NMR spectroscopy for Structural Biology
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Lecture: 42
NMR Analysis of Ligand Specific Parameters in a Protein-Ligand Interaction -I

So, good morning. This week we are we are trying to understand protein-ligand and protein-protein interaction. So, in the last class I explained that why protein-protein interaction is important because all the cellular communication happens through protein-protein interaction or some interaction of proteins with other molecules; and we looked at different methods that can be used for protein-protein interactions.

So, there are two important aspects of protein-protein interaction to understand are: what is the thermodynamics involved there and what kind of structural change happens. So, for thermodynamics, we looked at some of the techniques that can be used to derive the thermodynamic parameter like isothermal titration calorimetry (ITC) or Surface Plasmon Resonance (SPR).

These are the two widely used techniques for deriving the energetics or thermodynamics involved in protein-protein interactions. We also looked at some of the structural techniques that can give this the structural aspects of the complexes like X-ray crystallography, cryo-electron microscopy, these two are high resolution techniques. Other than that like a SEC - size exclusion chromatography, Analytical ultracentrifugation, the dynamic light scattering, static light scattering, some of these techniques can give you shape and size of the complexes that are formed.

So, you can decipher some of the characteristic of the complexes that forms upon interactions. And I tried to end why NMR is the better techniques because it gives both these parameters the thermodynamic parameter of these interactions as well as structural parameters of these interaction. So, NMR is uniquely placed to understand protein-protein interactions and also looked at what are the requirement for that.

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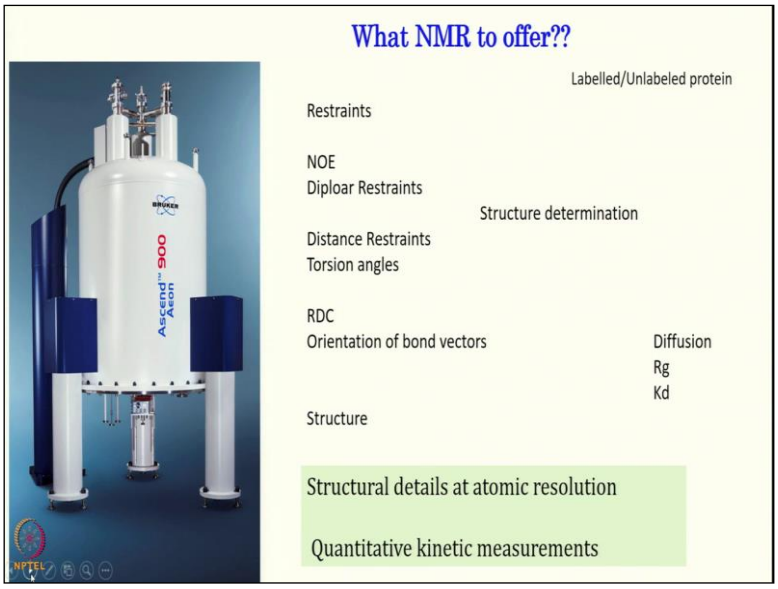

So, we will be starting from there, what are the requirement for the NMR spectroscopy and just to rewind a little bit, what actually NMR can offer either from the labelled protein like $^{13}C^{15}N$ labeled protein or an unlabeled protein. So, you can elucidate the structure using some restraints. Restraints can be NOE based restraints, nuclear Overhauser effect that measures the through a space distances between these two nuclei. You can measure the dipolar restraints using like using RDC

and all those. So from these you can measure the distance restraints, torsion angle restraints and orientation of the bond vector. So, using all of these restraints one can get the high resolution structure of the system in solution if you are doing solution state NMR right. And then we can also measure the diffusion using some of the like techniques that NMR has called DOSY where you can measure the hydrodynamic radii.

Some additional techniques can be used to measure the Rg like MD and all those can be used and NMR also can offer the dissociation constants. So, this we are going to now discuss little more detail. So, structural details using NMR can be found at atomic resolution and then we can also get the quantitative kinetic parameters for such measurement, therefore we can say that NMR is uniquely placed to offer the structure as well as the thermodynamics of the protein-protein interactions.

