I say the beta proton of threonine or beta carbon of tryptophan you should be immediately able to picturize that molecule in your mind and accordingly look at the spectrum. So, I would strongly recommend that you actually memorize or learn this by heart what are the different structures of different amino protein amino acids.

So, you can see this is always alpha which is the backbone here then comes beta gamma delta 1 delta 2 and so on; for leucine here it will be beta gamma delta is not there epsilon like that. So, this are the different side chain. So, we are only look for assignment for backbone up to now how do we assign the side chain. So, for that the first popular experiments is called H CCH COSY.

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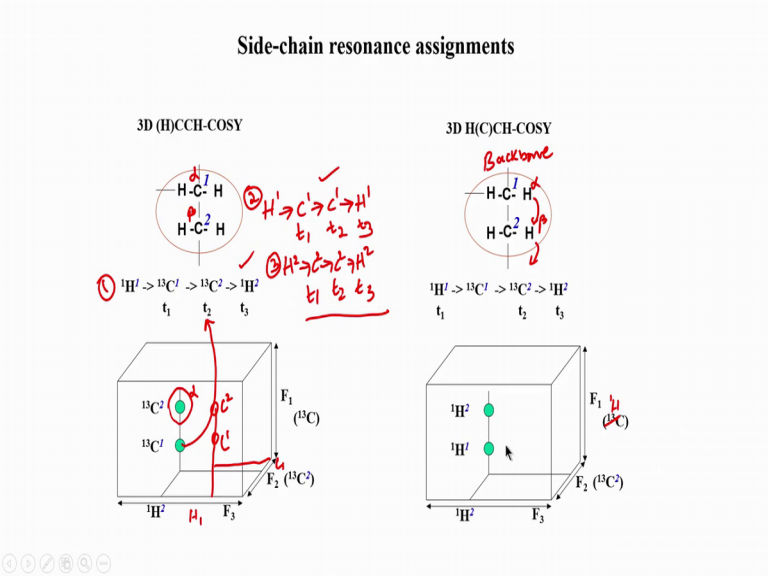

There are many different experiments again available in literature. In this course we will use this experiment for side chain assignment of proteins. So, here you can see how does this work, it works as follows. So, let us say this is a side chain of an amino acid ok. So, this could be beta gamma or gamma delta so on. So, in H CCH COSY as a name stands we correlate 2 neighboring carbons. So, gives please keep that in mind that we key correlate chemical shifts of 2 neighboring carbons ok. So now, how do you do that? So, this is what is shown so, we are put it in brackets here. Again this bracket means we are not going to get the chemical shift of this proton, but we need this proton we are going to use this proton to go from here to here.

So, let us say we start from any of these protons. So, consider this proton where the arrow is pointing, you go to the neighboring carbon that is directly attached carbon C 1 which is shown here. From C 1 you put a CC transfer COSY and go to CC that is why it is called COSY. COSY means you go to nearest neighboring carbon that is the directly at this curve; how do you do that there is a CC transfer. So, similar to what we saw in the triple resonance experiments. Now, once you come here you go to the proton which is attached to that carbon so, that becomes this H 2. So, how do we get the chemical shift?

So, you evolve this chemical shift first. So, after you transfer from here to here you evolve this chemical shift of this carbon. Then you transfer to the next carbon evolve that carbon again second carbon, then go to the third last to this proton and evolve that this is

nothing, but the direct dimension this is a final detection dimension. So, that is what is shown here that is the direct detection dimension. Now, you see the this is the peak pattern you would expect if you look at this kind of a cube. So, this is the 3D cube of a HCCH COSY here you can see that this axis is carbon, this is also a carbon be why because, there are 2 carbon chemical shift periods.

So therefore, two dimensions will be carbon and the third dimension will become the proton which is a direct dimension. So, this is the approach in HCCH COSY. So now, for a given H and carbon for a given these two pair here I will get the information of C 1 because, it is these two are now connected to this pro carbon. Now, one thing will happen is that this carbon to this carbon this is C 1 does not always transfer 100 percent to this ok. So, it remains some of the magnetization will remain on C 1 and that which remains on C 1 goes back to H 1 because this pathway now for example, let me write this here.

