Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules
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Lecture – 19
3D HNCACB and 3D HN(CO)CACB

In the last class we looked at this experiment 3D HNCA we were looking at that. We saw the peak pattern; we looked at see how the magnetization is actually divided from nitrogen into two pathways; one goes through the C alpha i another goes through C alpha i minus 1.

Now because of that we get the chemical shifts two types of chemical shift correlations; one is from between the nitrogen amide proton of associative i with residue i minus 1 C alpha, and the chemical shift of nitrogen and proton of i is correlated with the same residue i C alpha chemical shift.

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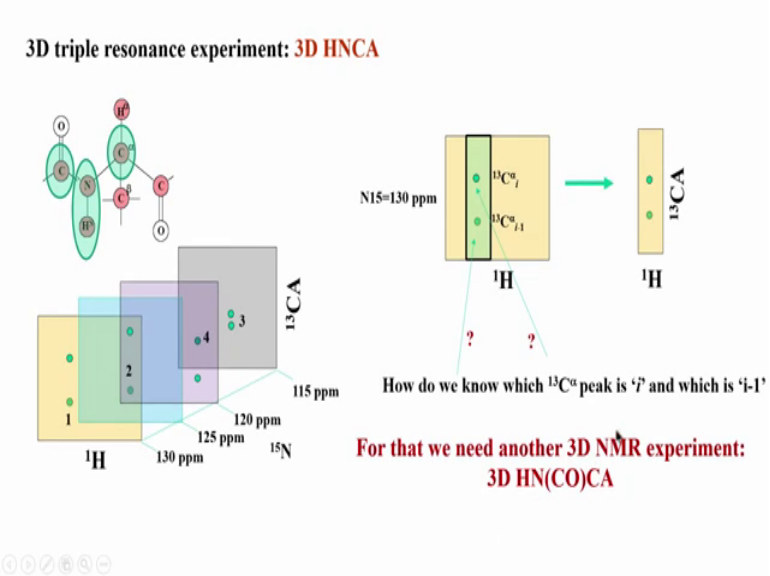

So, this is shown here again. So, this if you look at this particular slice which is taken at 130 ppm, we can see there are two chemical shifts for a given amide proton and a nitrogen chemical shift value.

So, this amide proton could be somewhere 8.5 or 7.5 and that and this together correspond to one amino acid which is shown here as i, but along this dimension C alpha

here we are getting two peaks. So, the question which comes to our mind is how do we know which is i and which is i minus 1 C alpha? This is what is shown here and that is something which is not easy to know directly looking at the spectrum.

So, we therefore, need to somehow distinguish or discriminate the chemical shift of i and i minus 1, we should know which is i and which corresponds to i minus 1. So, for that we need another 3D NMR experiment 3D HN CO CA. So, we will look at that experiment now. So, as I said there are about 7, 6 or 7 important NMR triple resonance experiment, which we will be looking at this in this course. So, the third experiment now the triple resonance we are going to look at is 3D HN CO CA.

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So, what is the advantage of 3D HN CO CA let us have a look how does it what information do we get.

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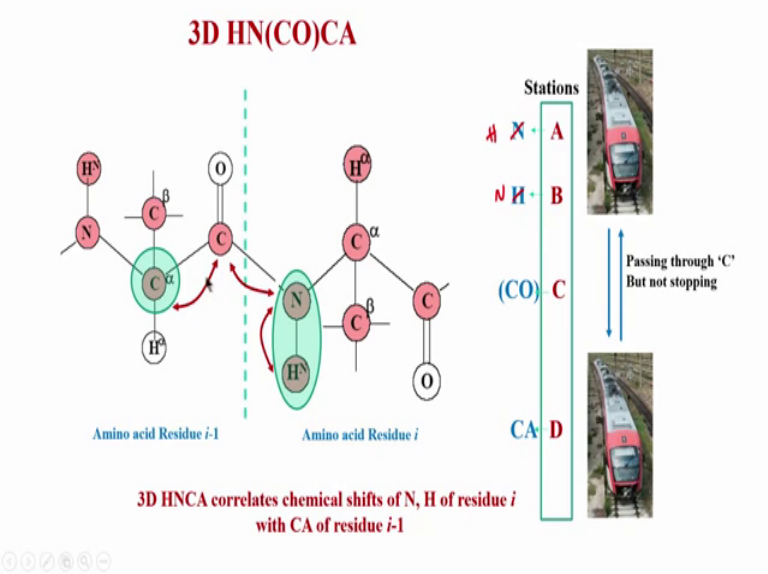

So, in a 3D HN CO CA we correlate the chemical shift of nitrogen amide proton of one residue i to its neighbour i minus 1 C alpha chemical shift. So, you can see this is exactly complementary to what we saw in the HNCA case. In HNCA we correlated this with this to this and this ok, but in this experiment HN CO CA we are exclusively going only to i minus 1, we are not going to self and how is that getting done? And that is getting done or that is being carried out by exploiting this coupling values.

