NMR Spectroscopy for Chemists and Biologists
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Lecture 58
Protein-Ligand Interaction- I

Welcome to today's lecture. Now we have given you glimpse of how to determine the peptide structure. And you have some idea why it is important. Next is what to do with this structure is known? So if you look at the whole drug industry, is after getting a target for protein. Because you know, proteins are workhorse for our body. Anything and everything that you imaging is done by protein. We are walking, done by protein. If our heart is beating, it is all protein's action. If our eye movement is happening, it is because of protein. So your oxygen is carried to different positions by this protein. So protein is very important target.

And therefore, whole pharma industry is looking for target, one target that can particularly affect a protein which is corrupt in a digit case. So therefore, the protein drug or protein-ligand interaction becomes very important, because proteins are proteins are communicator, proteins are worker, proteins are everything. So protein-ligand interaction is very very important for the pharma industry. But pharma industry actually, is generally does not work like our labs.

So in lab, we can take one thing and go in detail and study very slowly to what function is happening. But pharma industry many times requires a quick way to find it out whether this molecule will work or not? Should I invest so much time, money and energy in identifying the molecules? Therefore, a high throughput process is always desirable for pharma industry, to find what will work and what will not work; what should be discarded.

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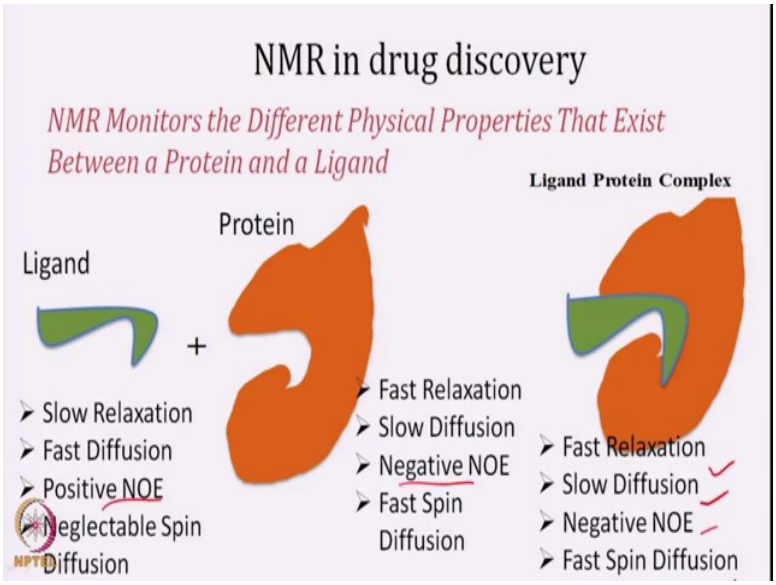

So NMR over the years has emerged as one of the important parameter in drug discovery. And today, we are going to monitor this aspect of NMR spectroscopy- how you can use the NMR in drug discovery especially suited for pharma industry. So as I said, it is all about protein-drug or protein-ligand interaction. Protein is bigger molecule, ligand is smaller molecule. How we can understand- if this ligand interacts with protein. And suppose we are given a soup of ligands, which ligand will interact with the protein. So that we can take this, optimize this to make a better efficiency drug.

So now, we got all the different parameters that can be used to identify or to monitor the property of ligands. So here, NMR can monitor different physical property that can exist between a protein and a ligand. And that can be used for finding a better optimized ligand in drug discovery protocol. So let us start.

As we said, ligand is a small molecule. So small molecules means it is a slowly relaxing molecule. And if it is slowly relaxing, then its diffusion is very fast. So in solution it is tumbling very fast. We had previously discussed small molecules that means it will have positive NOE. And then will be like there are not too many protons here, so spin diffusion is also going to be very very slow. So we have negligible or neglectable spin diffusion. Whereas protein, generally this is many KD, so this says few Dalton, 1000 Dalton or so. So it will be 100 kilo Dalton.

So proteins are bigger molecules. So bigger molecules, that means they have fast relaxation, okay and since they are big, so they are diffusing slowly in solution. They have relatively

negative NOE, bigger molecule. If you remember our previous classes, we have discussed that big molecule will have negative NOE and then small molecules have positive NOE. And there will be lots of spin diffusion because the proton network is very very dense. And that is how spin diffusion will be quite a bit.

So now suppose this ligand interacts with protein and make a complex. So now ligand is small, protein is big. So the resulting complex is going to be big. So if this resulting complex is big, we have a fast relaxation. It is similar like a protein. It will have slow diffusion because molecule, now overall molecule has become big. And it will have also negative NOE and it will have fast spin diffusion. So it will acquire all the property of a protein molecule.

