Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules
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Lecture – 38
Understanding Protein ligand interaction by NMR: T2 Filter

So, we were looking in the last class at the Protein ligand interactions how the fast and slow exchange gets manifested in NMR spectrum and as I mentioned that all depends on the chemical shift difference between A and B. So, in this class now let us continue with that and look at what happens to the protein signals, when there is a fast exchange or if there is a slow exchange.

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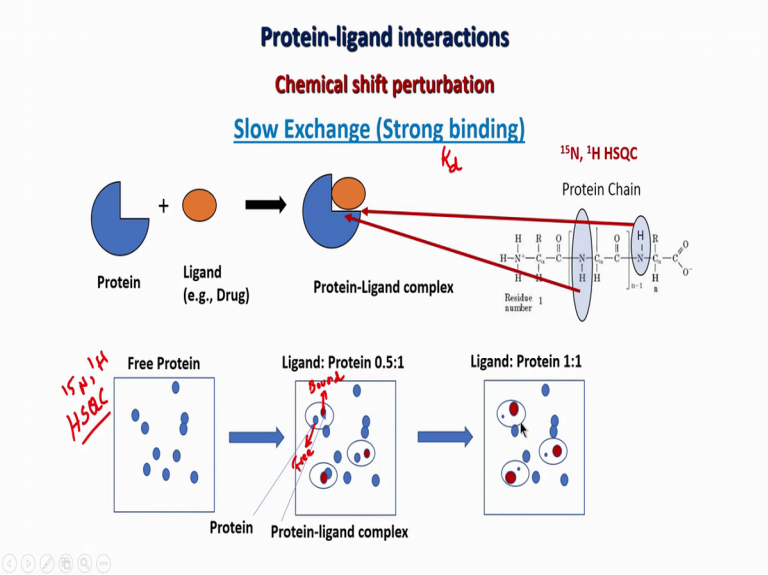

So, this is now indicated here. So, imagine this schematic figure which has been shown earlier also, that a protein interacts with a ligand to form a complex. Now what we are going to see is now the protein signals. So, protein signals how do we typically monitor? We monitor protein signals from HSQC. So, this is the protein chain and as we know now that each amide proton in the HSQC corresponds to one amino acid or one residue in the entire chain. So, the amino acids which are in the interface are expected to be the most affected in case of a binding.

So, let us consider a case where the binding is strong, again strong and weak binding is based on the K d value say the K d value and that K d value could be somewhere in the

micro molar to nano molar range for a strong binding scenario. So, in such cases we expect typically a slow exchange in the NMR time scale so, this is a very important point which is missed often is when we say slow and fast it is all depends on the NMR parameter that is chemical shifts ok. So, let us say now what happens see what happens in case of a NMR spectrum of a protein when we add the ligand. So, this is the latest this is HSQC spectrum of the free protein ok. Now I add so, let me write down here HSQC and is most like most typically proton nitrogen HSQC. So, this HSQC now I add the ligand to it so, I have directly added 0.5 is to 1 ratio means the ligand is concentration of ligand is 50 percent of the protein concentration in the sample.

So, you can see here now, some peaks will shift not all of them, not shift some peak will undergo I will show a new chemical no peak nearby not all will change. Why? Because we expect only the peaks at this interface to get effected, we do not expect the peaks other peaks in this part the interior of the protein to be affected. Because any structural change which is happening here is mostly restricted to the binding spot and they are not conveyed far away the whole protein is not changing its structure, only the local binding region is undergoing a small structural or conformational change. And therefore, only the amino acids which are at this interface of the protein ligand interface will expected to be change in or changing in their chemical shift.

So, here it is actually not a change, but a shift not a shift, but actually you can see there is a new peak which is a bound form. So, I can say that this is this peak is the bound form of the, the protein and this is a free form of the protein. So, you can see that this peak is not almost equal because point when I add 0.5 to 1, 50 percent of the protein has interacted with ligand and 50 percent of the protein is remaining free so, that is why it shows 1 is to 1 interaction.

