Multidimensional NMR Spectroscopy for Structural Studies of Bimolecules

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Lecture – 39
Understand Protein ligand interaction by NMR: STD NMR

In the last class we were looking at T 2 filter experiments in which we looked at how the T 2 relaxation values of the different molecular sizes can be exploited to look at ligand protein interaction. So, let us just quickly recap what we did in the last class.

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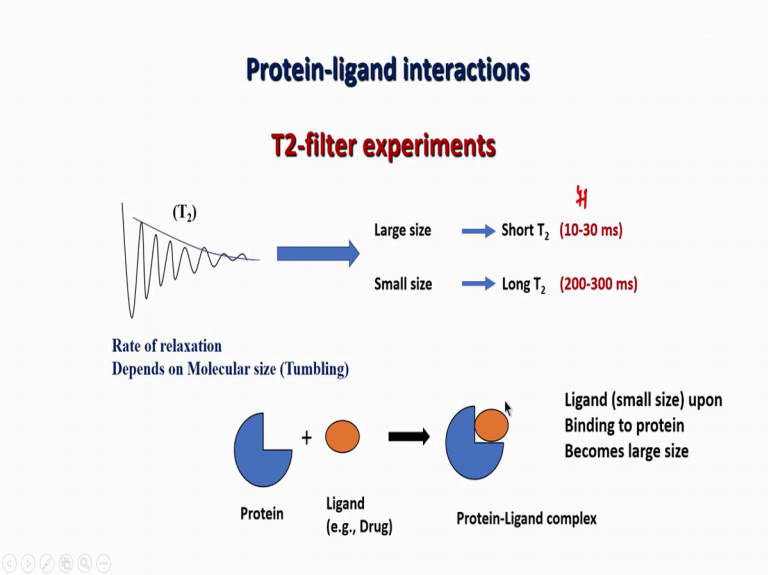

So, this is shown here that an FID decays with time and that decay is governed by T 2 relaxation. And this T 2 relaxation value depends on the molecular tumbling, and molecular tumbling is characterized by what is called as tau c, rotational correlation time. Tau c now depends on the molecular weight as we have seen earlier when we looked at NOESY. So, tau c is basically the larger the size tau c is higher when tau c is higher the T 2 is shorter. So, typically for small large proteins you will find the T 2 of protons will be in this particular range. So, let me clearly specify that protons, ok.

When you go to smaller size proteins or ligands it will be longer T 2. So, this is typically the order of 200 to 300 milliseconds this is for small molecule. So, our goal is to study the interaction of the small molecule that is the drug or a ligand with a protein. So, the assumption hypothesis here is that the molecule is small and is much smaller than the

protein, so therefore, when it binds to the protein the complex is becomes as much as the protein size. So, this is not so much in the size whereas, the protein is large. So, the ligand now becomes a large molecule because it now binds with the protein.

So, ligand therefore, is a small molecule here before binding and it becomes or behaves like a large molecule upon binding. So, this difference between the two states that is the free form and the bound form is what we are going to exploit or use to study the protein ligand interaction with T 2 experiment. So, basically that is what is said here is a ligand which is a small size upon biding to the protein becomes means it behaves does not literally become a large size it is still the same, but it behaves like a large size system because of its bound nature with the protein.

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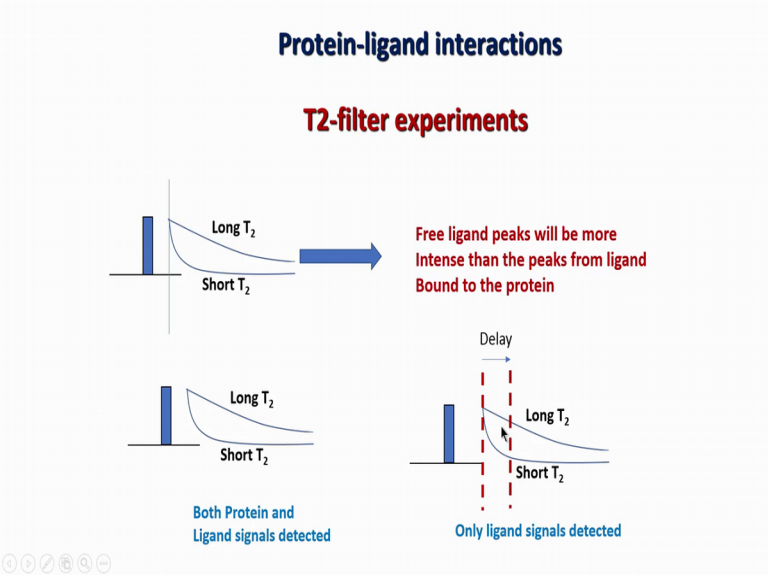