So, if you see carefully here this structure that is NCOCA it does not exist here, it is NCACO in this side is N COCA. So, very interestingly this is not the same as this part. So, if i transfer the magnetization from N to CO the magnetization will flow only to CO only here it will go only this direction, it will not go this direction because this co coupling is very weak. So, N to CO as I mentioned cannot be transferred like this because it is on less than a 1 about a 41 hertz where as this is 15 hertz. So, that is pretty strong and I can tune my inept delay to go exclusively or selectively to this side. Now once I go here, I can further go to the next atom by simply using this delay or this coupling which is 55 hertz.

So, you have to keep this coupling values in mind and this is about 55 hertz. So, if I have another transfer, I can actually exclusively go to this part. So, by doing that I am actually flowing the magnetization from HN of a residue i to N of residue i to C alpha of i minus 1. So, to just remind you again this is complementary to what we saw in HNCA where

we got both these chemical shift correlated to these two, but here we are correlating only the C alpha of i minus 1 to this pair NH ok.

So, let us see how can we think of this as analogy. So, the question is why this CO is in a bracket? You see here it is not shown a separately it is in a bracket. So, this is analogy I normally give is imagine that you have a train which passes through different stations, but let us say it does not stop at this station C, it passes through C, but not stopping. So, it goes via C while coming forward going forward and backward again it goes through C, but it does not stop.

So, we can now correlate this picture with this here how by correlating or thinking of A station as a nitrogen B sorry A as amide let me change this is H and this as nitrogen ok. So, I go from proton this proton I go to nitrogen then I go to carbonyl. What you see I am not stopping here I will not do anything to this nucleus I will not evolve this nucleus, but I will transfer to C alpha. And C alpha will be evolved then while coming back again I will come here, but I will not do anything to this nucleus, I will just simply come back using inept I will come back using inept, but I will not evolve this.

So, when I do not evolve this chemical shift I have no information of the chemical shift of this. So, therefore, we put that in brackets. So, I am using that CO as a relay, as a relay station, I am going through that, but I am not actually stopping there. Magnetization from nitrogen is flowing is going through CO, but actually it is only going because it wants to go further to C alpha and it does not want to stop there ok.

So, this is the analogy you can think of. So, any experiment in which you use a nucleus to as a stopping as a not a stopping, but as a relay nucleus so, that you can go from one side to another side that is put it in brackets and that is not evolved in chemical shift ok. So, this experiment is actually a 3D because it is correlating the chemical shift of amide proton with amide nitrogen with CA. So, CO does not have any chemical shift value in this spectrum ok. So, 3D HNCA correlates of C A i and i minus 1 whereas, 3D HN CO correlates only to i minus 1 ok.

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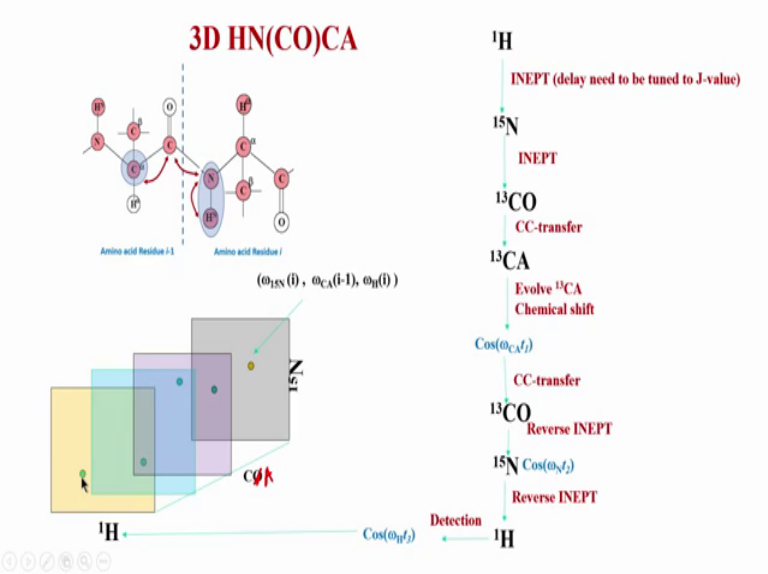

So, let us see how this experiment is designed. So, this picture is shown here again the magnetization flow. The magnetization flow happens from amide proton to nitrogen, nitrogen to CO of i minus 1 now and then C alpha. So, basically you can think of it as crossing the border.

So, this has a border, a dotted line is a border of amino acid i and i minus 1. So, the magnetization actually crosses the border goes to the previous carbonyl and then further goes to the next station C alpha, takes the chemical shift value of this and comes back. So, this is what is shown here in this picture here on the diagram on the right. So, we will look at that now. So, you start again from proton, amide, excite the amide proton which is as I told you is always done where using inept.