Similarly some other peak which is also in the binding interface may show a shift or a new peak will appear near its old peak and you can see that there is a now intensity of this peak is 50-50 or equal. Similarly, here another peak like here which was free here has now also showing a partner which is again equal in intensity. So, this is possible at this concentration. Now I increase further the protein concentration or ligand concentration and I make the ligand to protein as 1 is to 1, in such a scenario you can see here that the protein has completely disappeared the old protein peaks have gone for those in the binding regions. So, again these are the three binding region peaks as an

example and you can see here why is it so they have gone away because, the protein when I add 1 is to 1 the entire protein and ligand have formed a complex and the very little of the free protein is present. It will very rarely it will happened that everything of protein has interacted that typically happens when we add a lot of ligand.

So, when you add an excess of ligand then we can expect the protein peaks completely to be gone, but at 1 is to 1 even for a strong complex or a strong binding case typically protein peaks will not completely disappear the old protein I am talking about the free proteins they will still be a small population of the free protein, but you see the majority now at this 1 is to 1 ratio has is in the press form of bound protein. Now all the other peaks have not changed at all because, they are not in the binding interface, they are very slow in the lower end this side of the protein or interior of the protein away from the binding site. So, you can see this is how a typically a slow exchange will happen in a protein NMR spectrum you slowly titrate so, this process of adding ligand to protein we use our titrations.

So now, you can see we are not going to get any ligand peaks here and why is that because the protein is N 15 labelled we are labelling the protein by isotope labelling and we are added N 15 to it. And therefore, I mean it has become n 15 because of the labelling idea approach and the ligand is unlabelled it is free I mean it is not having any C 13 or N 15 labelled. So, therefore, when I add this ligand to protein, my ligand signals will not be there I will only get the protein signals. So, that is how we monitor the protein signals and in the slow exchange scenario this is what you will expect to see.

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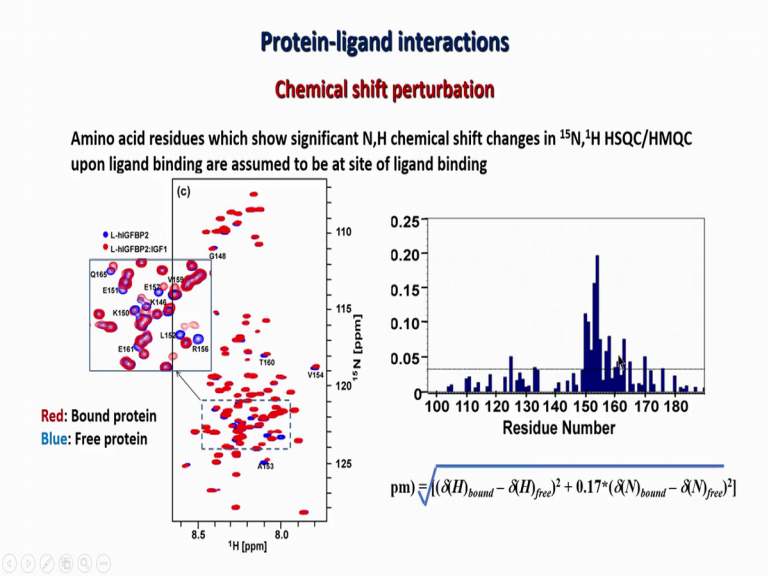

Now, let us see what happens if there is a fast exchange. So, this is what I was mentioned here, the signals which show significant shift in HSQC are expected to be the ligand binding. So, the slow exchange so, this is still the slow exchange scenario and this is A real case example which I showed earlier that you can see that there is A bindings region because of slow exchange.

So, you can see this is typically the case the red peaks have disappeared in the blue spectrum and the blue spectrum only shows the bound formed. So, there is no free protein left in the blue colour. So, blue colour is a free it has completely gone to the red is a bound form. So, there is no presence of both the peaks. So, red is completely different from blue. So that means, it is a slow exchange scenario because I have completely shifted from the free form to the bound form and this is what is captured in the chemical shift difference already we have seen this earlier.