So, now let us see how we can exploit this. So, a long T 2 will have a very long decay whereas, short T 2 will decay faster. So, what we do in T 2 filter is essentially this that it give a delay between after the acquisition. So, as soon as you apply the pulse, we do not start recording the signal we wait for a short delay. And this delay is why is this required? This is mainly required because we want to eliminate any signals coming from the bound form and only keep the short form, but if the ligand is bound to the protein then this decay will not be present and only this will be present which means the signal will disappear if there is a binding of ligand to the protein.

So, basically by monitoring the free ligand first where it shows some decay and then monitoring the ligand upon adding the protein the difference will tell us whether there is indeed any binding or not. We will see this shortly.

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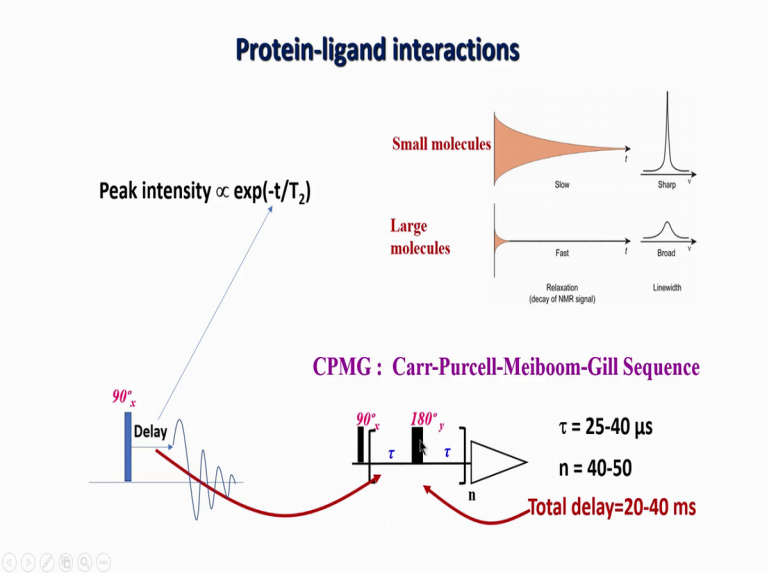

So, this is what we saw in the last class also that, what the delay essentially causes the decrease in signal because of this exponential damping factor, ok. So, if the small T 2 relaxation is short then this exponential will damp, means go down faster if this T 2 relaxation is long then this signal will not decay, ok. So, this is the idea here and this delay here which is shown here this is not actually a simple delay; that means, it is not simply a time gap. What is done is actually this sequence is applied this is known as CPMG.

In a CPMG you see this block here which is shown in the bracket that is repeated n number of times and this bracket this block consist of a tau period some delay tau, then a 180 degree pulse and then a tau. So, this is a symmetric means there is no chemical shift evolution during this period because of the eco. If you recall we have seen earlier in the case of inept and other experiments whenever you have a 180 degree pulse in the center of a element that is in between two equal delay periods then there is no chemical shift evolution during that time. But the coupling will take place and also the relaxation. Coupling we do not care right now because a tau is really a short period, but this decay definitely exponential decay will go on and that is what we want. We want the molecule

which are small to have a longer decay I mean to detect those the one which are short will be gone. So, this is idea.