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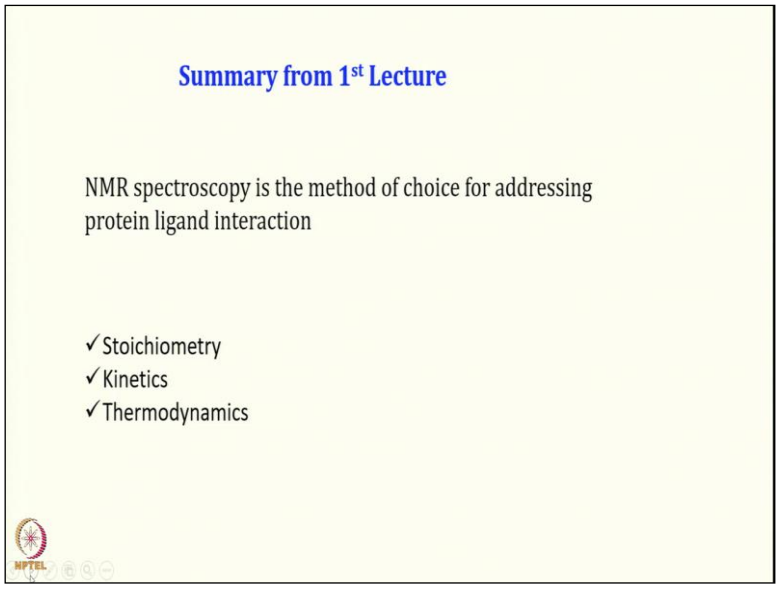

So, that is what the summary from the last slide. So, it can gives you stoichiometry with what ratio this true partner interact, what is the kinetics, what is the rate of the interaction, and what are the thermodynamic parameter involved in the interaction right. So, these are the some of the parameters that can be derived from protein-protein interactions (ppi).

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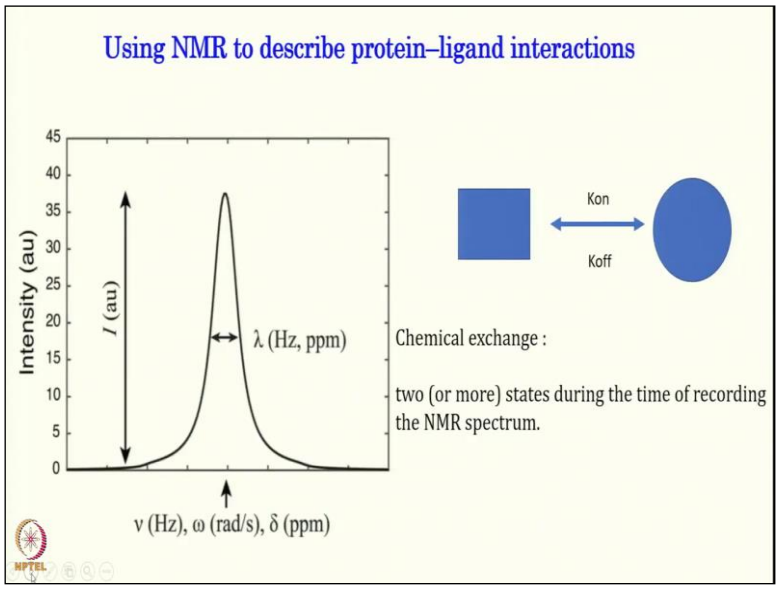

So let us start. How we can get all these parameter? What we can aim for from the signal that comes in NMR? So, let us take 2 molecule like a say here is one molecule and it interacts with small molecule like this. Now and changing its conformation something like this. So, there is a on rate of association, there is off rate of dissociation. So, that is what happening these 2 conformation are changing upon interaction.