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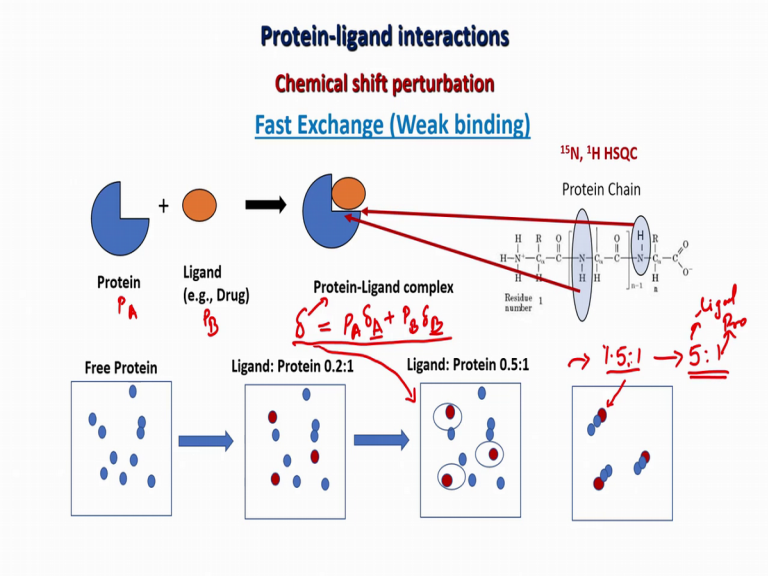

Now, let us see how a fast exchange case happens. In a fast exchange scenario, this is what will take place. So, let us go through this carefully. So, imagine that again we are looking at A HSQC spectrum of the free protein. So, this is a free protein now, now I add the ligand to this. So, now, let us say here instead of 0.5 I add a very small amount 0.2 of ligand is to 1 of the protein. So, in such a case what will happen is that this peak if you notice has actually moved it is not visible here we can show it here like this. So, when I have added the ligand to the protein this peak which was earlier the same residue which was the binding site and this they have actually moved from their original position.

But, there are not 2 peaks now there is only 1 peak and that has shifted means moved from its original position why is this happening? This is happening for this you have to recall our fast exchange part in the last lecture where we saw that in a fast exchange scenario when you add ligand or protein there is a movement or shift of the peaks because the population weighted average is the chemical shift value of the complex; that means, the peaks of this complex they come at the population weighted average. So, let me write that down again. So, our chemical shift of the complex is basically P A that is we can think of it is P A and P B as A population so it is P A delta A plus P B delta B so this is called population weighted average ok.

So, because of this the chemical shift of this complex which we are seeing here is neither A nor B it is somewhere in between A and B because A is P A is less than 1, P B is less

than 1 so between A and B somewhere the C value. So, this complex is now somewhere in between A and B. So, what is B? B is not seen here B will be only seen when we have completely added more and more of ligand. So, if I add more and more I go is to I go to 1.5 of ligand is to 1 then, I will see the completely bound form ok. So, till then I will only see movement of peaks means this is A spectrum which is superimpose means I have combined this 3.

And I can see this only for I am showing only the 3 binding region residues I am not looking at other residues because they are not moving at all. So, only for this 3 binding region you can see that they are actually moved ok, they will move like this up and down and this movement depends on the concentration of the protein to ligand. So, if the more is the ligand you add more towards the bound form it will move ok. So, this is the free form and the red colour is the bound, bound form typically comes when you had excess in fact, not just 1.5 sometimes you have to go to even 5 is to 1 for a weak complex; that means, five of the ligand and one of protein.

So, this is how typically NMR titrations are carried out you start from the free protein keep adding the ligand and go to verify access and if you see such peaks are moving of the protein then it is typical of a fast exchange scenario means the ligand interaction is weak and it undergoes a fast exchange. So, this is how the two different exchange phenomena were explained.

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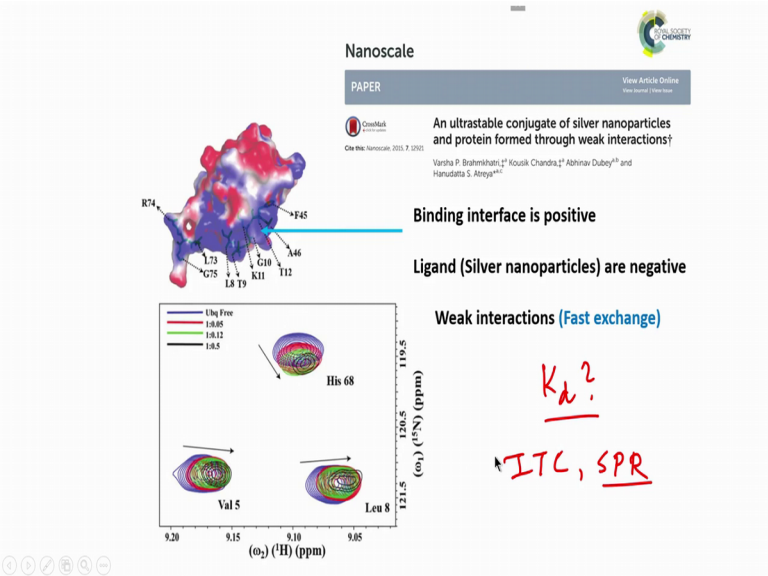

