Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules
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Lecture – 40
Understanding Protein ligand interaction by NMR: Transfer NOE NMR

So, we are looking at STD-NMR Spectroscopy. In the last class, where it basically idea is that, you study a weak interaction between a protein and ligand.

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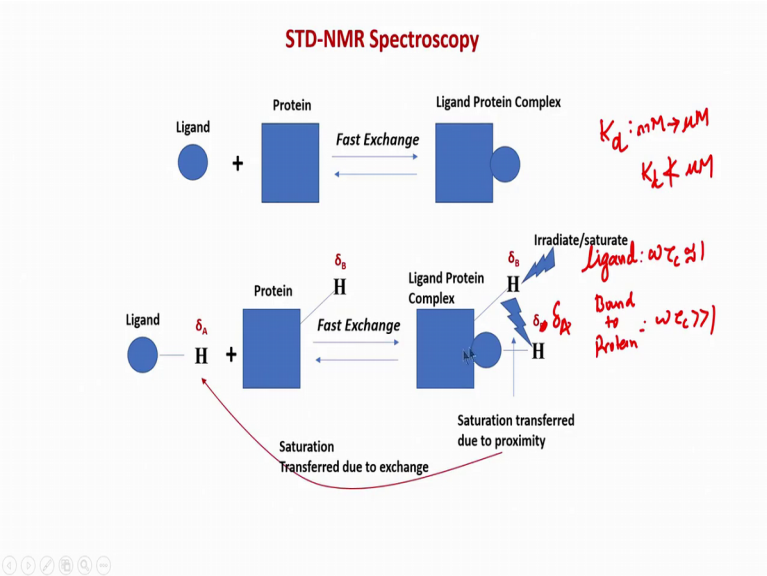

So, this was shown here, where we see that if your ligand is constantly exchanging with the free ligand ok. Because this ligand and this ligand are the same molecule, but now there is a fast exchange. So, when I irradiate a signal of the proton, in the protein. That irradiation is transferred to the hydrogen on the ligand because they will be suppose they are close in space they may or may not be we have to find some proton with which hopefully they are close. And once they are close they get transferred to this proton. Now this proton is nothing, but the same because they are exchanging.

So, what will happen? If you record the spectrum of the free ligand now you will see that this has decreased compared to no protein; when there is no protein and there is a protein the difference you will see that this ligand would have now reduced in intensity, because the saturation here affected the intensity here ok. So, the main criteria therefore is that the signal from the proton or the signal from the ligand should be weak. Because we are

not monitoring this complex, we are going to monitor the free complex. We are not looking at the proton in the bound form because the proton in the bond form will be very low in intensity.

The remember again tau c of the protein is very high. So, therefore, the overall signal of this system becomes very weak. So, therefore, we are normally we will not be able to study the bound form. But we can study the free form because there is a dynamic exchange. So, what we do practically therefore? Take a large concentration of this ligand, because this is weak interaction and the protein concentration is much less compared to the ligands. Let us see this in this slide here.

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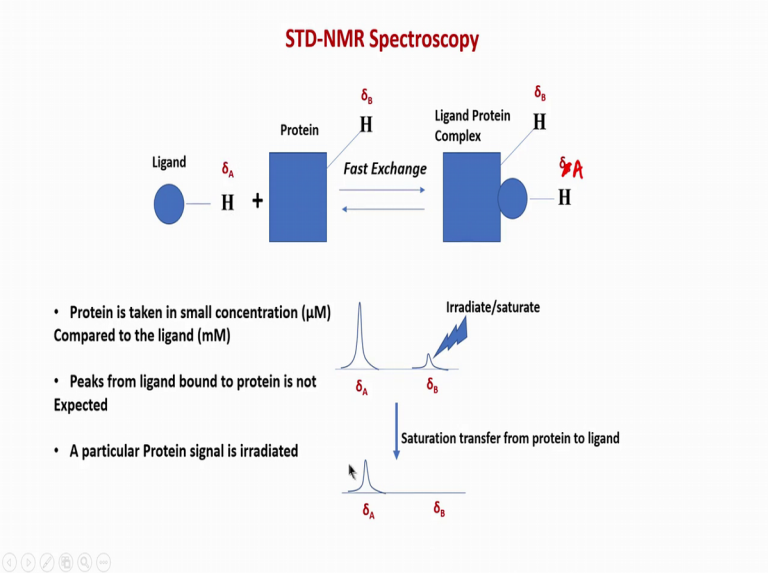

So, this is the same figure here. Again there is a small error this should be delta A ok. So, what typically we do is we take the protein in a small concentration. I mean at small concentration typically is micromolar and the ligand is in millimolar. So, you see there is almost of course, not thousand times. Protein will be roughly around 30 micromolar to 50 micromolar. And ligand will be around 1 millimolar. So, there is a factor of 20 or 30 is to 1 ratio between the ligand and the protein. That means, this is really in very high excess amount compared to the protein ok.