We transfer it to nitrogen according to tuning it appropriate J value then from nitrogen, we use inept to go to CO. So, if you recollect this part actually is similar to HNCO. HNCO we did the same thing. We went from proton to amide, amide proton to nitrogen with inept and there from nitrogen again we went to CO for inept, but now there we evolve the CO chemical shift, but here see we do not evolve the CO chemical shift, we directly go to the next atom by a simple C C transfer module and this some module is nothing but like a cosy transfer. Like an cosy in proton proton, they transfer by just J decoupling because these are homonuclear remember this is both are carbon 13. So, this a homonuclear C C transfer and that depends on the coupling value. So, we have to tune

it to the coupling of these two nuclei and the coupling constant for these two if you remember is 55 hertz. So, 1 over 2 times 55 hertz if I use, I can go from CO to C A then we evolve the C A chemical shift. So, we are now capturing the CA chemical shift and that is captured as cosine or sine omega CA t 1 and then you go back all the way.

So, this is now the job is done. Now you have to go back to the original proton. So, for that going back again you go back first to CO by C C transfer here, it is exactly the same now. It is going to be the reverse then from CO to N you go by reverse inept, and a nitrogen once you come you can now capture the chemical shift of nitrogen because we need chemical shift of nitrogen that is evolved during this period.

And then you go to proton by reverse inept and finally, detect the carbon and hydrogen chemical shift and that is your direct dimension. So, now, we can see the three chemical shifts what we have got here; we have correlated or we have connected the chemical shift of amide proton to nitrogen to C alpha of i minus 1 that is important to remember. Now if you look at the peak pattern how what kind of peak pattern do you get? You see this is similar to what we saw in the case of HNCO, but here now this axis.

I am sorry this is a mistake here it should be CA because we are not evolved the C alpha chemic CO chemical shift, CA is what was evolved. So, this is CA dimension ok. So, for every slice here I am getting only one peak and what is that chemical shift? That is a CA of i minus 1 for a given nitrogen.

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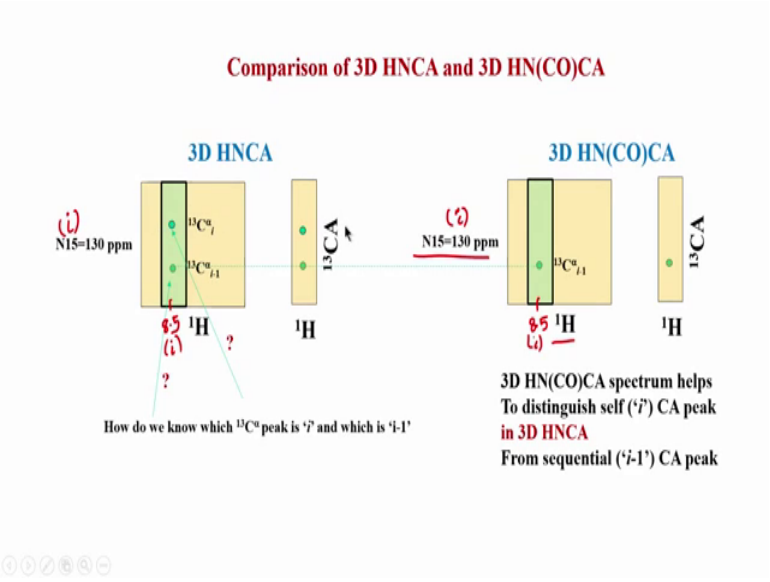

So, let us go further and see. So, look at this again HNCA if you look at this HNCA spectrum, we saw that we get two peaks in the C A dimension for a given amide proton and a nitrogen combination.

So, this is one amino acid. For each amino acid we will get two peaks; one corresponds to i and i minus 1 and we also noted that we do not know which is i which is i minus 1 that information was not given is not available from the spectrum. So, therefore, we did this experiment HN CO C A which helps to distinguish at the self, self means i residue i here from i minus 1 which is called sequential. How does it do that? Look at this here. So, you can see this is the 3D HNCO C A spectrum which is what I showed in the previous slide.

We are taken a slice of that for the same combination. You see this combination should be same as this combination; that means, if this is 8.5. So, let me write down this here suppose this is 8.5 this should also be the same 8.5. So, as a same location of this and this I am able I am getting now only one peak. So, now, if I compare this strip, this strip with this strip I can now match the two strip and you see I can see the line here, you can see the line here. This now tells me that this corresponds to i minus 1. Because this experiment this spectrum exclusively gives me only i minus 1 whereas, this spectrum gives me both i and i minus 1.

So, by comparing these two in a computer, I will be able to distinguish which is i minus 1 which is i. So, in this case it turns out this peak is i minus 1 because for the same strip same combination of these two values the same amino acid i, I am getting now this as i minus 1. So, remember again let me write it more explicitly this is a residue i, this is residue i, this is residue i and this is also i. For a given residue i, I am getting now i minus 1 in this case and i and i minus 1 in this case.