So, this is now let us see an example of a real case. So, this is back to this particular paper which I showed earlier and there the silver nanoparticles were added to protein and it was a weak interaction and you can see here how the chemical shifts are moving, the peaks are moving when more and more ligand is added. So, you can see here this blue colour is a free protein that is ubiquitin, free ubiquitin now if I add more and more of my ligand that is red is 1 is to 0.05, green corresponds to 0.1 and black is 0.5 you see the peaks are moving.

One more thing if you notice here the peaks are also becoming weak you can see, this is weak in intensity, this is small black is weak and why this weak the weakness is coming because the complex is a large system. So, therefore, the intensities become less because the T2 relaxation will we broaden the peaks. So, whenever you have a large complex T2 is short and if T2 is short the intensity of the P or area is also small. So, that is why it is happening, but for our discussion right now what is important is that the peaks are shifting like this when I add more and more of my ligand to the protein.

So, in this case my protein ligand was a silver nanoparticles. So, this is what we saw earlier and the interactions are weak because it is electrostatic interactions typically electrostatic if it is sometimes are weak not always and if there is a weak interaction in NMR it undergoes fast exchange type of scenario. So, now, having seen fast and slow exchange, now the next question which comes to our mind is how do we measure this binding affinity this K d value how do we measure because finally, that is what is important for us if you are designing a ligand or a drug molecule you want to know the strength of the interaction.

So, how can we determined by NMR remember there are non NMR techniques like ITC Isothermal Titration Calorimetry or you can use what is called Surface Plasmon Resonance SPR these are well known techniques to find the strength of interaction between ligand and protein. So, normally NMR is not so popular for measuring binding affinities. NMR is mainly helpful for characterizing whether it is fast or slow or weak or strong, but never the less we can still measure with NMR.

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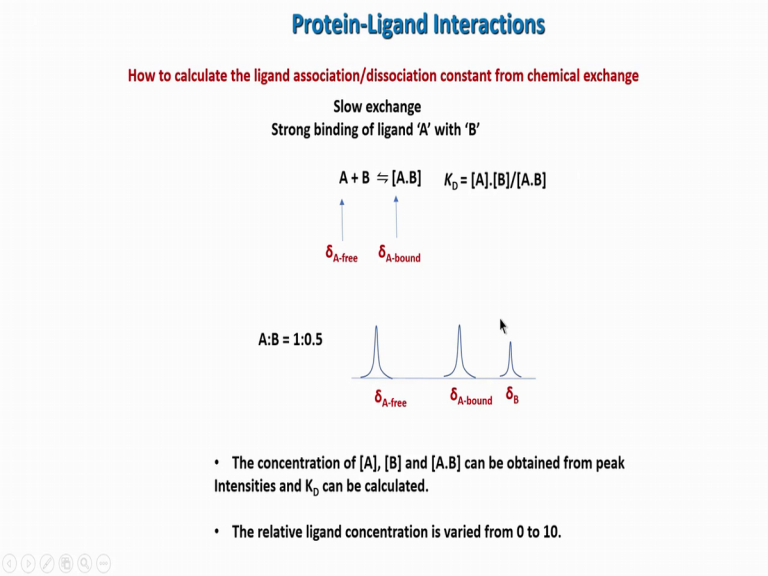

So, how do we calculate that let us see for the two scenarios, one is the fast exchange case and another is the slow. So, in a slow exchange situation is very simple you have a population of free form initially you have fully free form, you slowly add the ligand or protein and then the bound form signal will appear. So, in a slow exchange case, the intensity of bound is different. So, this is now I am showing here the peak corresponding to B and peak corresponding to A, but what happens is there is a peak corresponding to the bound form which is somewhere coming here.