So, now what happens is the peaks from the ligand bound to protein is not expected. Because for two reasons; number 1, it is in a very small quantity. So, it is this protein ligand complex, will also be in micro molar concentration because of the protein itself is

only in micromolar. So, this will be equal to the protein or less and that is one reason. Second reason you will not expect to see the signal from the complex that is in bound form is that the t 2 relaxation of this system will be very short because the tau c of this system will be very high. So, if you recall in the previous lecture I talked about omega into tau c is much greater than 1 and that happens for a large protein ok.

So, therefore, these two reasons make the resulting no signal getting observed from the bound ligand; remember protein is of course, very weak in intensity or further we will not get any bound form because that population is even less because of this dynamic exchange. So, this spectrum will be dominated by the free ligand ok. So, what we do is, we first record a spectrum of the ligand alone. Then we add the protein and then we get the signal from the protein. So, this is just a very brief schematic drawing of a case where the ligand signal is separated from the protein. This is normally happens all the time.

But very sometimes it can happen that you are ligand signal is overlapped with the proteins signal. Because protein is of lot of protons, proteins is not just one proton. So, this is normally huge spectrum where everything will come from 0 to 10 ppm and ligand also comes from 0 to 10 ppm. So, sometimes or many a times it can happen that your signals from the ligand overlap with the protein signal. So, in such cases this very difficult to interpret the data. We want the signal of the ligand to be isolated means, separated from the protein signal. So, in such scenarios STD-NMR is useful experiment.

So, as I said many of many a times this ligand will have aromatic signals, or very downfield shifted amide signal and so on. That will help us to separate the ligand signal from the protein signal. And typically the protein signal which is irradiated now. Remember in the previous lecture we discussed this that we have to irradiate, a signal of the protein which if it is close to the ligand proton will transfer that irradiation. And that irradiation of this proton of the protein is called as saturation ok. So, this is a standard nomenclature used in NOE. So, we saturate the signal of the protein, not all the protons of the protein a particular signal. So, which one typically we do take? We take methyl signals.

So, this protein will have methyl peaks. And the typically the methyl peaks of a protein, will come somewhere between minus 1 to 0 or 1 ppm. So, they are really off field means they are really separated from the ligand signals. So, ligand will may or may not have

methyls. But let us say it does not have methyls, close to the protein. So, the protein will have methyl signal somewhere around 0 ppm. And that is what is irradiated, and the hypothesis or the hope is that this will have some protons nearby from the ligand. And that will transmit that saturation to that. So, that is why we use the word saturation transfer you are transferring the saturation from one signal that is one proton of the protein to the proton of the ligand.

Now, this explains the term saturation transfer. Now difference is there in this name that is we will understand that now. So, this is what is shown here. So, you irradiate the signal of the protein, and that now helps in transmitting the irradiation to this signal of the ligand. So, what will happen is that this saturation transfer from protein will take place to the ligand. And that will result in the ligand signal now getting slightly reduced. Not fully gone because, this is a very high population. Here there is no there is no NOE there is no saturation of from interaction between these two. So, this signal is still there what has gone from here, will be some amount of the bound form which is also present here.

So, because there is a dynamic exchange, this saturation of this hydrogen results in affecting this proton signal and that proton signal is gone in intensity, gone down in intensity. So, you see now if I subtract the spectrum without irradiation before and after irradiation. So, this is after irradiation, and this is before irradiation. I will get a signal from the ligand which is reduced in intensity. This minus this will be somewhat reduced. And that is basically the idea of difference here in the name. I take a difference with and without saturation, and the difference if it is not there, if the there is no difference then there is no interaction because whether I irradiate or not if there is no difference in the signal of the ligand.

It means there is no interaction of the ligand with the protein, but if when I subtract and if I see a difference, before and after irradiation. It will immediately mean that there is some interaction, because of which this change has happened. So, this is why we take the difference of the 1 D spectrum before and after irradiation and therefore, that is called as saturation transfer difference spectroscopy STD-NMR.