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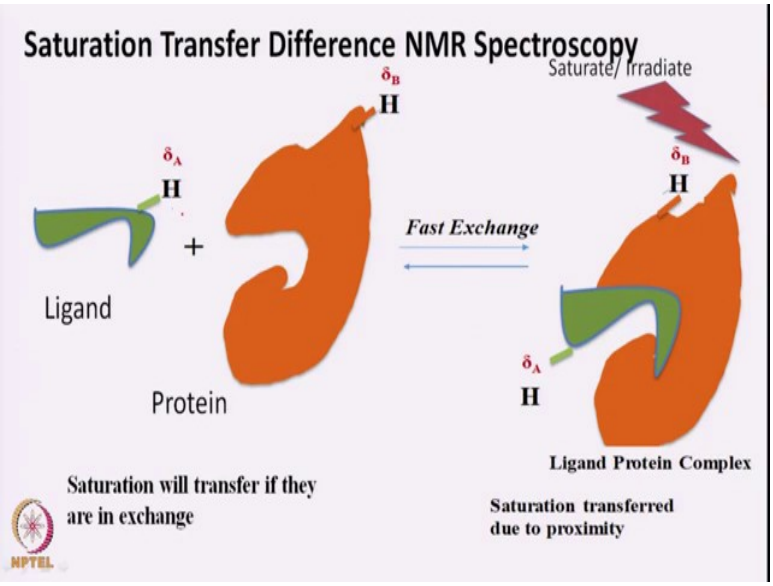

Now, what we are going to do is concept that we have discussed earlier. So if you remember, we have said that if 2 spins are somehow interacting through a space. You perturb this spin; the effect of this perturbation will be seen on that spin. Like polarization transfer we had discussed earlier. The similar concept is going to be used in something experiment called saturation transfer difference NMR spectroscopy. Or in short form it is called STD- saturation transfer difference.

So what we are going to do- suppose our ligand, which has some protons and protein which has some protons. So chemical shift of this proton H is δ_A and this chemical shift is δ_B. Now, if suppose this is binding in a micro molecular range. And it is fast exchange. So ligand is binding, going off in a micro second time scale or fast exchange. So that is the complex that we are going to do. So now, suppose we do some experiment or some trick.

Suppose the are two are interacting. And we saturate selectively this signal of proteins. The effect of that situation will be seen on the chemical shift of ligand, okay. We are saturating protein signal. We are seeing effect of that saturation on ligand signal, if these two are interacting. And this precise concept is used in a technique called saturation transfer difference.

So, now situation will be transferred if they are in exchange and they are in proximity. If they are not in exchange or if they are not interacting, they are not in close proximity, then effect of saturation on protein will not be transferred on the ligand. And they will be not interacting. So that is what we are doing.

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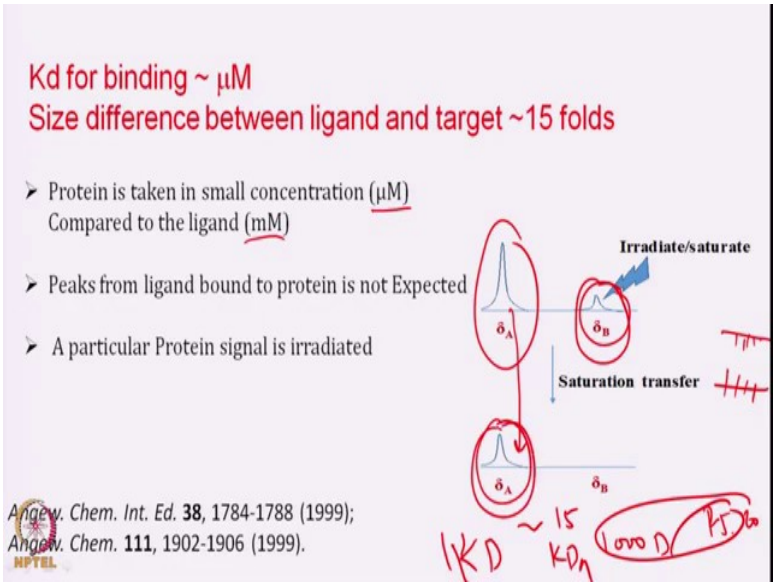

So the K_d of such kind of process should be in μM. And size difference between ligand and target should be at least 15 folds. What I mean, if your ligand is say 1000 Dalton, your protein should be more than 15 to 20 kilo Dalton. If it is more, it is better. Otherwise, the effect will not be seen. So 1 K_d is ligand and 15 to 20 kiloDalton is protein, then we can do this experiments which is called STD. And binding of this should be around micro molar range. So you have some idea of binding.

Now protein is taken in a very small concentration. You do not need very large concentration of protein. You just need large concentration of ligand. So if you take protein concentration say in few μM and ligand concentration in mM, we can do this experiment. Now what we are saying, since this concentration is very low, so we will not get any signal coming out of the protein. We will only get the signal coming from the ligand.