And what is the values of delay? Typically, tau values, this tau here is of this order 25 to 40 microseconds. So, when you multiply this by n, n is the number of times you repeat this block is typically to a 40 into 25 is around 10 millisecond or it can 20 millisecond or it can be 40 millisecond because this comes two times. So, this is how a CPMG block is put in a 1D experiment. So, this is a standard 1D experiment. So, instead of directly detecting after this pulse like in a standard 1D we are inserting this element or a block so that it can allow the signals to decay without chemical shift evolution.

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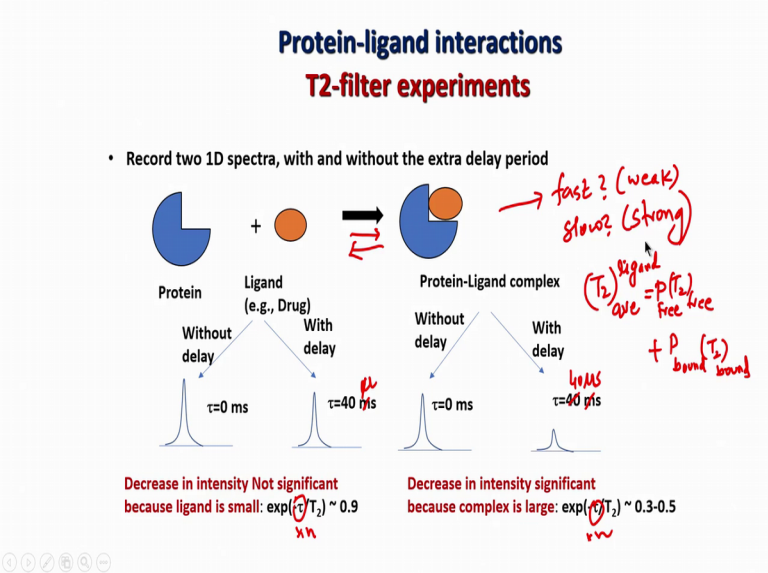

So, now how do we do these experiments practically. So, we record two spectra two 1D spectra with and without the extra delay period. So, as I mentioned in the previous slides that when you record with the delay period your signal from the ligand bound to the protein will be gone and when you record without the delay period your only the free ligand should come. So, if there is no binding of the ligand with the protein then with and without should look the same. And if there is a binding of the ligand with the protein then with and without will look different because in the time when the protein binds a ligand binds the protein the delay period will decrease the signal.

So, let us see how schematically we can expect to see the spectrum. So, this is that interaction we are looking at and now we can see without the delay that is when I set the

delay value tau to 0, ok. So, if you go back here this is this tau delay sorry and this is set to 0. And remember this 180 is a very small I mean the duration pulse. So, we will ignore we will not consider this time of the pulse. So, let us assume this is also 0. So, when tau is 0 then the entire block is 0; that means, there is no delay and that is how we actually execute without delay.

Now, with delay let us say we use tau value of sorry this is microseconds, microseconds, and then the total delay let us say is about 40 milliseconds then you see there is a decrease in the signal to noise. Means there is a decrease in the signal intensity because they have T 2 relaxation. So, you see the ligand even though it is a small molecule if its T 2 is not 0 therefore, it will also decay whether it is bound or not a free ligand also will have a T 2 and that T 2 will cause a slight decrease during this gap during this tau period when CPMG is applied. This decay is now not so much because it is a small molecule.

Now, when it is bound to the protein, ok; so, this is the decrease in intensity is not significant because ligand is small. So, typically again this varies from molecule to molecule, but typically for a small molecule the T 2 based on the T 2 you can expect about only a 10 percent fall, means this signal which is on the right here will be about 90 percent of the signal, 80 to 90 percent it will not be. So, much decrease because T 2 is long for a small molecule.

Now, what happens when the protein binds to the ligand or ligand is added to the protein? Again, we record two spectra with and without delay. Now, without delay tau equal to 0 you will get some peak, ok, but this peak will be broader than this peak because of the larger complex. But now when I add the delay the T 2 the tau has really decreased, the signal has really decreased in time, again this tau I would put it as 40 microseconds, ok. So, this is not in milliseconds this is tau is 40 microseconds, but the total CPMG could be in milliseconds, because we have a n times this number. So, it could be that n is equal to 50 or 600, then it becomes 20 to 10 to 20 milliseconds.