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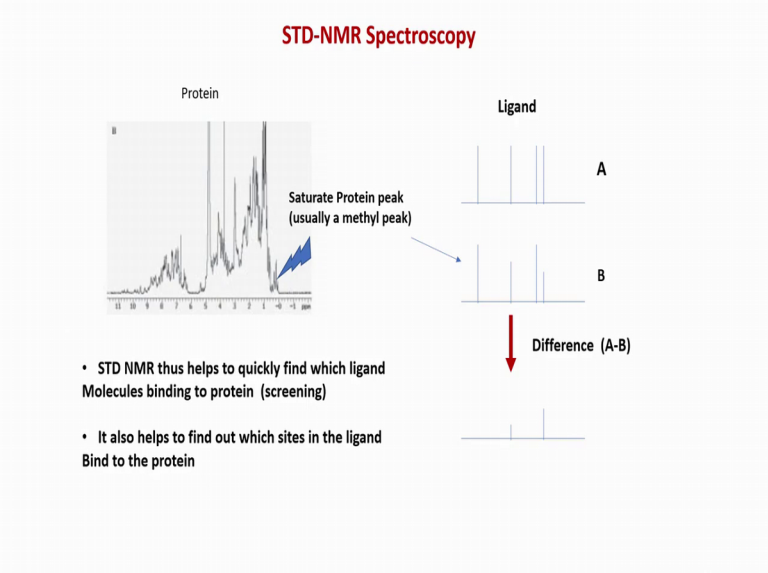

So, this is an example now. So, you can see this is a spectrum of a protein. So, a protein spectrum is really complex. It goes from minus sometime ppm, here it in case is 0 to 10 ppm. So, what we do is we select some peak in this zone, methyl peak. Why do we choose that peak? Because that peak is isolated number one because the ligand may come here or ligand may come here somewhere else. So, ligand peaks are not coming hopefully here. And secondly, methyls are always in the hydrophobic core of the protein and ligands also sometime bind in the deep pockets of the protein.

So, if we expect the ligand interaction in the deeper hydrophobic pocket. Then the methyl signals irradiation will immediately affect the irradiation or signal of the ligand which is also sitting in the hydrophobic pocket. So, that is the expectation and that is what is done here. So, you can see that when I have a free ligand or before irradiation. Let us say this is the signal from the ligand, now ligand signal will always be stronger than protein because, if you recall we use as I said 20 to 30 times of ligand compared to protein. So, this signal will be much 20, 30 times smaller plus the t 2 of protein is also very short.

That will result in a very fast relaxation of the protein and that will cause a decrease of the protein signal. So, what we normally see is only the ligand. Now, after irradiating the signal of the methyl peak of the protein. If there is an interaction between the protein and the ligand as I have mentioned there will be a decrease in the proton signals of the

ligand. This is called saturation transfer, because saturation in saturating a methyl proton of the protein will cause a decrease in the intensity of the ligand protons. Now, if I take a difference between A and B. You see I will get some peaks which are affected because of this irradiation. Not all ligand peaks may be affected because again remember proximity is a key here.

The ligand proton should be closed in space to the protein signals a protein hydrogens, if there is no signal if they are not closed by, that irradiation will have no effect. So, suppose there are some protons of the ligand which are close to the protein protons. That is methyl protons they will be affected; that means, their intensity is goes down here and here, but the remaining protons of the ligand will not be affected. So, if I take a difference or subtract, I will get the peaks of the ligand which are bound to which close to the methyl peak. Not only that this difference automatically tells me, that there is an interaction going on.

Because, if there was no interaction between the ligand and the protein or binding, I would not have expected any difference, this minus this would be simply 0\ because both would look the same. But the fact that there is a interaction or binding immediately is indicated by change in the intensity. So, STD-NMR thus helps to quickly find, which ligand molecules bind to the protein. So, this is again used for screening a large number of drug molecules initially when you do not know which one binds strongly we expect all the molecules to be weak binders.

But initial screening will help to filter out those which even are weaker than the regular compounds and then take the one which are binding and further optimize it to make it stronger. So, not only that as I said it also tells us which sides of the ligand are close to the protein because they are the one which are affected. So, this also helps us to find out which sides in the ligand actually bind or are in the binding interface of the protein. So, this is basically the idea of STD-NMR.