So, that means there some exchange is happening there and we can define this exchange phenomena called chemical exchange, where two or more state during the time of recording of NMR spectrum changes. So, we can define as a chemical exchange and we are going to use this term again and again. So, it is a simple two or more state are in exchange when we are recording the NMR spectrum, that is the chemical exchange.

So, typically when we record an NMR spectrum you get peak like this. Now this peaks, has few property which can be deciphered to understand what is going on upon interactions. So, if there is a free protein, you get peak like this, but when it forms complex you can also get peak like this, and just comparing these peaks you can learn a lot about what is going on. So, let us analyze this peak in a simplistic term.

So, when you record a peak what we get? A resonance frequency, which you can measure in hertz or radian per second or in simple term chemical shift that is in ppm. So, that is a that gives the position of the peak, the resonance frequency is the position of a peak. Next parameter what we get is intensity of the peak, how high it is. And the third parameter that we are going to get is a linewidth, how narrow or broad it is.

So, three parameter just by recording a simple 1D NMR spectrum we can get the chemical shift right. So, chemical shift is given by omega, that is can be denoted in ppm, radian per second or hertz and intensity gives the height of the peaks and lambda. So, what these three parameters basically denotes in such case.

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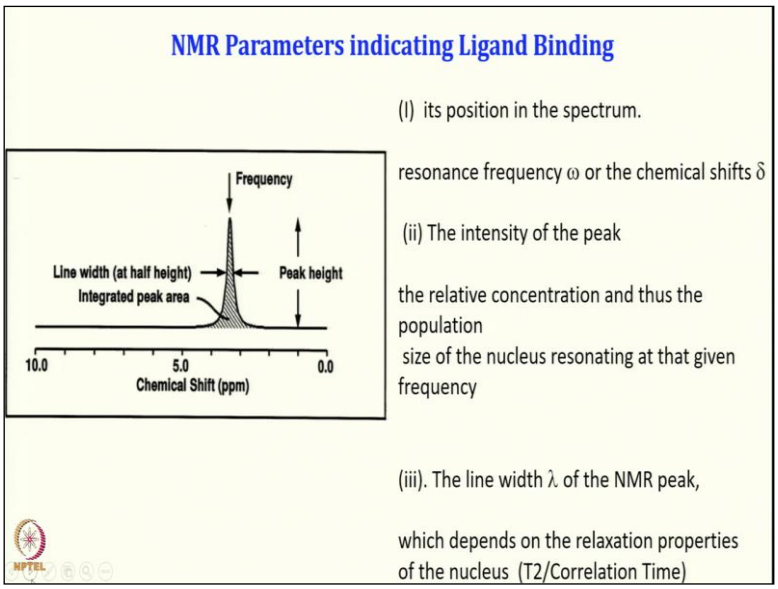

So, let us see first; first and most important chemical shift; what it gives the frequency with which it is resonating. So, position of the spectrum, resonance frequency omega or the chemical shift tells about the chemical characteristic of that particular nuclei. So, when it is in free form, it has one chemical characteristic; when it is in bound form, it can change its characteristic. So, resonance frequency may change upon complex formation.

So, this is the first readout that we can have. Next one is intensity of the peaks. Now intensity tells about how many molecules contributing for that particular intensity. So, peak height or the intensity tells about the relative concentration thus can tell about the population and size of the nucleus resonating at that particular frequency. So, how many molecules contributes to that particular frequency that is given by the peak height.

The third parameter is a lambda, the linewidth right. So, linewidth in the in the NMR spectrum depends upon relaxation property of that nuclei which is T2 or the correlation time what it tells. So, suppose molecule is like is small. So, that means its relaxation time will be of one kind, molecule forms complex, it is now slowly tumbling, that is the correlation time how fast or how slow it can tumble.

So, it is a slowly tumbling its peak will be broad. Now the linewidth that we are getting here is telling the characteristic, whether it is a small molecule or it is a big molecule. So, these three parameters just from recording 1D spectrum we are deciphering what is the resonance frequency, how many molecule contributes to it, and whether it is relaxing fast or slow, what is the linewidth.