So, when I apply this delay the protein ligand complex will show a very fast very small intensity that is because of the rapid T 2 or rapid decrease in its intensity and this is almost like very high rate of decrease and again typically based on the T 2 of a large protein and tau value of 40 microseconds multiply with n. So, I have to actually also this is remember, this is not just the tau this is not just the tau it is tau times the n and this is

also tau times multiply by n because we have applying it n times. So, the total delay is actually tau into n.

So, based on a typical T 2 of a large protein you can expect that the signal intensity would fall by almost 30 to 50 percent. So, this is this is the scenario for a large protein ligand complex and this is how we can actually detect whether a ligand is binding or not. So, this is what typically people do in pharmaceutical companies, they will screen a large number of ligands with the protein. So, we for example, let us say you are initially you are trying to discover a new drug, but you do not know which molecule will bind to the target protein. So, you start screening or you will start adding one by one a lot of ligands so you quickly can record, these spectra these spectra typically take only a few seconds to record. So, they are very sensitivity is very high. So, in a few one in a one day you can screen thousands of compounds automatically these all are done by a sample changes software which are automated these days. So, we just have to do in a robotic manner.

So, each ligand is added to the protein and you record these spectra. This is of course, recorded only once for one ligand, so you and for every ligand one has to record these two and for every ligand one has to record these two with the protein. So, there are about four such spectra. But we can see very quickly you can find out if the ligand is binding or not because this decrease will not be so dramatic if there is no binding. So, by just adding the legend and quickly screening for a large number of molecules your initial hits for a drug molecule can be obtain and then it can be further fine tuned, the ligand molecule can be synthesized and so on for better binding faster or more accurate.

Now, there is one more thing, how do we find out whether this binding or this interaction is it fast or slow. So, this is a very important point. The question is the interaction fast, means weak or is it slow, that is strong. So, you want to if you want to find out whether this is the case then we have to we can do the same experiment, but to distinguish between a fast binding case or a weak fast interaction or fast exchange case weak binding or a strong binding case what is done is you slowly increase the concentration of the legend from very small value that is from 0.1 is to 1 and you keep increasing up to high values that is 2 is to 1, 3 is to 1 or even 10 is to 1 means 10 times ligand compared to 1 times protein.

So, when you do that when you go from a small small concentration of the ligand to a higher concentration by titration your T 2 now will keep changing if there is a weak interaction. Why because a T 2 value will be now a population weighted T 2. So, this is very important point that an the T 2 average of the ligand of the ligand is actually a population weighted, means population of the free form of the ligand into T 2 of the free form of the ligand plus population of the bound form of the ligand and T 2 of the bound form of the ligand, ok. So, this is called population weighted T 2.

So, if you have a weak interaction between the ligand and protein as we increase the population of the bound form; that means, you add more and more of your ligand then the T 2 will shift towards this number, because this population will be less now, because more and more ligand is weighting bound. So, your population of the bound form will increase and the overall T 2 that is average what you can see in the spectrum will also increase sorry decrease, because T 2 of bound is less than T 2 of free T 2s bound is a larger protein size a complex compared to free.

So, basically the T 2 depends on what is the ratio of the population. So, if you have a very tight binding case, in a tight binding case or a strong binding case we do not expect any free to be there in the system because as soon as I add the ligand if it is a strong binder it will immediately bind here and nothing of the free will be there in the sample. So, if there is no free ligand present in the sample then this part will be 0 because population of free is 0. So, your average T 2 now, is actually nothing, but the T 2 of the bound form. So, that is what is shown in this picture here that I am getting a large decrease because there is no free from left. So, the entire decrease is only because of the bound form.

But if it there is a weak interaction then the population of free is not going to be 0 because this now will become an equilibrium. So, this will be in equilibrium both ways, it will go in both directions. So, your free population will be there present all the time. So, when there is that free population present all the time this expression this equation you can see that this will not be 0 and therefore, you get an average value and that average value means it is also has contributions from the free T 2, which means the total T 2 will be somewhere between this T 2 free and T 2 bound. It will not be completely like a T 2 bound neither will it be as small as or as long as T 2 free.