So, one has to therefore, understand this carefully that the combination of HNCA and HNCO CA helps me to distinguish a self peak from a sequential peak and this is very useful for us when we go to assignments of proteins in the second half of this course. So, I would suggest to read this carefully understand this carefully using the different textbooks that was recommended.

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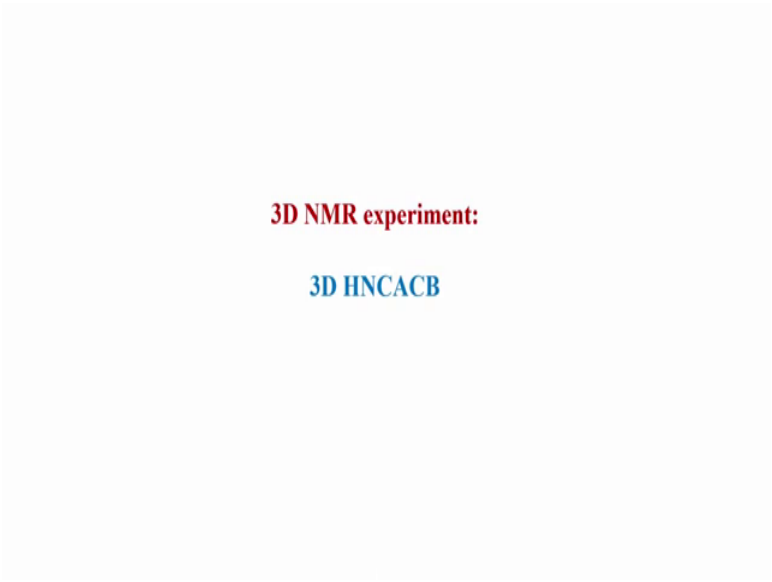

So, let us move on to the next experiment which is 3D HNCACB; this is a very (Refer Time: 13:42) experiment for assignments and it will build on our knowledge from HNCA.

So, we have seen what is an HNCA experiment. So, as the name suggest we are going one step further and we are going to correlate C beta and C alpha to N and H. So, all there is nothing in brackets here if you notice carefully, remember if we have something in brackets then it becomes that nucleus is not evolved in chemical shift, but here we are not here we are evolving all of them. So, this is a HNCA CB experiment.

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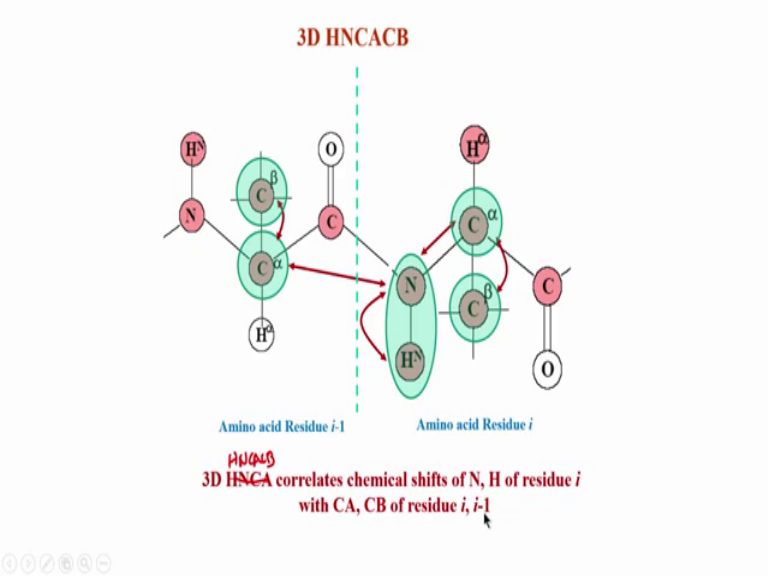

Let us see how this works. So, this is how it is done. So, you have a nitrogen amide and amide proton and a nitrogen. So, similar to HNCA, we transfer the magnetization here. From here it flows in the both direction like we saw in the like analogy to also a river; the river splits into two pathways. So, here the magnetization flow like a river, splits into two pathways. One goes to C alpha which is the same amino acid i. So, this is going from i to i and this is going from i to i minus 1 directly. And from here in HNCA CB we go one step further we take it to C beta and we also take it to C beta here ok.

So, this is additional step compared to what we saw in the HNCA case. In HNCA we did not go to C beta we stopped here and then we came back to nitrogen, but here we will go one more step extra so, that we get this chemical shift also in addition to this chemical shift. So, we are getting more information in HNCA CB compared to a 3D HNCA. So, it is useful experiment because we get this very import C beta chemical shift also information for a 2 amino acids neighbouring amino acids ok.