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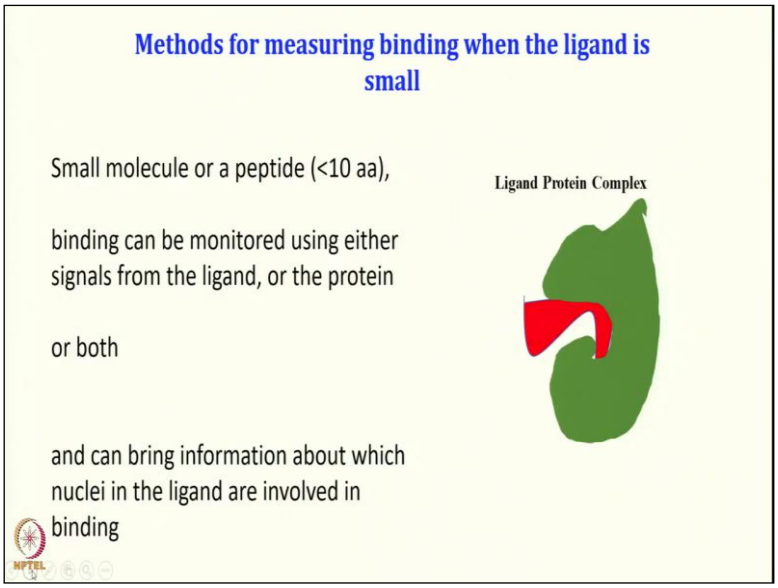

So, using these, actually we can already start understanding the protein-protein interactions. So, so let us divide this protein-protein interaction into two part. First part where we are looking at the protein, another part where we are looking at the small molecule, ligand. So, here is our protein P, and here is our ligand L. So, these two are forming ligand-protein complex, ligand generally is a small molecule, which can be a peptide or even a small molecule; peptide means less than 10 amino acid.

Now in this case when we are understanding protein-ligand interaction, we can either probe on the ligand side or we can probe on protein side or we can probe simultaneously both;; that is that is the ability NMR has that we can probe both of these. It can bring the information about which nuclei in the ligand are involved in the binding. So, as we mentioned like we will be dividing this protein-ligand interaction into two, one first is ligand-based detection and another is protein based detection.

So, for few slides, initially for one or two lecture will be focusing on ligand-based observation and then we will shift to protein-based observation. So, let us start with a ligand-based observation.

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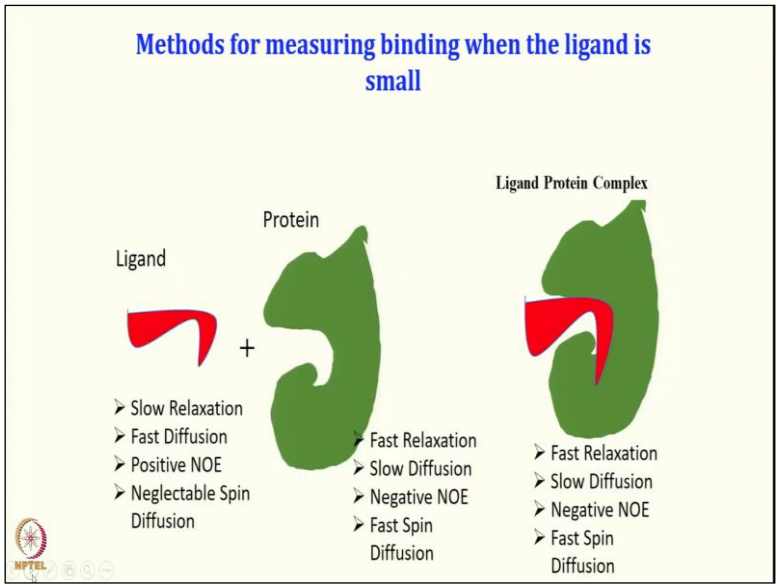

So, here ligand is typically a small molecule, protein is big molecule. Now because of this ligand has some property its small molecule protein relatively is bigger molecule it will have some property and when it forms complex it will have its own third property. So, if the ligand is small, it will slowly relax, that means lines are going to be sharper, it will has fast diffusion, translational diffusion small molecule.