So, you can see that what will happen is this will not be so dramatic it will probably come a little bit higher, ok. But as we increase the population of or if we increase the concentration of the ligand then you are going to move from this free form to more and more of bound form, and therefore, your T 2 actually starts changing as you increase the ligand concentration. So, this is basically the overall idea in a in a T 2 filter experiment that you are able to capture not only whether the ligand is binding or not you are also able to figure out whether it is a weak binding case or a strong binding.

So, again remember what is the range of the K D value, when you say this is a weak binding the K D that is the dissociation constant we saw in the last part where we looked at the other experiments, like chemical shift perturbation, we saw that the K D value is what is characterizes the interaction of ligand with protein and weak interaction means the K D is somewhere from millimolar to micromolar. Whereas, when it goes from micromolar to nanomolar we call that as a strong binding case, ok.

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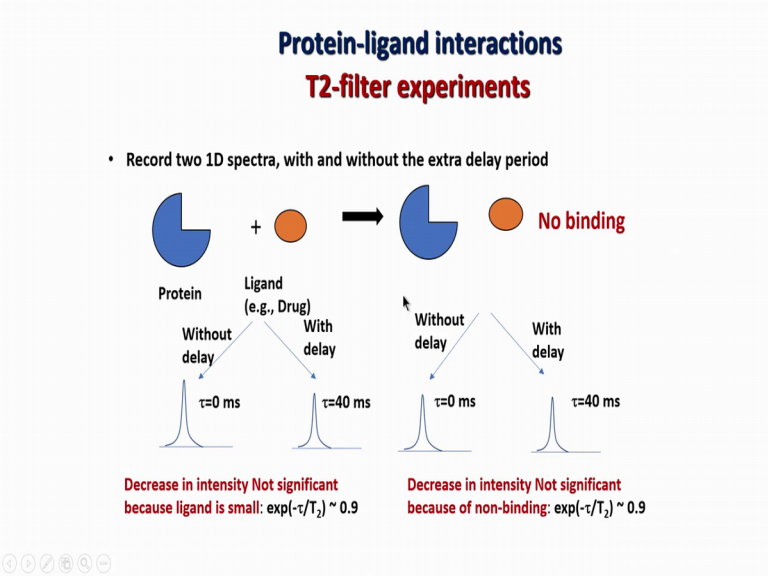

So, now let us say that there is no binding. So, this is what is shown here, if there is no binding we saw already that there will not be any change in the intensity, ok. So, then your intensity will remain the same. That means, here what we saw in the previous slide here we are assuming there is a binding, ok, but suppose there is no binding at all I mean this ligand just does not bind to it, then in that case this state that is ligand and the protein is the same as this ligand and this protein on both sides. So, there is no binding. So, in

such a case everything looks the same that is before adding the protein you have this scenario again remember this correction this is a total delay not tau value tau is roughly about microseconds total delay I would have taken into n, tau into n would be 40 millisecond. So, total delay with and without delay you will get this, but when you add the ligand to protein again you will get the same thing because there is no binding.

So, by observing this we can again say that there is no binding of the ligand to the protein. So, this is again useful for screening a large number of drug molecules when we study the discovery, means screening of new drugs for a given target protein.