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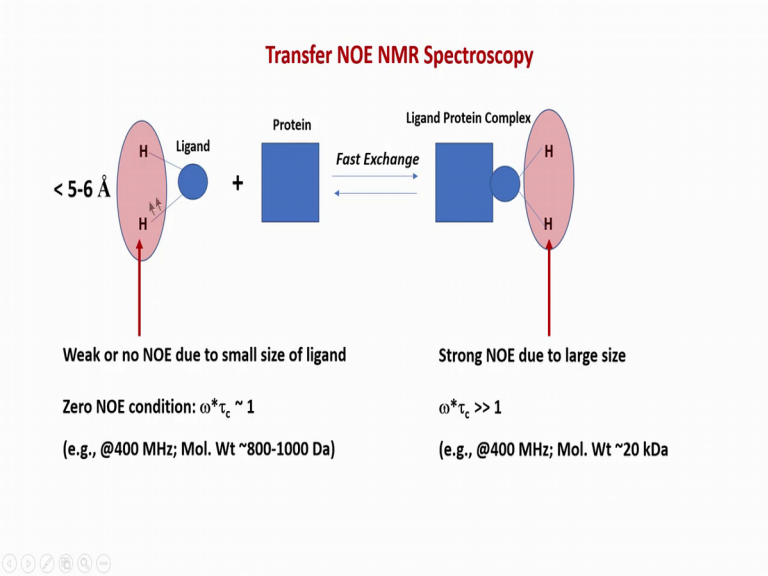

We will look at another technique which is closely related to STD-NMR. This will be the last part of this course where we look at how NMR can be used for weak interactions. So, again and remember that strength of NMR is weak interactions. So, we are essentially able to study weak interactions which normally you will not be able to study with any other technique. So, again let us consider a case of weak interaction a weak binding. So, imagine or consider assume this ligand which bind to the protein and now there is a fast exchange between these two states which means there is a weak interaction.

Now let us say that there are two hydrogens in the ligand which are less than 5 armstrong in space means they are closer in space and they are less than 5 to 6 armstrong in distance between the two ok. So, let us assume again another assumption is, but then ligand binds to the protein this distance does not change much ok. So, that is assumption we will of course, that can change here, but let us assume that this ligand is a weak interacter. So, when there is a weak interaction the distance may not be so much or structure may not be so, much affected for the ligand and that this distance remains the same or similar need not be have to be exactly same.

So, when there is a freely ligand I remember again this is something I showed in the last lecture for a small molecule typically this condition happens depending on your omega value which is your spectrometer frequency, and depending on the tau c value which is a

rotational correlation time. So, for a if you are working at 400 Megahertz NMR spectrometer and your molecular weight of your ligand is somewhere in this range. Then this omega into tau c becomes equal to 1 then there is no NOE here. You see therefore, you do not expect to get any NOE effect, in the ligand free ligand because of the small size.

But now here there is a strong NOE effect because of the large size. Because this complex is now behaving like a, the ligand behaves like a large molecule because it is attached to the protein. Even though the attachment is not strong it is a dynamic exchange. Whenever it is attached to the protein this complex now is becomes large and the ligand behaves like a large molecule so, that will cause result in omega into tau c, becoming much much greater than 1 for the same spectrometer. So, we are not going to change, but molecule now is larger because it has been bound to a bigger protein. So, this is 20000 Daltons compared to 1000 Dalton, 20 times or 30 times sometimes the protein size is even bigger.

It is about 100 Kilo Daltons then, the NOE is much higher. Now, second assumption which this is one assumption, assumption means where hypothesis is that there is a increase in the tau c of the protein which we know and that will cause higher NOE. Another assumption is this distance does, not change much. Means compared to the free form. So, if it is 5 Armstrong here we expect it to be close to 5 here as well ok.

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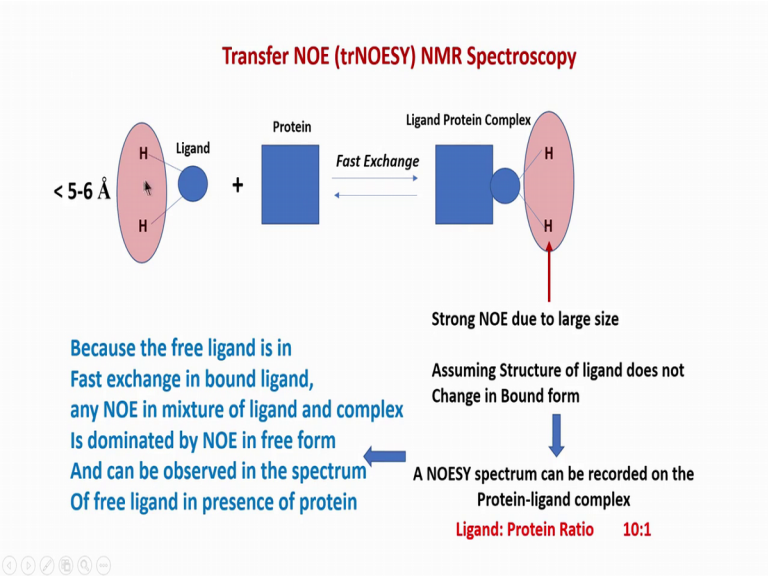