Now say this is my signal from protein. And this is my signal from the ligand; I just plot it for you. But this signal, we are not seeing it; just for representation I plotted it. Now we are irradiating this signal from protein, which is delta B is irradiated, saturated. So saturation means, you are making the population here and here equal. So that means this peak will disappear after saturation.

Now since these 2 protons are in exchange and also in close proximity, the effect of saturation particularly at this position, you are going to see here. Therefore, the intensity of this peak will decrease; intensity of this peak delta A will decrease. So now, by saturation of peak of a protein effect is seen on the ligand peak. And that actually will show up in reduced intensity of the ligand. And this method was developed by a group in Germany and published in Angew Chem. So this is called STD. So let us discuss what actually we are doing here.

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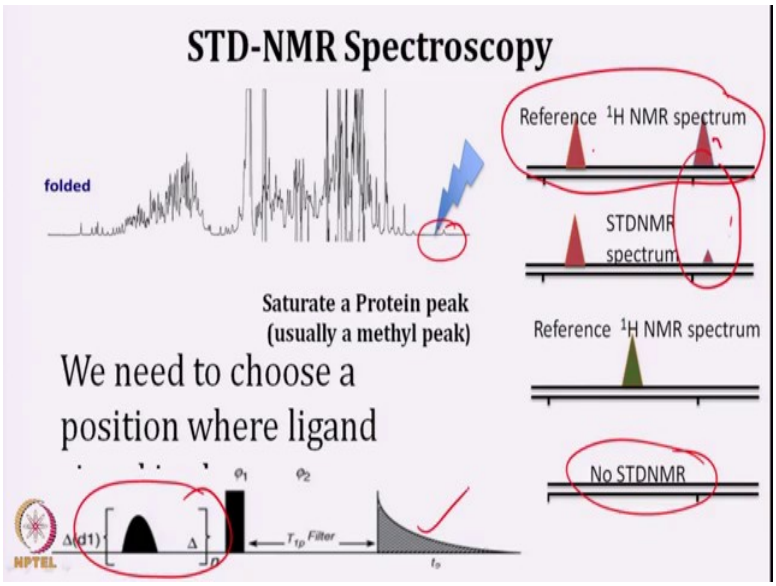

So we are saying, say this is protein signal, if we take in high concentration, so this is protein signal. Now we have to saturate a signal which is not a ligand because we are selectively saturating out protein signal. So suppose we are saturating somewhere here quite up shifted like -1, -2 ppm, and effect of the situation we are going to see on the ligand signal. So this is say our reference spectrum for the ligand to equal intensity peak we are getting.

Now we saturated the protein signal. And all the protons of the ligand which were in close proximity of the protein, there intensity will decreases. So here if you look at, this intensity has decrease, this has not changed. Now if we take a difference of these 2, then we know that

okay, this we will cancel it out and only signal from here will appear. So we know that this proton is interacting, this proton is not interacting.

Similarly, you can take another example. Here is the reference spectrum. If there is no STD, means no interaction, then you do not get any effect. And therefore, it would take a difference; you do not get any signal, okay. So that is what you see. If it is interacting in the difference spectrum, you will see some peaks. If they are not interacting, that means there is no peak. That is what easily just by recording 1D spectrum of ligands in presence of protein, you can find it out whether this ligand is binding to particular protein or not.

So things to remember- we are collecting 2 experiments. One is reference spectrum, where we are not saturating protein signal. One, where we are saturating protein signal and recording this spectrum. Now, we are taking the difference of these two reference spectrum and the saturated spectrum. And if the peak appears, that means we are; this ligand bind to that protein and we are getting therefore a signal.

So we need to saturate protein peak. Usually this is methyl position and that we can find it out just by knowing where the ligand signal is not. The pulse sequence is something like this, okay. So, 2 experiments are done- 1 saturated 1 non-saturated. And this is done by, for saturating. Then you just apply a 90˚ pulse. Then, this is for removing the protein signal. And essentially record it.

So saturation followed by 90˚ and then recording in one experiment you do not saturate and in another experiment you saturate. Take the difference and the difference peak will tell whether it is binding or not.

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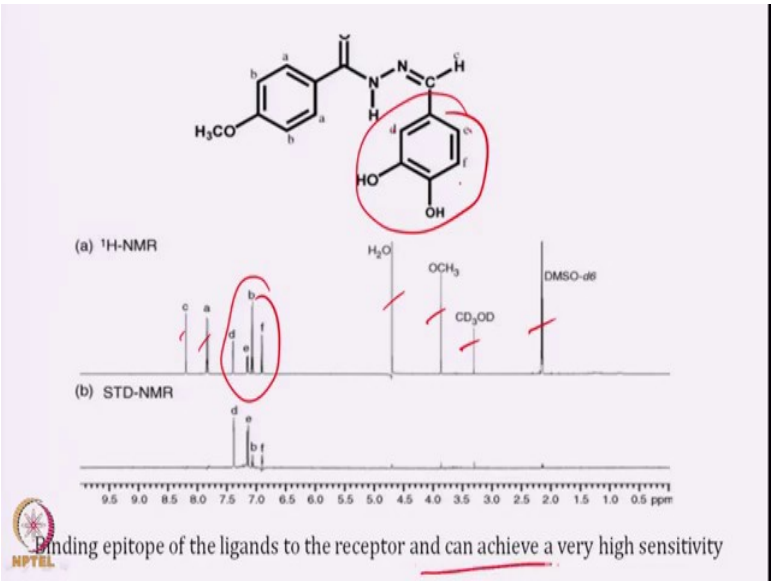