So, let us see how this experiment is constructed. So, this is what I should write a CACB. So, this is HNCA CB correlates chemical shifts of NH of residue i with C alpha and C beta of residue i and i minus 1 both ok.

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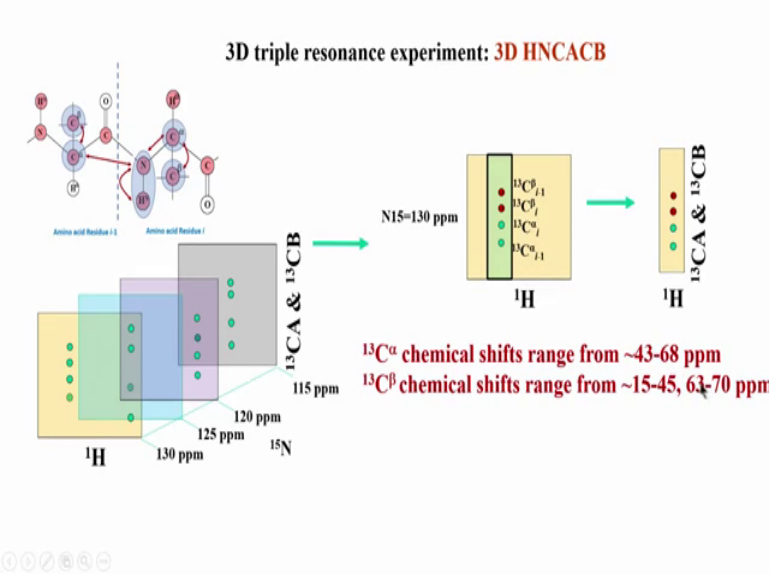

So, this is how the spectrum now will look; this looks slightly complicated compared to what we saw. So, now, you can see here this axis is both C alpha and C beta because we have detecting both of them in the one of the dimension.

So, earlier we have restricted only to C alpha. So, if you recollect, we C alpha chemical shift range was given as 43 to 68 ppm, but C beta has a longer range we will see that shortly. So, how does the peak pattern look? So, what you see now look at this here; for every slice for every strip or every peak for a given amide proton and nitrogen for example, if I look at 130 and suppose this is 8.5 which I shown in the last slide you can see that corresponds to one amino acids.

So, for every amino acid i you will get four peaks in this axis and this 4 peaks correspond to C alpha i minus 1 C alpha i C beta i C beta i minus 1. Why we are getting four? Because two are from here if you look at this picture this side two are from the i and two are from i minus 1 ok. So, four together now one good thing in HNCA CB is the colour the peak pattern the face of the peak, the colour in the spectrum real spectrum will be the C beta will have a opposite face mean if I make this as positive they will appear as negative in the intensity.

Or I can say this is as red colour if you display in blue colour it will display me in a different colour. So, this is just a display thing, but the point is that the C alpha chemical peaks corresponding to C alpha of both i and i minus 1 have one particular face or one

particular intensity but this will be opposite negative. If you call this as positive this will be negative if you make this as negative this will became positive. So, C alpha and C beta therefore, can be distinguished, but the problem of distinguishing C alpha of i and i minus 1 still remains, because these are coming in the same colour same face that i cannot distinguish that we will see how to do that shortly.

Similarly, here C beta of i and i minus 1 they have the same colour, I cannot distinguish between these two, but there are ways to do that which I will show you, but in general total number of peaks now coming for a given strip there are 4. So, what is the chemical shift range here? What is the values we are looking at? So, that is given here. So, far a C alpha the we saw already this is 43 to 68 and C beta now is a bigger range, it comes in two parts two zones what if 15 to 45.

So, we can say this starts from 15 goes up to 45 and then between 45 to 63 normally you do not get any C beta very rarely you will see and that now is C alpha region more or less. Slight overlap is there between C alpha because some isoleucine violin proline C alpha has come below 63 they overlap with the C beta of some other amino acid such as serine and threonine. So, this zone 63 to 70 is typically for serine and threonine these two amino acids where as all the other amino acids come between 15 to 45 ok.

So, now this although they overlap slightly here, there is some kind of a overlap, but still the colour is let us say the phase of C beta is opposite compared to C alpha. So, anyway you can distinguish till which is C beta which is C alpha even if they come in the same region, but more or less you see there is a regions exclusively for C alpha and exclusively for C beta.