So, now given these conditions, what will happen in a Transfer NOE? So, this is called Transfer NOE spectroscopy. So, transfer NOE is basically similar to STD saturation transfer. Here we are transferring this NOE effect from here to here. Because the broad idea here is, that this molecule has no NOE. But this molecule has the same ligand becomes has shows an NOE effect. But it does not show an NOE effect here. So, by having a large excess of this in my sample, and a small amount of this my population of this is going to be small. But whatever NOE effect is shown here, will be manifested or seen in the NOE between here because this is a transfer between here to here because the ligand is in fast exchange ok.

So, whenever there is a fast exchange this NOE effect here, shows up in this molecule. Even though this molecule is not having any NOE. So, we will see this, this looks like a contradictory statement or confusing statement, but we will see how nicely this shows up in a spectrum. How we can get the spectrum of this molecule like this ok? So, what typically is done is a NOESY spectrum can be recorded on this complex, I mean the entire complex. Once you add the protein and as I said the ligand is in large excess, compared to protein. Typically we take 10 is to 1. Means the ligand is 10 times higher in concentration, compared to ligand ok; sorry ligand compare to protein.

So, ligand is 10 is to 1, 1 is protein. Now what happens is, because the free ligand is in fast exchange with the bound ligand, any NOE in the mixture of ligand and complex is dominated by the NOE of the free form. So, this is what the point is that any NOE effect I see in the ligand complex form will be basically this NOE effect because there is no NOE for this. And it can be observed in the spectrum of the free ligand. The NOE effect here is seen in the NOE of this spectrum, even though this one on its own does not have any NOE effect ok.

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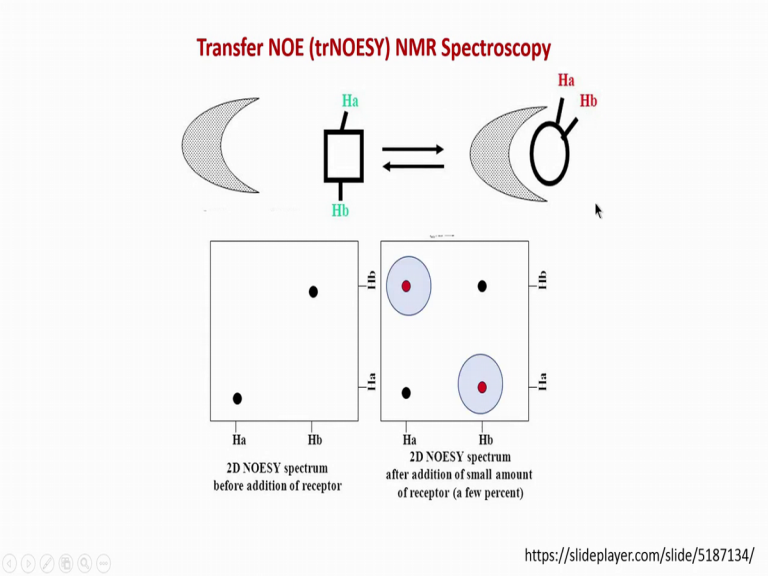

So, let us see in the spectral spectrum how it looks it looks like this. So, this is let say there are two protons of the ligand and this is the interaction. When the protein shown here in a gray. Now these two hydrogens of course, here we are showing there is a difference means this as shortened here. But normally we assume that the distance between this and this need not change. So, we can see here. So, when I record before addition of the receptor or the protein. Again this is been taken from this slide here, from this website. So, we can go to this website if you want to have more details.