So just for an example, I am showing it. Say here is my ligand which is binding to a protein. So, in first experiment we recorded proton NMR spectrum and we get the chemical shift for each of these protons right, *ABC* you can see here. Now; then we saturate a signal from protein and then subtracted that signal from here. So if you look at this, this, this, this, they are anyhow coming from solvent. They are almost vanished. And here also, this peak and this peak vanish.

However, *DEBF*, these peaks appears that means, this ligand interact with a protein at *DEF*, these 3. So this moiety interacts with the protein. This moiety does not interact with the protein. So precise, in beautiful way just by recording 1D spectrum, we can find it out binding epitope of the ligand to the receptor. And that can be achieved with a very high sensitivity in a very quick manner, just 1D, 1D means few seconds. In few seconds we identify which moiety of the ligand is interacting with the protein. That is what is STD.

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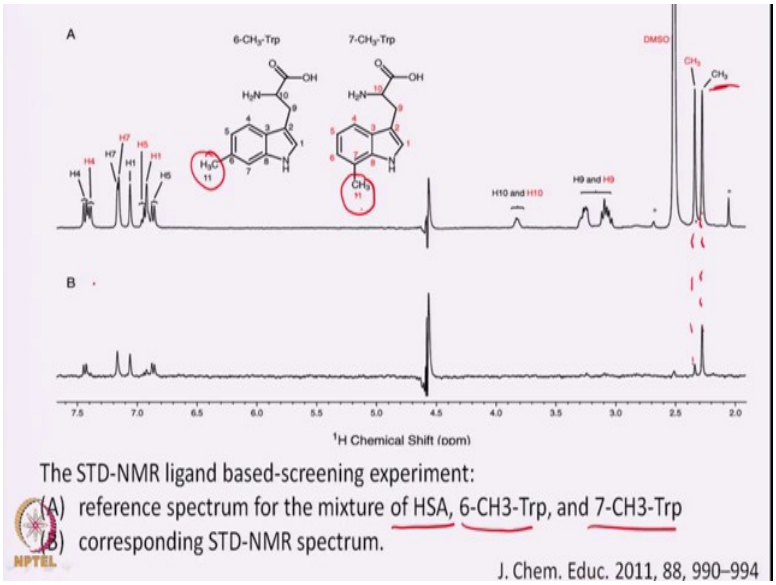

For another example, let us take a mixture of these two. And we want to find it out which one binds. So if you look at here, the mixture has only, like only 1 difference okay. So difference is essentially here, this. Rest everything, if you look at is same right, all the moiety is same. So only this CH_3 is shifted here. So 6, 7 change is there.

Now these 2 ligands, you are given a mixture and you want to find it out which one of them is actually binding to the protein- whether methyl at 6th position or methyl at 7th position. That is what we have to identify, which one is binding with what. So we recorded a spectrum. The 1D proton spectrum and we can identify the radar coming from this 7th methyl tryptophan and 6th is coming from 6th methyl tryptophan.

We record a spectrum. We identify these peaks. Then we record a STD spectrum, means by saturating protein peak, recording 1D spectrum. And we find that here are some peaks which are appearing. So here if you look at these peaks are appearing. Now we can find the difference is a mixture of protein which is HSA and 6th methyl tryptophan and 7th methyl tryptophan and then, corresponding now you can find it out the spectrum.

So if you look at here, this is bind. So if you look at this methyl seems to be strongly interacting, like 6th methyl tryptophan seem to be strongly interacting with HSA compared to 7th methyl tryptophan. And one can get other protons as well. So this 7th does not interact, 6th interact.

So now from the mixture of these 2 compounds, just they are isomers; position is different- 6 to 7. One can find it out that which one interacts with the protein, which does not interact. And that is, just within a few second one can identify ok. So now that was, we started with one. We now looked at a mixture of two. Similar thing you can do with the mixture of three or even four.