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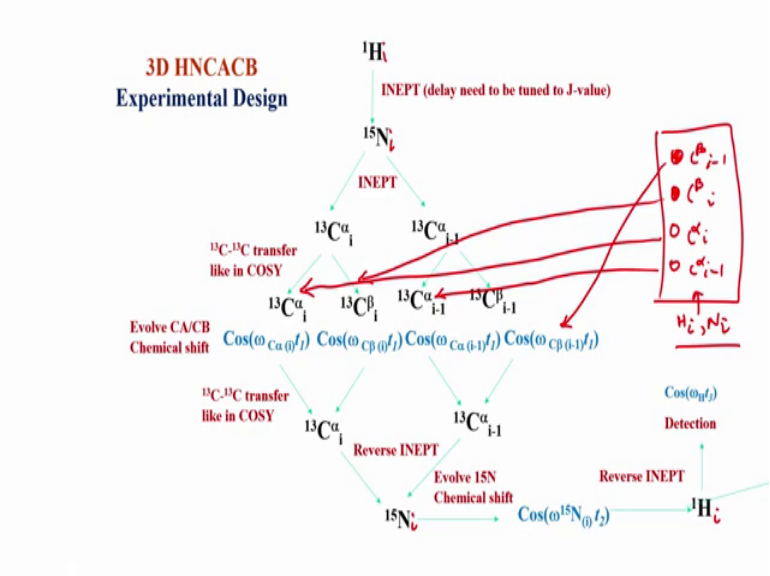

So, now this experiment is built like this.

So, this was I said is a very complicated experiment, but idea is simple this is if you have understood how an HNCA HNCO CA and HNCO these three experiments work you will understand this as well. So, this is more closer to HNCA. So, in a HNCA we saw that you start from an amide proton, you go to nitrogen 15 the same amino acid i with a inept then we saw that the magnetization splits into two pathways. So, same thing happens here it splits into 2 and the two are one is i and i minus 1. Now here further what we do is we add a C C transfer step like in a standard cosy homonuclear cosy.

So, why do why do you do that? Because you can go to C beta now the next amino atom, but you see as I showed you in cosy in the 2 D NMR part, we can never go 100 percent to we can try to go if uses specific delay value, but we normally use a delay value such that it goes it remains on itself this and something relay is 50 percent goes to C beta. So, about that 30 to 40 to 50 percent goes here and remaining 40 to 50 percent or 60 percent remains here ok.

So, that depends on the delay period what we use, but normally we use a delay period such that there is a 50 50 transfer. Similarly, the i minus 1 use the same concept it will also go to it will keep some magnetization in on its own here like this and some 50 percent will go to C beta of that amino acid i minus 1. So, now, at the end of this step we will have four atoms sitting in the or excited by the pulse and they will be all in the x y

plane after this transfer. So, now, we x i once they are excited we can evolve those chemical shift for these four and each are independent. So, each of them will now independently evolved as different chemical shift ok.

So, these are this is one pathway going independently of the other pathway which is this path way. So, these are four different pathways flowing independently ok. So, you can see now here that now I have independently four chemical shift now I reverse the hole operation I go back to my original proton. So, how do I go back? I put the same transfer element transfer step what was put here. So, it goes back into c alpha.

So, whatever 50 percent came from here to here it goes back similarly what came from here goes back again its 50 percent of 50 percent we will not going to detail of that, but this goes back into C beta to C alpha. Similarly these two which have come originated are come from here they merge again back into the C alpha i minus 1 once these two have come we use another reverse inept to go to 15.

So, we are reversing this whole process here and then here we evolve the chemical shift of nitrogen because we need that value we need that chemical shift and once we evolve that goes as N 15 i t 2 remember we are starting we are considering an amino acid i. So, this is i. So, I can actually write here to be more clear this is i this is i ok.

So, from i we are splitting into i and i minus 1 and from i minus 1 we are coming back to i. So, this is again a residue i and then finally, we get the residue we go from proton sorry from nitrogen we use another reverse inept and we come to proton and that proton this is we come by simple inept concept and then that is what is detected finally, as a in the 3D ok. So, this is the final detection period and they are captured we capture the proton chemical shift. So, you see now we are correlating many chemical shifts. So, let us see this carefully

So, we are correlating one pathway. So, each pathway is one type of chemical shift correlation. So, let us start from this pathway here on the leftmost pathway that is which is flowing like this arrow is showing and in this pathway we correlate this chemical shift amide with nitrogen with C alpha.

So, this is one peak the second peak is this, this and this combination. Third peak is this combination this amide proton correlated with this nitrogen to C alpha minus 1 then

finally, C beta minus 1 and n i and h i. So, this is what I showed you as four different peaks each peak is basically one pathway.

So, I can say one this is see let us say if this is C beta i minus 1, these peak is coming from this pathway; if this is C beta i this is coming from this pathway and this is C alpha i it is coming from this pathway, and C alpha i minus 1 comes from this pathway. So, for a given H i N i combination, I am getting this four chemical shift values. So, this is how an HNCA CB works that for a given amino acid H i N i I am able to get four different chemical shifts.

So, this is a lot of information very useful for us for protein assignments as we will see later on. But, now the question remains is how do I distinguish all this. So, one thing is as I said the phase this phase will be different compared to the phase of the alpha. So, we just colour it differently ok. So, in this way.