So, it will be fast diffusing and it will have a positive NOE and there will be minimal spin diffusion like there are not too many spin. So, spin diffusion will be minimum on the ligand side. Now protein since it is a bigger molecule, it will have fast relaxation, like here the lines will be sharper, here lines will be relatively broader okay, it is a bigger molecule. So, slow diffusion, it will have negative NOE. Since there are too many spins in protein,

so there will be fast spin diffusion. When it forms a complex, now complex is a bigger molecule and even if we are looking at the ligand molecule, it will have fast relaxation because now it has form a complex right, it is a tighter complex. So, lines will be broader it will slowly diffuse, it will have negative NOE and of course it is in the vicinity of many spins therefore it will have fast spin diffusion.

So, these are the parameters are going to change even if we are looking at the small ligand molecule, because now upon complex formation the overall size has increased. So, fast relaxation, negative NOE and fast spin diffusion will happen right.

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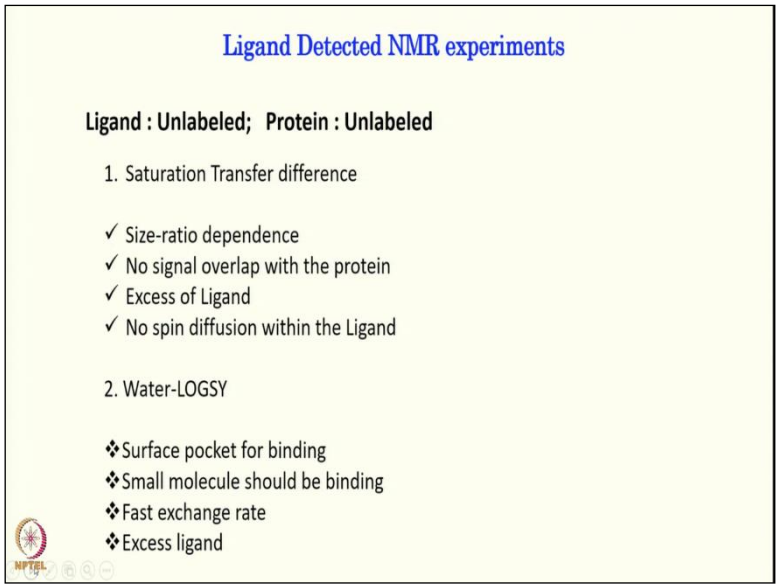

So, let us delve deeper into how we can design or how we can understand the ligand-detected NMR experiment. So, here assumption is our ligand is unlabeled and protein is also unlabeled right. So, we are not going to isotopically label any of these, this is smaller one, protein is bigger one. Few of the experiment that has been developed and I am going to discuss in detail. One is called saturation transfer difference another is called Water LOGSY. These two experiment I am going to explain in detail.

Now saturation transfer difference as name suggested it is a difference in the saturation that that the molecule can absorb right. So, that is a like 20 years ago this technique was developed and this is widely used for throughput screening of the binders for a receptor or a protein molecule, this is quite widely used. So, we are going to discuss in detail how saturation transfer difference works and how we can use for screening the binders.

The declaration here for STD it is a size-ratio dependent. So, there has to be size ratio difference like of size ratio consideration for ligand and protein and another important phenomenon that we have to take care that there should be no signal overlap with the protein. So, ligands should have once range of signal, protein should have another range of signal; at least where we are saturating it there should not be overlap then only we can understand the STD and no spin diffusion within the ligand.