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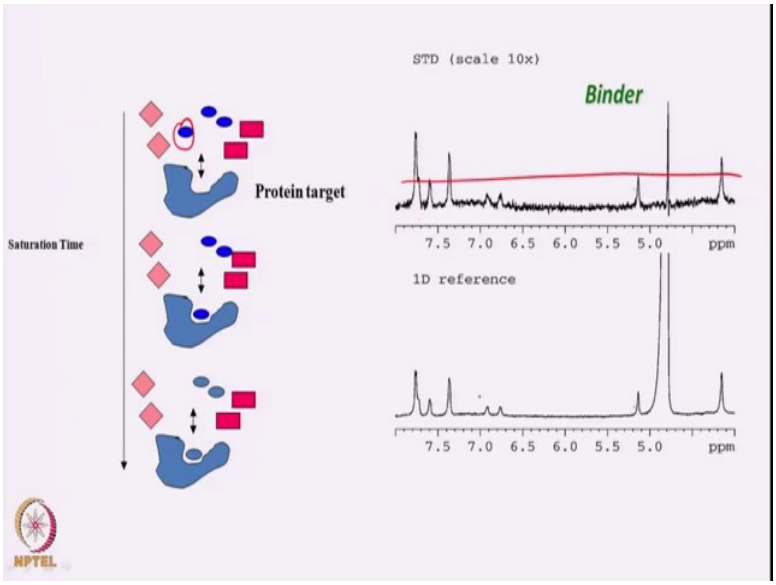

Suppose we have a mixture of these many compounds. And then one of them is binding depending upon what is the activity site and how ligand can fit it. So we keep increasing the saturation time. And now what will happen that a binder will show the STD peak here and non-binder will not show. So non-binder there will be no peak coming from these two sets. Only for this will be shown here. So a binder will show a peak. Non-binder will not show a peak. And therefore, even from the mixture of the compound, you can find the appropriate ligand.

So see; now this is, this can be used as throughput screening method for finding it out which ligand is binding to protein and which does not bind to protein. And now that can, even was a ligand. And if you look at the non-binders, like here say there are just flat peaks that means something like this is not binding. Reference spectrums for these guys are like this and reference spectrum is very well spread. So here you can see each of these peaks are presenting a STD spectrum.

Again reminding you, STD spectrum is a difference spectrum where we are saturating and where we not saturating. So non-saturation minus saturation is giving peaks, means these molecules like blue molecules may bind.

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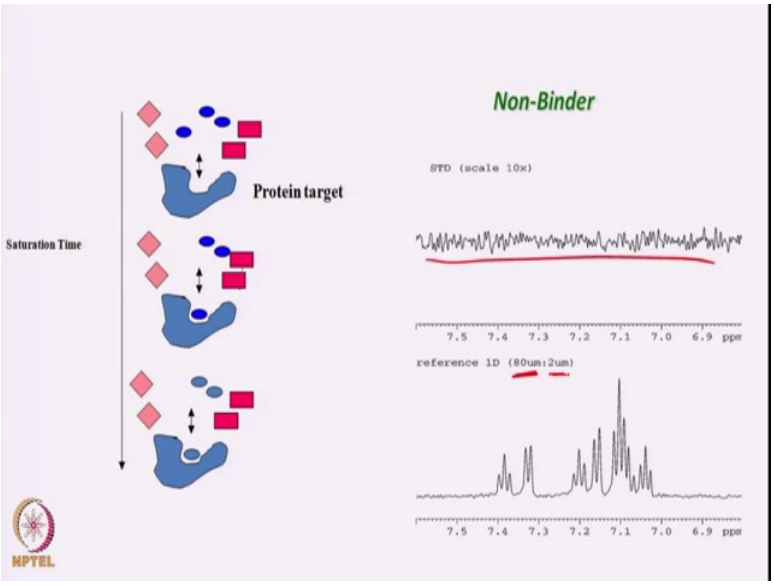

And here, the red molecule or pink molecule is not binding. Therefore you do not see peaks in these regions, where the reference spectrum has a peak and this does not have. And if you look at, this is just a very small molecule, concentration of the molecule we are taking- 80 nM of say binder or the ligand and 2 mM of protein. That is what you need just for finding it out. So with a very low concentration of ligand-protein, now we can and from the mixture of the compounds, one can find out a binder and a non-binder. And therefore, it is high throughput method for screening of a drug molecule.

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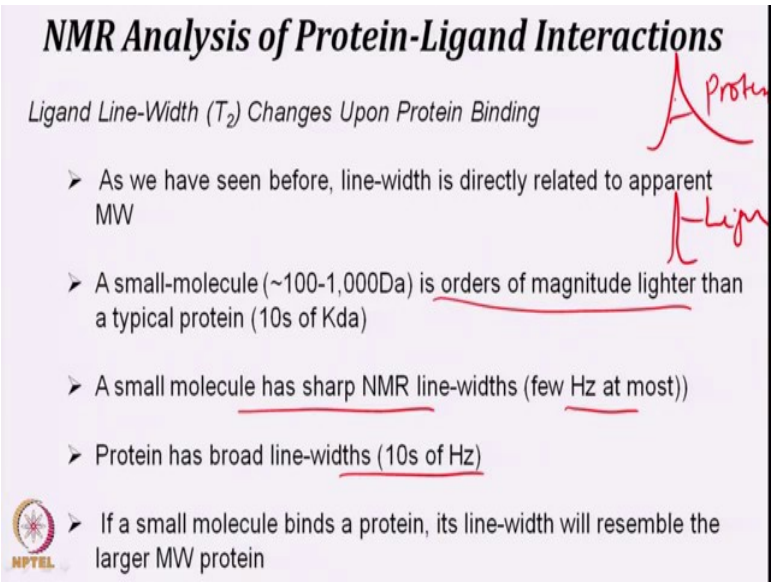