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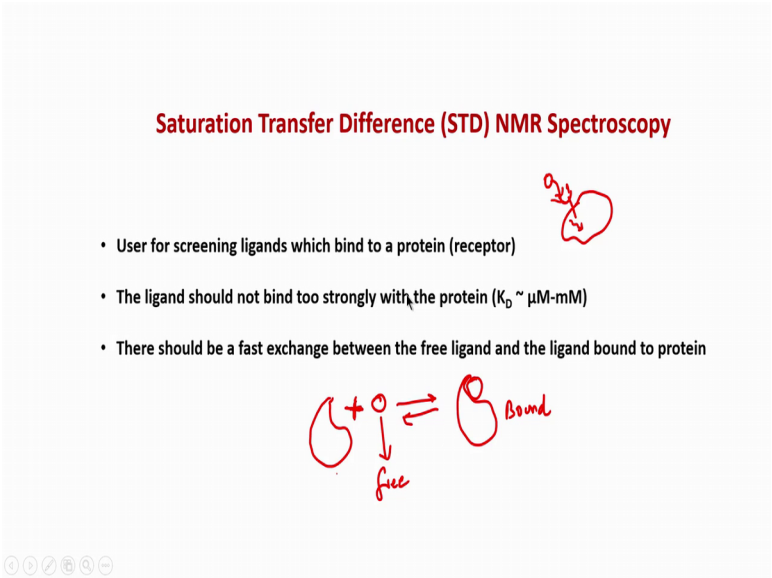

So, now let us move on to the next topic of again how to study ligand protein interactions with NMR. This is the one of the very popular technique in NMR spectroscopy and very routinely used in drug discovery. So, let us see what are the three major things, one thing is it is used for screening ligands which bind to a protein. Typically, you know in a drug industry majority of the drugs target what is known as a receptor. What is a receptor? Receptor is basically on the cell you have a we have a protein which is sitting across this membrane.

So, the ligand, ligand binds here and anything whatever binding happens it is transmitted inside the cell the information not the ligand, ok. So, this information of ligand binding to the receptor is transmitted and lot of interactions or any further development takes in the cell. So, drug blocking this interaction is what typically the drug molecules or drug

industry is based on. So, typically they design a small molecule ligand which will bind to this receptor similar to the natural ligand which is present in our body. But this fellow, this particular ligand will have a much stronger affinity than the one which we have in our body. So, therefore, it continuously blocks the receptor and the action will not take place because the natural ligand that is a which is supposed to do the job is absent or is not allowed to bind, ok.

So, now in STD NMR we try to characterize not the strong binding ligands, we first try to screen which are weak binding ligands, because once you have a weak binder then you can slowly optimize it further and make it a strong binder. So, therefore, initially we start only with a weak binding ligands and only STD NMR that is saturation transfer difference we will shortly see what this means, but this basically is completely relies on weak binding situation. You cannot study a strong binding case, strong binding there are as I showed in the previous slides there are many other techniques not only NMR you can use non-NMR techniques to study strong binding. So, weak binding NMR is the only technique. In fact, one of the best application of NMR is to study weak interactions and that is come that is brought out very nicely in STD NMR, ok.

So, therefore, when there is a weak interaction there should be a fast exchange between the free ligand and the ligand bound to protein. So, this is similar to what we showed in the earlier slide. If we have a ligand then this equilibrium should be always present. This is bound form and this is free form, the ligand is in the free form. So, this exchange, this dynamic this should be plus. So, this dynamic exchange between the free and the bound should be high then only one can use STD NMR spectroscopy. So, let us see how this what is the principle behind this and how does it work.

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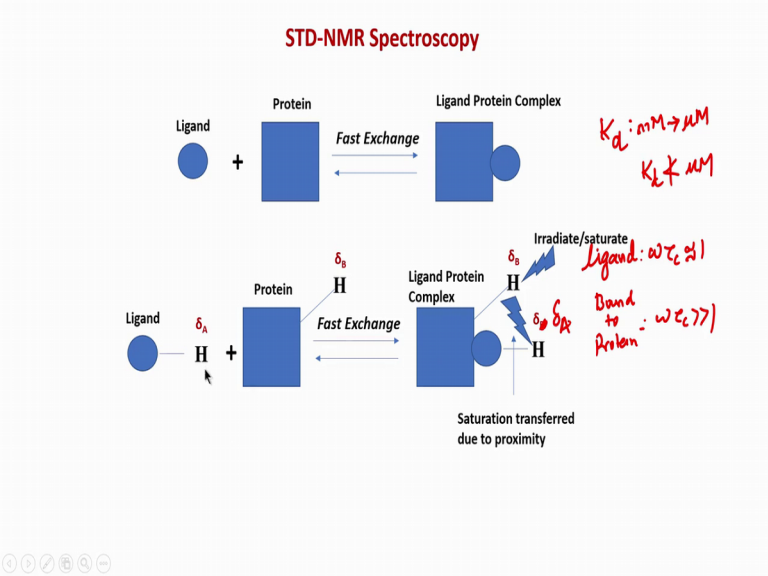