So, typically small ligand should be there. So, minimally spin diffusion should happen for STD to happen. Now Water-LOGSY is another technique, which basically exploits the surface pocket of for binding right. So, if a molecule a small molecule binding to a receptor there is a

surface of binding right. So, it exploits basically what is at the surface and the small molecule actually should tightly bind to receptor.

And there should be fast exchange rate and ligand should be accessed then only Water-LOGSY can be used for understanding it. So, Water-LOGSY a little bit later I am going to come back but these are the prerequisites for Water-LOGSY.

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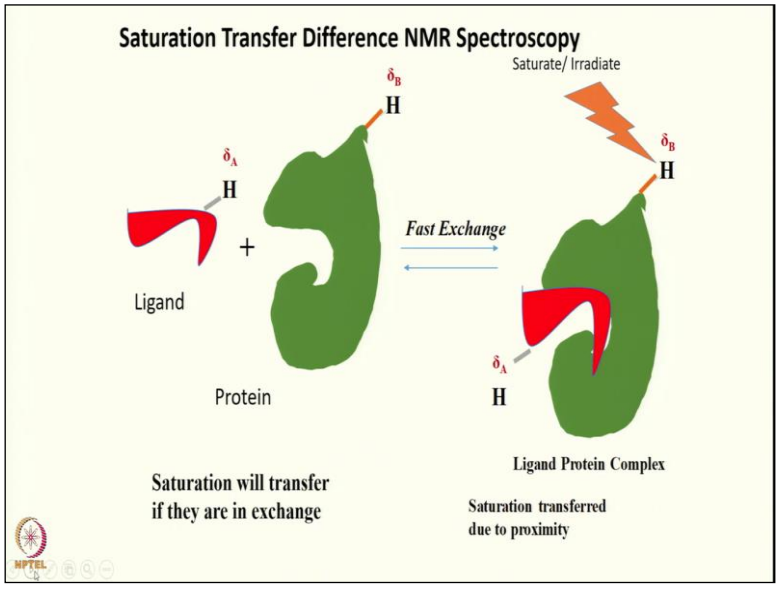

So, let us start with saturation transfer difference spectroscopy, STD NMR that is what in short it is called. So, here is our ligand molecule, here is our protein molecule, ligand has some protons which is resonating at δ_A, protein has some protons which is resonating at δ_B and these two are basically in fast exchange that means their binding should be in order of loosely called its order of micromolar range right. So, Kd should be 10^{-6}.

So, this is one of the prerequisites for STD to happen, another is there should be difference in the molecular weight. So, typically this should be few Dalton and this should be kilo-Dalton. There has to be in fast exchange when they forms complex that is a protein-ligand complex. Now saturation will be transferred. So, what we are doing, we are exclusively saturating the signal that is present in the protein molecule.

And since the saturation gets transferred, it will be transferred to ligand molecule and that is what we are going to take a difference. Once we are saturating protein molecule what is the signal of the ligand molecule, and when we are not saturating protein molecule what is the

signal, we take the difference and that will tell whether ligand is binding and or not binding and if binding where it is binding.

So, essentially you can find it out epitope that binds to the receptor molecule. So, how saturation will transfer, we are saturating on the protein molecule. Now that saturation can only transfer if they are binding. So, they are in close contact, close proximity. So, binder will see the effect of this saturation that is happening on the protein molecule, we saturate by irradiating these resonances and that can be transferred to the protein molecule.