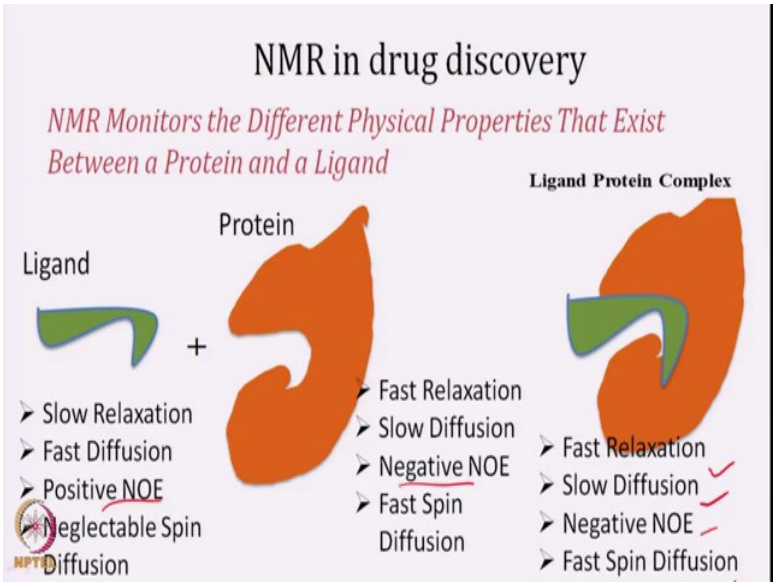

Now other thing that we have said can also be used for finding it out whether it is binding or not. So if you remember, I said there are many parameters that can be used like here. So one thing was ligand being a smaller molecule, relaxes slow. Protein being a bigger molecule relaxes fast. So if that is the case, can we use this relaxation property to identify whether this molecule is binding or not. That means relaxation property which one, T_2, the transfer relaxation rate.

And transfer relaxation rate, if you remember, it is encoded in the line width. If a molecule is relaxing fast that means lines are broad. If it is relaxing slow, lines are sharp. So here is a line for protein and here is a line for ligand. Now we are going to exploit this phenomenon of line shape to find it out whether this is, the ligand is binding to protein or not. So as we have seen before, line width is directly related to apparent molecular weight- High molecular weight, broad line; small molecular weight, sharp line.

So small molecule like 100 Dalton to 1000 Dalton the order of magnitude lighter than the typical protein of kilo Dalton, kilo Dalton protein will have a broad line,a small molecules sharp line of few Hertz. And then proteins have broad line, of say few like 10s of Hertz or 100 of Hertz. So if a small molecule binds to a protein. Now as we have seen- so this whole molecule will now tumbles slow and behave like a bigger molecule. Therefore, the line will become broader. So line sharp for smaller molecule, protein line broad and complex will be line broad. And that is what we have.

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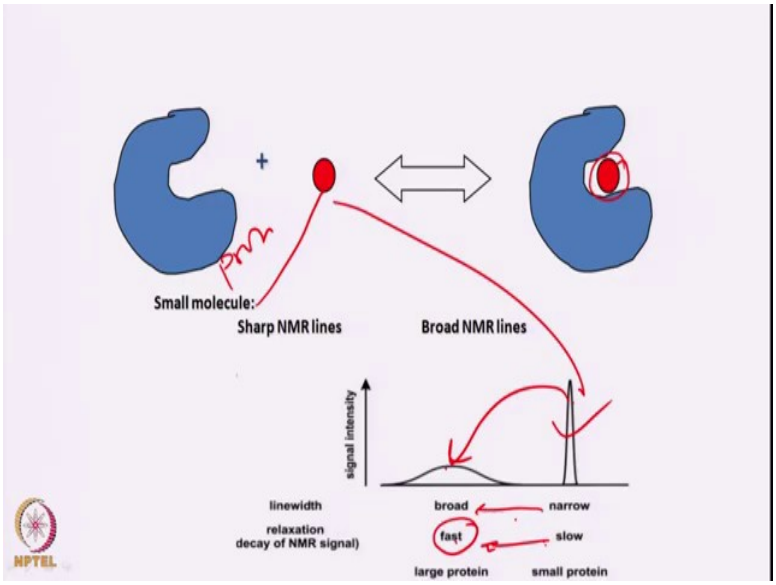

So if here is my small molecule. This is my protein. This will have sharp line like this. And typically this should have broad line like this. So now, if the complex formation happens, because of complex formation; now we are looking at the line of say ligand which was earlier sharp line. Now because now it is slowly tumbling, it gives to broad line.