So, in this particular scenario 1 is to 0.5 half of A has got converted to A, B complex so half is free half is bound so, they are equal in intensity. And in such a case the concentration of A, B and A, B complex you can see from here I can get all the intensities measured and then if I measure the intensities in NMR you should remember the intensity or area of any peak is directly related to the population of that species or that molecule, therefore, if I measure the intensity or area of A B and A B complex that is bound form I can simply put it in this equation and then I can determine the K d value. So, what I can do is vary the concentration of A and B and then try to get a fitting to a linear regression and try to get the K d value.

So, for slow exchange it is a simply a matter of titration and then calculate the different areas of the 3 species, what are the 3 here again free A, free B and bound A B. So, 3 peaks will come ok. So, this is the case for slow exchange.

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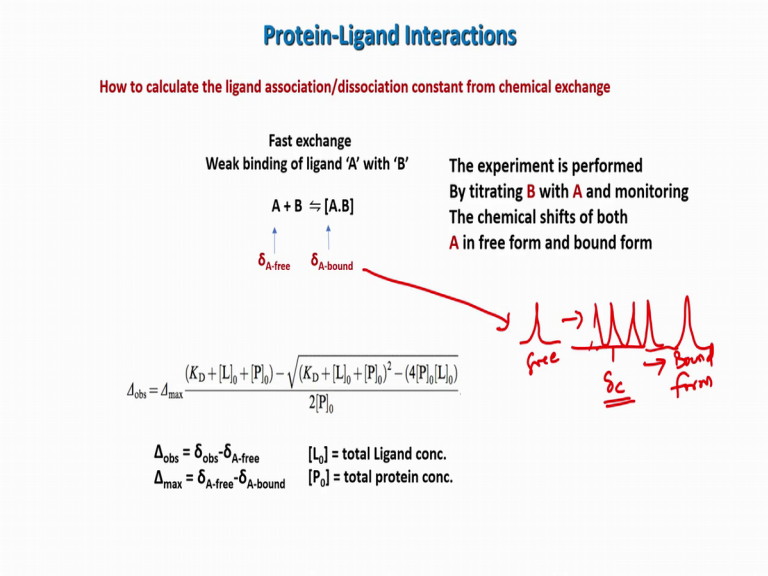

Now in a fast exchange how do we measure, it is slightly complicated. So, in a fast exchange scenario let us consider again this reaction or this complex. So, I have this A form which is free I add B to it and A B complex is formed. So, in NMR I will have to monitor these two chemical shifts A and free and bound, but ha ok, but the experiment is done by titrating B with A; that means, suppose I am monitoring A then I titrate means I add slowly in steps B molecule to A and then chemical shift of A in free and found bound form is monitored.

So, as I said in the in the case of in the slow exchange in the fast exchange typically the peaks move. So, you will have a peak which is a free form then it will start moving towards a bound form. So, this is the free form then slowly it will move like this because it is moving towards the bound form because this is this is the population weighted average. So, when you look at a population weighted average the population of bound for increases bound form increases when you are adding more of B and in such a scenario the peaks starts moving towards the bound form. So, this movement is what is captured in this delta observed value.

So, if you look at this equation delta observed on the left side is at any given time at any point when you have added a required amount of B you can find out what is the peak of the weighted average that is observed value minus the free form, free is when no B is added; free is when no B is added ok. So, that is the difference at any point when B is

getting added to A what is the observed weighted average chemical shift that minus the free form of A is this value here.

Then you have what is called delta max, delta max is basically this simple difference between free and bound this is a completely bound form; that means, when I keep adding it will keep shifting to bound form and after a long time it will settle down at the bound form then it will not change because now the bound form is maximum so it is now form completely formed. So, therefore, that is my final bound delta bound.

So, this minus free will give me the maximum possible shift which can happen and then other numbers here refer to the chemical the concentrations because we know the total concentration of ligand, how much we start with we know the total protein, what we have added and if you put in these values you can find out the K d. So, K d is just simply fitting our values of these 4 parameters to the equation and extracting the appropriate K d value. So, this is how K d it is extracted from fast exchange case in NMR.