But the basic idea is suppose I record the 2 D NOESY spectrum before adding the protein; that means, for the free ligand. I do not expect any cross peak between A and B. Because there is no NOE effect the omega into tau c. Maybe close to 1. So, therefore, the NOE effect is 0. So, therefore, there is no cross peak between the A and B. But moment I add the receptor or a protein and I record the spectrum again, you see there is a cross peak coming now because the cross peak is not from this population. The cross peak is yeah is dominated by this population. But we are having more of this. So, the actually the whole spectrum is actually this of the free.

But the NOE effect is coming from the bound form. So, it looks as if the NOE of the free has now appeared. But actually it is not the NOE of the free form it is the NOE of the bound form which is appearing on the spectrum of the free form. This is that is like a transfer you transfer the NOE effect from here to the free form. So, that is why we call it

as a transfer NOE NMR experiment ok. So, this is very interesting experiment it helps us to find out the structure of the free form. Now from the NOESY here, remember NOESY is for structure determination of either organic molecule or protein molecule.

So, from the NOE spectrum no earlier I could not have got structure of the ligand, in the free form because there was no cross peak. So, no information of the distance remember distance comes from the intensity. But now I got an NOE cross peak, and based on that I can convert it into distance and then distance between A and B I can find out, in the bound form. Because again remember this NOE is not from the free it is from the bound. So, I can get the distance between these in the bound form, but the idea is it is appearing in the free form. So, the distance as I said assumption is they should not change much, but even if they change my information is still coming from the bound form.

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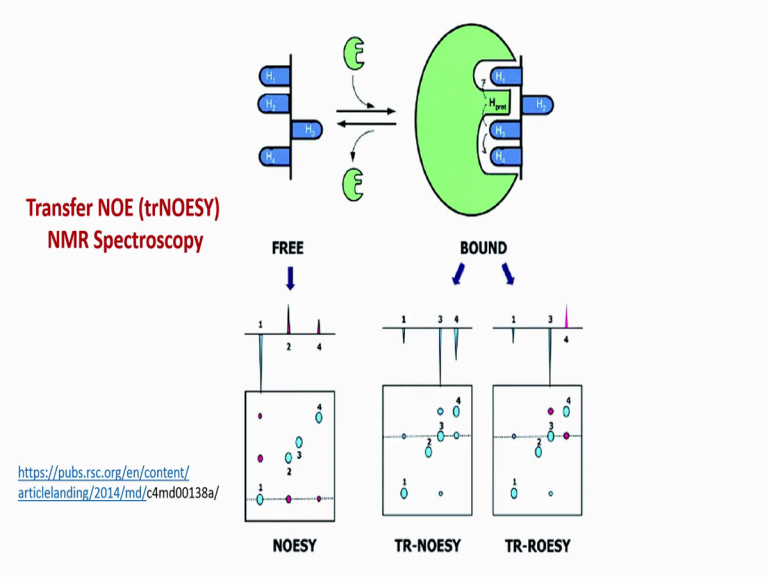

So, this is what is shown here. So, this is another example taken from this website now. So, here you can see again this is a ligand which is having some structure like this. There are different hydrogens H 1, H 2, H 3, H 4. Now in the free form it is showing some spectrum like this. And there is a negative NOE, remember NOE can also be negative, because omega into tau c can be less than 1, or then it can show an opposite NOE or more than 1 sorry.

Now, but the point here is between H 3 and H 4, here I am not getting NOE here you see between 3 and 4, there are no cross peaks. Why? Because 3 and 4 are although they may

be close, they may not be having NOE effect. But if I add a ligand to this now 3 and 4 are come close to each other. And because this is a large complex, the NOE becomes much stronger compare to here. And we can see now I can see a cross peak between 3 and 4 here also this is just a two different experiment. But the idea is between 3 and 4. I am now getting a cross peak which earlier I was not seeing between 3 and 4.

Not only that I am also able to see this small peak 1 and 3. We will see this between 1 and 3, this and this that was not there here in this case. Why? Because 1 and 3, now have an intermediate proton from the protein molecule, which will give me the transfer of NOE from 1 to 3; so, 1 to 3 also showing a small cross peak. But now between this proton has gone on this side. And therefore, there is no cross peak between 2 and 3 or 1 and 2. 1 and 2 have become far away. So, we can see between 1 and 2 there is no cross peak which was there earlier here ok.

So, basically this is the idea of transfer NOE experiment. Wherein you look at the bound, you look at the NOE in the bound form. But what you are monitoring is the signals from the free form because the signal is much higher in the; ligand is much higher in concentration. And therefore, that helps us to integrate get the indirect information, by looking at the free form we are getting the information of the bound confirmation.