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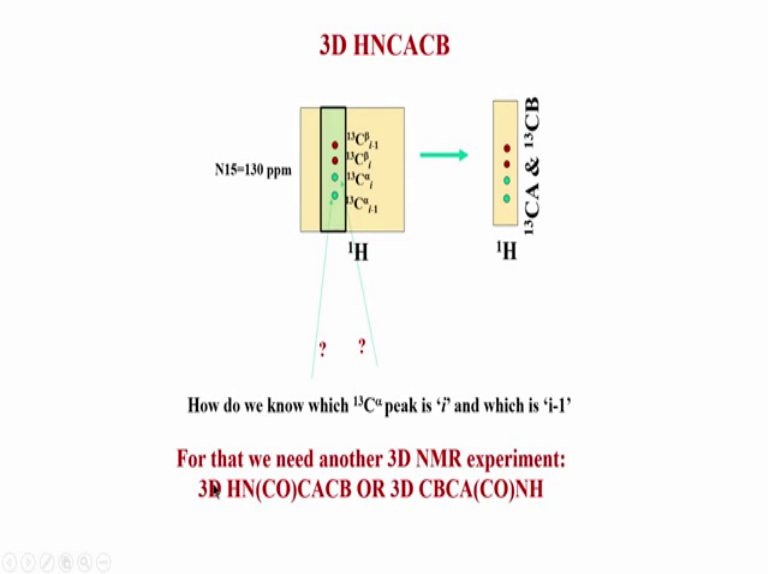

But we still have this ambiguity that which is alpha i and which is i minus 1. So, this is what is shown here similarly we do not know which is beta i which is beta i minus 1 ok.

So, therefore, this confusion is similar to what we saw in the case of HNCA CB sorry HNCA 3D HNCA we had the same problem, that we could not distinguish those two we could not distinguish. So, what did we do in such cases? We took another experiment. So, similarly here we now need another 3D experiment, which brings us to this

experiment either you look at this or this both has the same information. So, now, I will show you this 3D HNCO CACB which gives me a complementary information compared to what you get HNCACB. So, combined together I will be able to distinguish.

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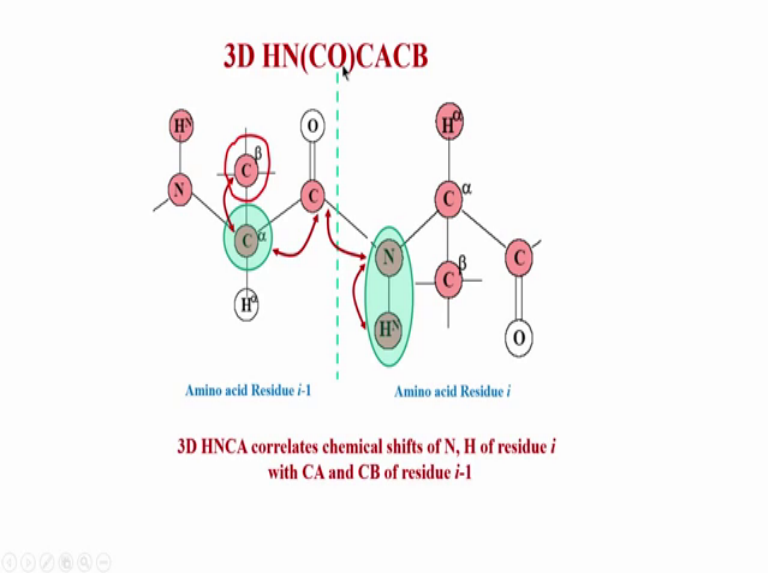

So, let us start from HN CO CACB how does that experiment work. So, you can see this is similar to now HN CO CA. So, if you recollect HN CO CA we went from HN, we went from CO to CO from n and from CO we went to C alpha, but here now we are going one more step further and we are getting the chemical shift of C beta as well. So, you see this is a longer transfer, we went we are going longer and longer compared to what we did in the HNCOCA, but we have to go longer because we need to get the chemical shift of beta. But again look at this here it is exclusively selectively going to the i minus 1 it is not going to i because NCOCA does not exist here it is NCA CO.

So, therefore, this transfer happens exclusively from i and it crosses the border, which is the border here and it crosses the border the magnetization and goes to the neighbouring amino acid from neighbouring amino acid it goes to C alpha and from there it goes to the C beta station. You can think of it this as stations then it comes back. Again if you look this here we have put this in brackets and; that means, that actually I do not get the chemical shift of this, but actually I will get all other chemical shifts.

So, this also can be put it in circle because I am getting a chemical shift of that ok, but I do not get capture the chemical shift of C alpha sorry CO. So, it is put it in brackets here ok. So, let us see how that experiment now works.