So, one pathways this, the second pathway is H 1 going to C 1 it remains on C 1; that means, during t 1 and t 2 the same carbon is evolved then it comes back to H 1 during t 3. So, this is pathway number 1, this is pathway number 2. So, both of these are present in the HCCH COSY. So, you can see here if you look at this peak here this is correlation is C 1 C 1 H 2 C 2. So, this is C 1 H 2 C 2 that is this combination. So, this peak is basically this pathway, but if you look at this peak here it is C 2 C 2 H 2.

So, we can say there is one more pathway here H 2 going to C 2, going to C 2, going to H 2, so, this is t 3 this is t 2 this is t 1. So, you can see this combination here will give me C 2 C 2 H 2 correlations and that is basically this C 2 C 2 because, in this axis also it is C 2 in this axis also it is C 2 and in this axis it is H 2. So, this pathway is what basically corresponds to this peak. So, similarly so what we are doing here we are going from H 2 to C 2 to C 1 to H 1 or H 1 to C 1 to C 2 both ways, so, both sides you will get.

So, you will looking at H 2 here if you look at H 1 somewhere here you will get again 2 peaks. So, if I look at now H 1 here, if I put H 1 here I will again get 2 peaks, the same 2 peaks will come. This will be C 1 and this will be C 2 and this peak here were correspond to C 1 ok. So, like that you for H 1 also we will get a correlation similar to H 2. So, this is basically the transpose concept this is kind of a diagonal peak and transpose peak. So, we can see that this is how see this is a diagonal peak it is a anti diagonals, but this is a diagonal and this two are the cross peaks.

So, this is how HCCH COSY helps us to assign the side chain because, if I know the chemical shift of this carbon suppose this is alpha. So, let us say this is an alpha carbon this is an alpha carbon. So, from alpha I can get beta correlation. So, from beta I can go to gamma and from gamma I can go to delta and so on. So, we will revisit this experiment again when we look at the other real assignment for a protein. Now, let us look at another version of this experiment is nothing, but the same experiment all we have done is now look at this here carefully. What we have done is evolution is now on proton rather than carbon. So, here if you look at here the evolution was on carbon.

But not on proton so, we had put proton in the brackets. Now, the carbon the second carbonate that is this carbon the transfer is put in brackets. Because, that is now not evolved you see there is no evolution of chemical shift here instead the evolution has come to this proton. So, this proton this carbon this carbon, so, this is HCCH the same experiment, but what is happening will look at this here instead of getting carbon chemical shifts I am getting proton in this axis. So, this actually axis is wrong this should be proton; it is a proton carbon axis here. Because, this proton this proton and this these are the 3 dimensions of the cube and the correlation depends on which are frequency labeled.

So, frequency labeling proton carbon , so, H C H now depending on which is 1 which is 2 you will get those correlation. So, similar to this exactly similar to this I will have another pathway. So, in that pathway I will get H 1 C 1 H 1 because this t 1 will shift to H 1 in this case. Similarly in the second part where here H 2 t 2 H 2 the H 2 will the labeled not C 2 now. So, this t 1 will move to H 2 then I will again get H 2 C 2 H 2 combination which is this H 2 C 2 H 2.

So, you can see that by simple transfer of magnetization we are able to correlate 3 chemical shifts and that is helpful because, if I know the assignment of this proton for example, then from here I can get the assignment of this proton. From here I can go further down suppose this is the side chain this side is backbone. So, let me write that suppose this is the backbone and suppose this is H alpha; now I could go to H beta assignment then I can go further like this to H gamma and so on. So, essentially we start from an known chemical shift which is the backbone or C alpha and from there we go to side chain. So, that is how with the side assignments are carried out.