So line width increases because of binding from narrow to broad. So it was earlier fast tumbling. Now it becomes slow tumbling. So tumbling also changes, line also changes. And that gives an idea, now this ligand is binding to protein. So upon complexion, more ligand lines will become broader. And one can identify just looking at the line that whether this is binder or non-binder.

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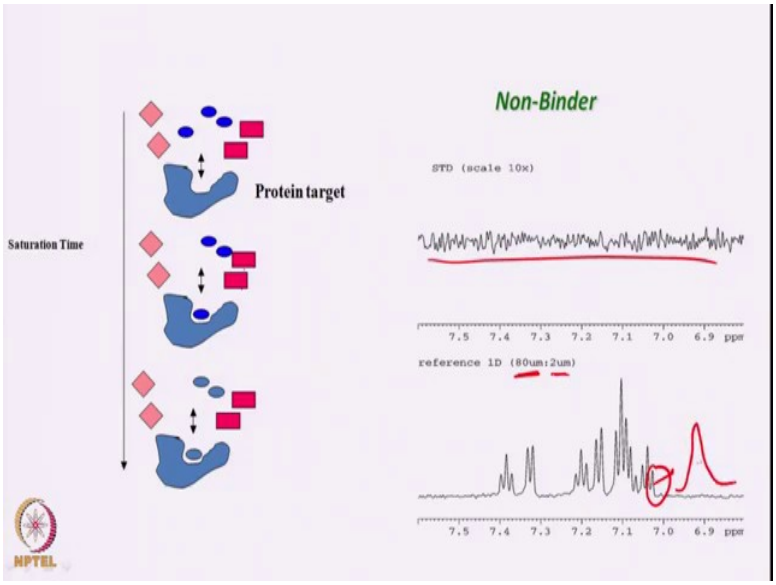

So like here is non-binder- lines remains same. If, suppose this was binder, lines becomes relatively broad. And just looking at the lines, one can find it out whether it is binder or non-binder, just by looking at the lines one can find it out- binder or non-binder okay.

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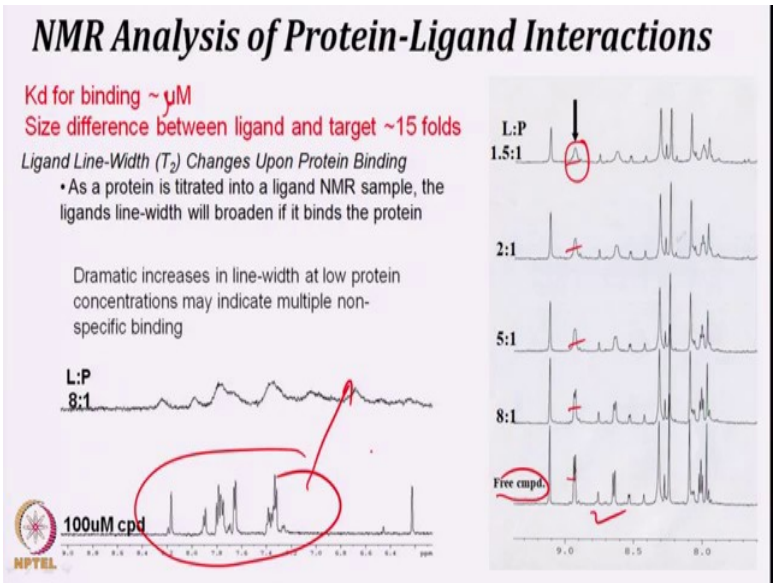

So now we just we had looked at NMR analysis of ligand-protein interaction. Here if you concentrate it, say free compound, we have a free line. So K_d should be, in typical in µM range; your µM range K_d should be. And size difference between ligand and protein, as I was saying it should be minimum 15 folds. So ligand line width will change upon binding. And as you keep titrating; so you have say, 500 µM of ligand and just 5 µM of protein. Now if you keep titrating it.

So if you keep titrating, what will happen? You just look at this line, follow this line. Now this line was quite sharp. Here two lines are sharp. If you increase here, ligand to protein; now if we are coming one to one, line becomes really broad. So this says that now this protein-ligand interaction shows dramatic increase in line width happens. Now at the low protein concentration, it means that binding is not there. So if you increase here, say ligand and if you go to ligand-protein concentration, line becomes dramatically broad and that says that this ligand is interacting with a protein.