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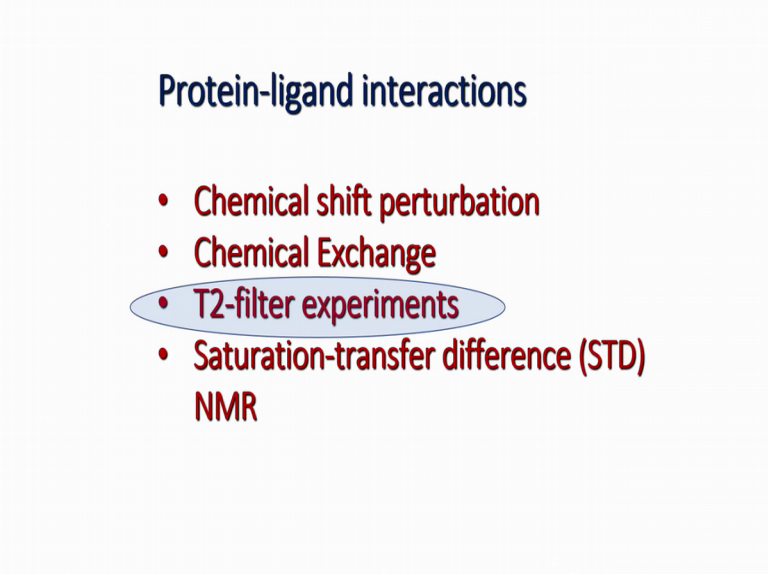

So, now let us move to the next topic that is how to monitor protein ligand interaction with what is called as a T2 filter experiment. So, let us see how that works.

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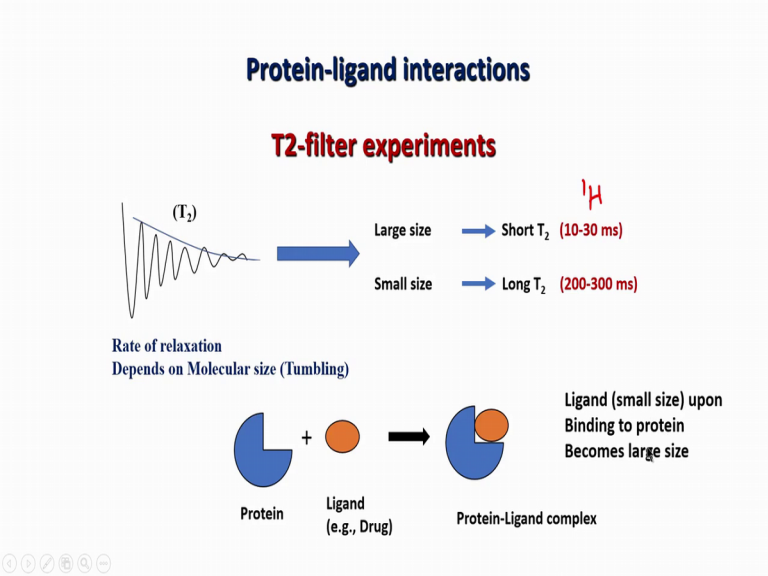

So, T2 filter now as the name suggests, it depends on the T2 concept of T2. Now what is T2? T2is what we have seen much earlier in this course that it is what is called transverse relaxation. So, if you see this is an FID, an FID decays with time and that is because of T2. So, there are two types of relaxation in NMR T1 and T2, T1 relaxation is the recovery of the magnetization to the z axis and T2 is the decay of magnetization or dephasing of magnetization in the xy plane ok.

So, our experiment our idea now will mainly depend on the T2 relaxation and T2 depends on the size of the protein of the or the ligand or any molecule and this is very important. Because, as you increase the size T2 shortens becomes smaller and why does it become smaller because of this tumbling whenever the rotation of molecule is slowed down; because of increase in size there is a lot of interactions which dampen or reduce T2 ok.

So, this is well known you can refer to many of these books of for T2 how it depends on the on the tumbling or on the correlation time, that is autocorrelation time of the molecule. So, in generally if you have a lot size protein ok; so, let us say you have 10 kilodalton or 15 or 20 kilodalton higher proteins the proton T2 so this numbers are for hydrogen. So, let me write now this is for proton typically will be of the order of 10 to 30 milliseconds, but if you go to a small size like ligand which is like a small molecule its T2 will be pretty long it could be in this range sometimes even longer.