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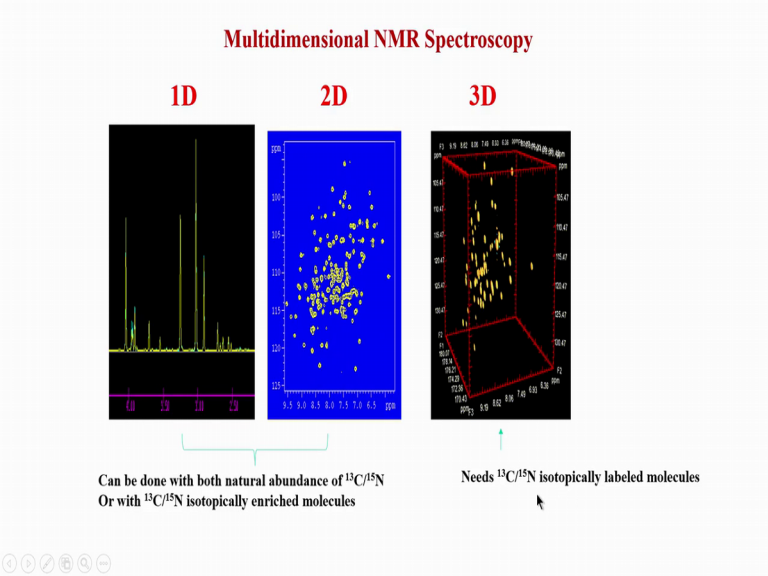

So, this is the 3D experiments that we I mean the generally the pictured you will form. So, the we have seen in this first half so, we come to the end of the first half of the course. So, this is a 1D we started with, we looked at the 2D HSQC spectrum and other different experiments. This is how a 3D will look like you can see just like a 3 dimensions peaks are dispersed in the 3D.

One thing is important to understand here is that you this two experiments 1D and 2D can be done with natural abundance, means I do not need to label the protein. I can do the standard amino a protein without any labeling isotope labeling, but a 3D needs labeling. So, this is something we have been telling in the beginning and now I repeat this that you need to labeled because, with natural abundance to do a 3D will take a long time. So, there for normally we never do 3D without labeling the protein; we will see how to label the protein.

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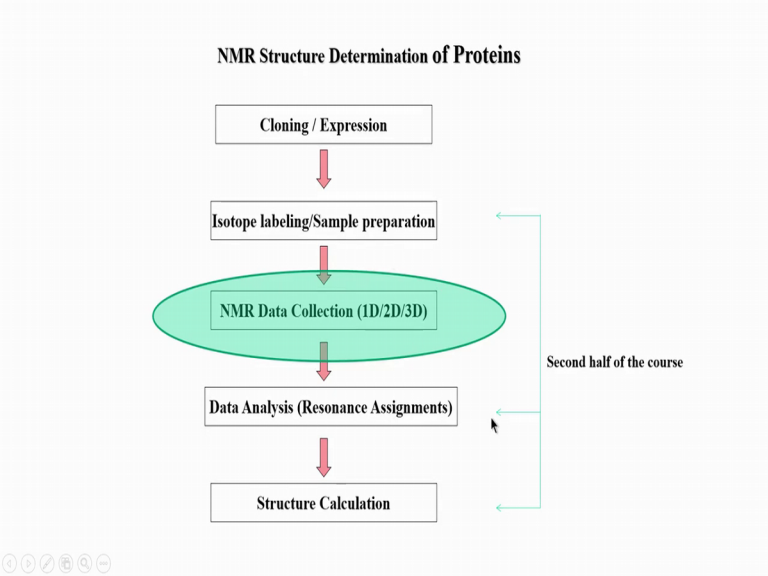

So, this is the how where the whatever we have learnt up to now fits in the overall picture. So, this is the to typical flowchart which is used for the structure determination of protein. So, you start from protein expression or cloning well this is the biological step experimental step, we will see that. Then you will do label the protein, then you collect NMR data and analyze the data and do the structure calculation.

So, this is what we have seen up to now we have only covered the data collection theory part. So, the second half of the course we will now begin with what is isotope labeling, how do we assign the protein, then how do we calculate the structure and once we have the structure etcetera how do we study its interaction with ligands and so on. So, this brings us to the end of this first part of the course. Now, we begin from the next class with isotope labeling and other things in proteins.