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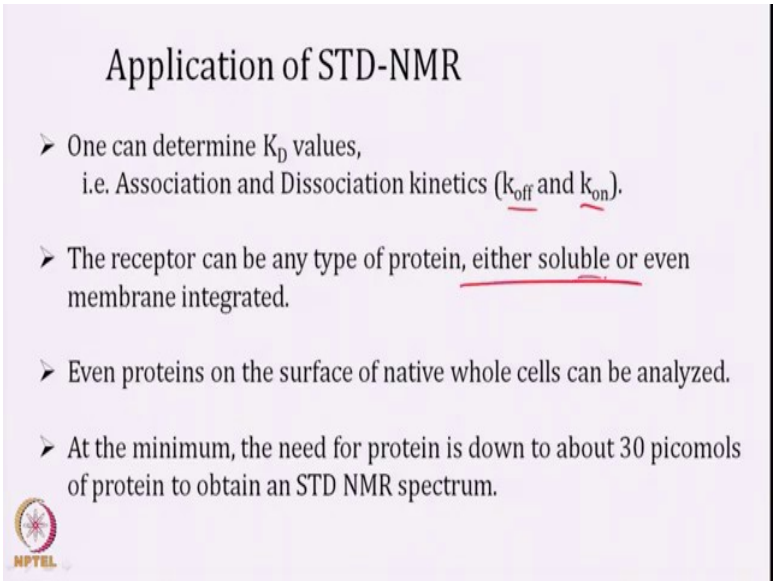

Great, so application of STD, so what all you can do by saturation transfer difference, you can essentially determine the K_d of the ligand, dissociation constant. So you can find it out, association constant, dissociation constant kinetics- k_{off} and k_{on}. And then one can find it out, any type of receptors in protein, either it is soluble or even membrane integrated. You can find the ligand for a receptor, whether it is integral membrane protein or a soluble protein, one can identify.

And even proteins which are embedded on the surface of whole cell, you can analyze by STD NMR and find it out whether there is ligand for that or not. And one good thing is that you need very less protein concentration, just say about few μM or even pM is good enough for obtaining the STD NMR signal, okay. So that is what you can do by STD NMR application. So I hope this concept can be used in pharma industry to identify it.

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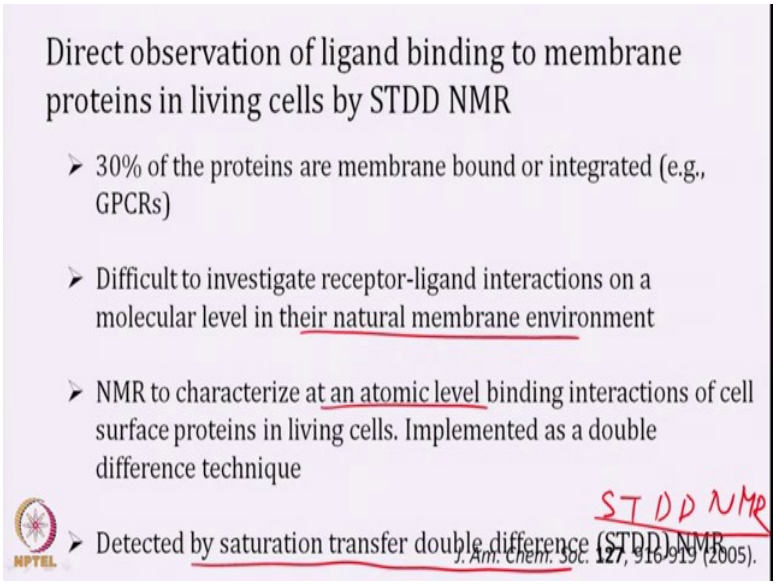

So I will go further and then we find it out, now can we direct detect the ligand binding to a membrane protein in living cell. So this topic, because this is very very interesting, you see, the soluble proteins are okay, you can find it out. But billions of dollars revenue is just by targeting G-protein coupled receptors GPCRs. They mitigate the all signaling and 30 percent proteins are actually membrane protein. Many of them like GPCRs and all those. So they are difficult to investigate, what is the receptor ligand interaction because these proteins are not easy to purify, for making it soluble and looking at the binding affinity. They are difficult to purify, difficult to reconstitute and get it for structure determination.

But STD offers very quick methods without perturbation of the protein environment, to find it out what will be ligand-protein receptor interaction. So all the difficulties associated with membrane protein can be tackled by STD NMR. So what one can do here, we can use the atomic level binding interaction of cell surface protein in the living cell. But we are looking at the ligand all the time. And this is done by something called saturation transfer double difference, STDD. So this is STDD NMR.

So now I am going to explain you what actually STDD NMR is –saturation transfer double difference for studying the membrane embedded protein or protein immobilized on the cell surface and finding the binding epitope on the ligand, how we are going to do this. So this is very interesting. Hope to see you in the next class. And I am going to explain in detail, how you can use this STD technique to finding it out these kinds of interaction of whole cell. Thank you very much and looking forward to seeing you in the next class.