So, this is what is shown here let us say that you have a ligand which is bound to the protein or which is going to bind this is what we want to study. And assume that there is a fast exchange which means there should be a weak interaction what does it mean weak interaction, again I just repeat from the previous slide K D should be in the range of millimolar to micromolar, not more not less than micromolar, ok. So, K D should not be less than micromolar. So, this is typically the condition for studying that STD NMR.

So, now, let us say that this is the case. Now, imagine that there is a proton here on the ligand, some proton call it as delta B chemical shift and suppose there is a proton on the protein, ok. So, this is shown here. So, imagine that there is a proton on this ligand and there is another proton on the protein. Now, when it binds to this molecule this is the same proton. So, this should be A, delta A, ok.

Now, what happens is in the bound form if you recall our NOESY theory or NOEZY principle we saw that in some small ligands the NOESY effect is 0, because of omega into tau c becomes equal to 1 and in the protein case omega into tau c is always higher than 1. So, let me write down here in a case of ligand omega into tau c can be equal to 1, ok, but in the bound form bound to protein that omega tau c becomes much higher than 1. And why is this happening because, the tau c of a protein is much higher than tau c of a ligand. Remember, tau c depends on the molecular weight and we also went through a formula quick formula to estimate tau c there we saw the tau c is basically molecular

weight in kilo Dalton divided by 2. So, if the molecular weight of the protein which is very high results in tau c becoming large.

So, when tau c becomes large then your omega into tau c also becomes large much bigger than this number one and omega is same because we are doing all the experiments in the same spectrometer, but in the ligand case it may happen that your tau c is not very it is not NOE effect is not there because of the small tau c value and therefore, the NOE becomes almost 0. But depends on the ligand size again typically in pharmaceutical companies people study the molecular weight of around 500 and for that kind of a molecule this will happen, ok.

So, now when I irradiate suppose in the bound form, suppose I irradiate the protein what happens is this protein signal this when I irradiate the protein signal this irradiation is transferred to this proton. And how is that transfer takes place? This transfer happens because of NOE that is Nuclear Overhauser Effect and that effect can happen only if they are close to each other again. Just this condition is not necessary I mean is not sufficient it is a necessary condition, but sufficient condition means there should be also a close distance between the two protons, ok. So, this is very important.

So, suppose there is some proton in the protein which is close to the ligand proton. So, if I irradiate that protein signal that is a proton belonging to the protein, then that irradiation gets transmitted to the signal or to the proton on the ligand. And this transmission takes place because as close in space and not only they are close in space they also satisfy this condition, ok. So, this is what is shown here saturation is transferred due to proximity.

Now, when this ligand exchanges with the free ligand, because remember free and bound are in fast exchange. So, then because of that fast exchange there is now this signal the free ligand is also affected, because this proton is a same proton as this proton. Only thing is here it was in the bound form and here it is in the free form; so, therefore, if I decrease the signal of this peak. How do I decrease by indirectly by irradiating a protein peak. So, a protein peak irradiation decreases the protein signal and this decrease of protein signal is transmitted to the or results in the decrease of ligand signal because of NOE and that decrease of this ligand signal is now transmitted to this signal because this

and this molecules are same. So, any decrease here is also similar to a decrease here. They are the same molecule.

So, now by monitoring the free ligand I can figure out whether there is a interaction going on or not, because the signal from the bound ligand maybe very weak. So, the idea in STD NMR is that the bound signal of the ligand is very weak or very small because it is very large system, large complex, but the free ligand is available in large quantity and it is as sharp signal; ligand from the spectrum of the ligand will be this free ligand will be very sharp. So, by exploiting that we are able to study the interaction with the protein.

We will continue this in the next class. And look at a how is this manifests or how this shows up in the quality of the spectrum or how the spectrum can be interpreted.