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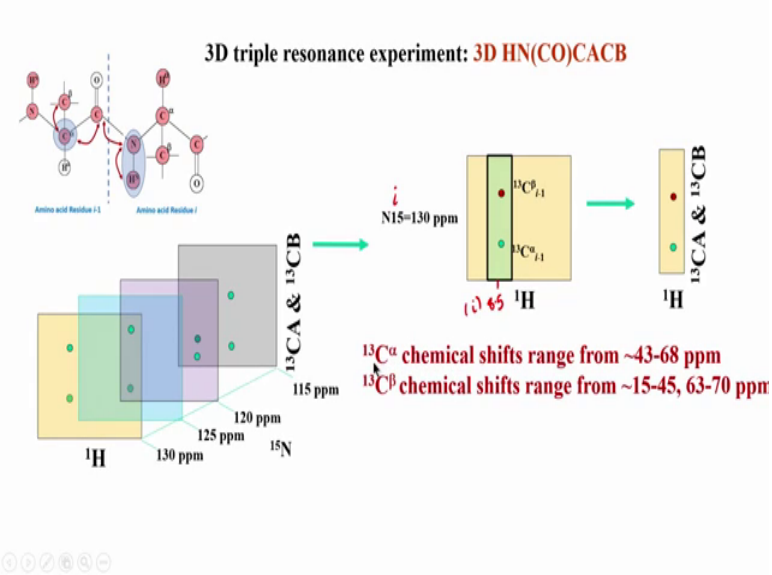

This is how this is of same picture which was shown in the previous slide, where going from HN to CO to CA to CB. Now in this 3D experiment is very same as HNCA CB in terms of the axis, but now you should notice we are not getting four peaks here we are getting only two peaks and why is this two peaks here? The two peaks are basically corresponding to only C alpha i minus 1 and opposite phase again for C beta.

For C alpha C beta are opposite in phase here as well, but for a given amino acid i if i call this as i and a given amide proton i, let us say this is 8.5 this is residue i; for these i i combination I am now getting i minus 1 chemical shifts of C beta and C alpha. So, this is the beauty of this experiment that I am able to only exclusively get i minus 1 from here whereas, in HNCA i was getting both. So, you see HNCA CB and 3D HNCO CA CB are complementary and their need needed together chemical shift wise is the same the C alpha always remains in this range this does not depend on experiment it is a inherent nature or the range of C alpha similarly C beta also always remains in this range.

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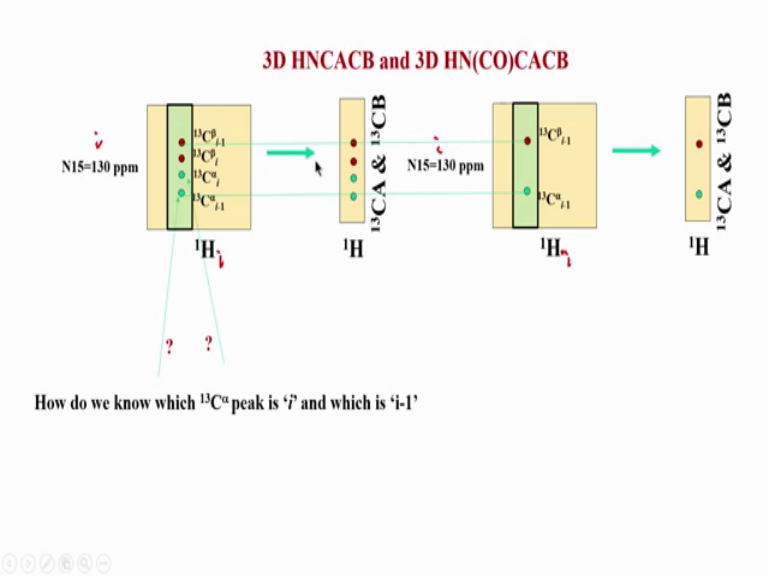

So, now, you can see here the question we asked is how do you distinguish C alpha from C alpha i minus 1 which you could not similarly beta from i and i minus 1 now with this experiment HNCO CACB I am able to do that because now if you look at this same combination.

So, these are both i this is i this is i and this is i. So, for a same i amino acid i, I am able to get i minus 1 here whereas, here i got only i, I mean i got both i and i minus 1. See if i draw a line you can see this line here it is matching with i minus 1 here.

So, based on this combination if i keep these two spectra side by side or this strips if i keep them side by side in my computer I will be able to figure out which two correspond to i minus 1 and which two corresponds to i. Now when this is i then the positive phase will be i C alpha and the negative will become automatically C beta. So, we look at this combination of positive negative for C alpha and C beta and we look at the combination of HNCO CACB and HN CACB for distinguishing i from i minus 1. So, in this manner we can actually separate two residues i and i minus 1.

We will go in the next class two more 3D experiments, before we wrap up the 3D NMR part we look at some experiments how to assign the side chains which are very important. So, right now if you really if you recollect we have only look at backbone NMR chemical shifts of NCACB and CO, but we have to now look at how side chains

are assigned. So, in the next class we will continue with 3D and finish it with some side chain 3D NMR experiments.