So, there is a big difference almost an order of magnitude difference between the T2 of a small molecule and a large molecules. And this is what we have to exploit or use in this experiment T2 filter. So, how do we do that let us see, so let us say we have a protein again the same scenario we have a ligand which binds to the protein now this ligand is a small molecule and when it is bound to the protein it behaves as a large molecule because now protein is bound to the ligand. So, ligand is no longer free like this, so its rotation or tumbling is happening along with the protein so that means, its tumbling is also getting slowed down.

So, if stumbling is getting slowdown its T2 will become short and it will be as much as the size of this complex. So, therefore, you can see between a free and a bound form there it could be a big difference in the T2 of the ligand so, looking at ligand only there is a possibility so ligand small; is a small size molecule, but upon binding to the protein it becomes large. So, how do we know use this idea let us go further.

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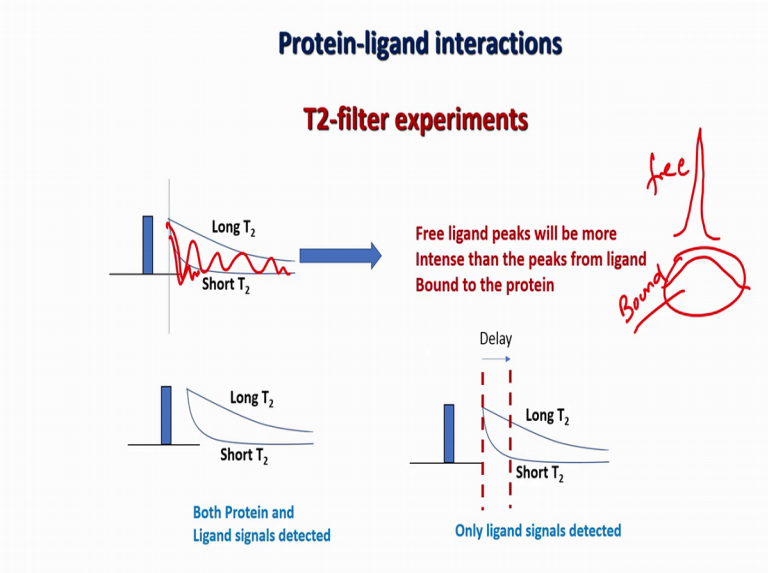

So, this is what is shown in terms of a decay. So, let us say I have a 1D NMR experiment, in a 1D NMR, I simply apply a 90 degree pulse and then I start recording the FIDs. So, this is FID now we can see that if I have a molecule which is as long T2 the FID will live longer, but if I have a molecule which is having a short T2 its FID will decay very fast. So, you can see that the FID of the short T2 molecule of the sorry the

molecule having short T2 that is proteins will be much short lived means decay faster compared to the FID of the molecule with long T2.

So, free ligands will have therefore, a more intense peaks because if you do a Fourier transform, you will get a ligand which is very sharp we will see this again in the next slide whereas, a protein will be broad because a protein has a short T2 and a ligand has A long T2. So, the line width or the sharpness of the peak will change when the ligand and between the ligand and the protein. So, what we can do is, we can do the following trick suppose I put a delay. So, suppose I apply a pulse and then after the pulse I do not record the signal immediately, I suppose I keep a delay period delay means some time and that time, in that time what will happen is my protein signal would have decreased considerably, but the small molecule signal would not considerably decrease.

Therefore if I detect a spectrum or record a spectrum after this delay, I will most likely see that if I have a free ligand, the free ligand now will not show much decrease the free it will be probably 10 to 20 percent. But, as a signal from the bound form that is ligand bound to the protein will be rapidly gone to almost 70 80 percent down. So, that is basically the idea which we use in T2 filter is that you essentially look at the size dependence you assume; you assume that the T2 of a small molecule is long and therefore, it is longer lived and therefore, in the spectrum it will show a stronger peak and whereas a short large molecule that is a complex so, you can think of this as a free ligand and this is bound.

So, the bound ligand is now showing a broad peak because it has A shorter T2. So, by monitoring the T2 relaxation of the free ligand and of the bound ligand, I can then distinguish whether there is a change or not. If the ligand binds to that protein, then I should expect that the ligand will show a short T2, if the pro ligand does not bind to the protein, then the ligand T2 should not change. So, by just simply monitoring the T2 of the ligand in the free and bound form I will be able to distinguish whether the ligand is binding or not.

So, we will continue in the next class and we will see how this can be exploited further to characterize the ligand to protein interaction.