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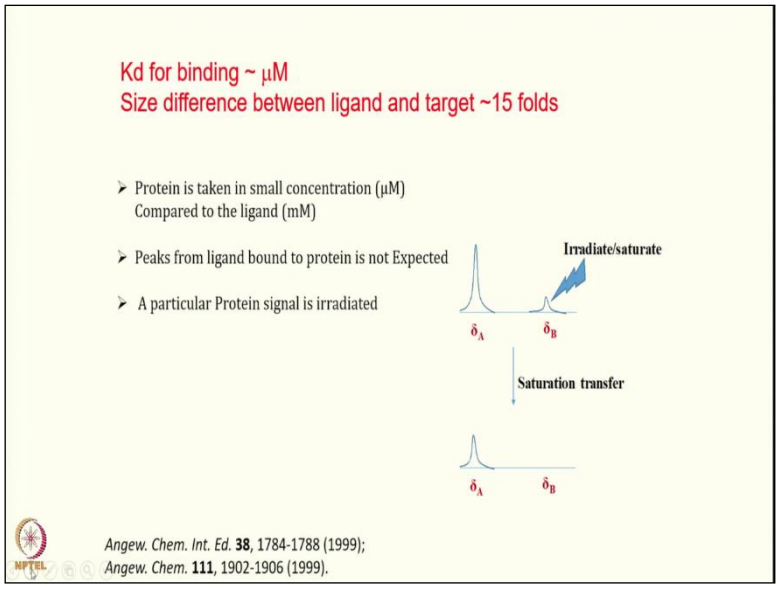

So, as I mentioned the Kd for binding should be in micromolar range and size difference between the ligand and target should be about 15 fold right. So, if you are taking 100 Dalton of ligand it should be 1500 Dalton of the protein molecule or bigger even is better right. So, that is one of the prerequisites, another is they should have a micromolar binding. So, typically while doing experiment we take small concentration of protein which is in micromolar.

And ligand should be in millimolar range, because we are detecting on the ligand. So, ligand generally should be high excess. So, that we can get exclusively signal coming from the ligand molecule. Now peak from the ligand bound to protein are not expected that even may not come and a particular protein signal is irradiated. So, what we are doing as we saw while doing the experiment.

We are irradiating the atoms present in the protein molecule and the effect we are observing on the ligand molecules. So, suppose these two peaks, this is from ligand molecule, this is from

protein molecule, you can see this intensity is high and little bit sharper, this intensity is low because we have taken low concentration, lines are broader.

Now we irradiated the protein molecule and since they are binding, the effect of that irradiation is being seen on the ligand molecule. So, you see intensity of the ligand has decreased and this of course has vanished. So, this was published by Meyer group in Angewandte Chemie some 22-23 years ago and this has become really popular in industry for drug discovery and design right.

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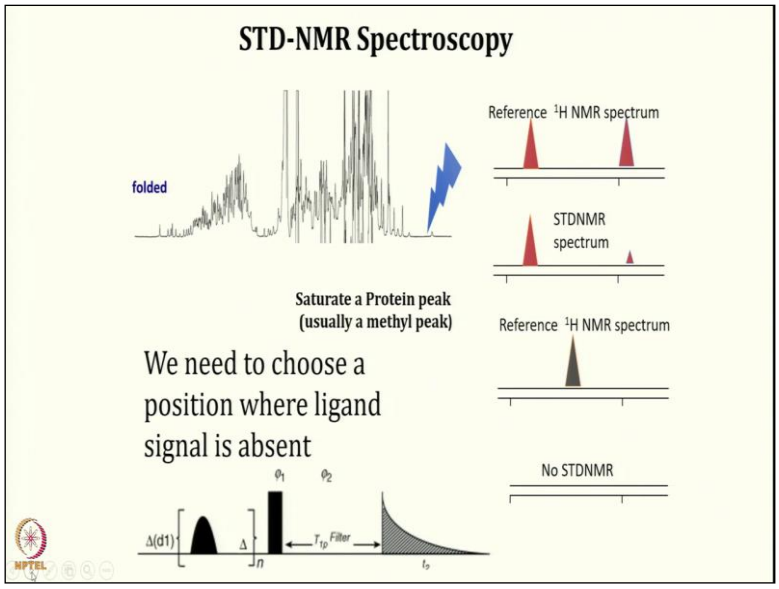

So, what we are doing, we are selecting a signal in the protein that can be irradiated like this is a typical protein 1D spectrum, you see there will be some up field shifted peaks which can be selected for the irradiation. So, typically methyl peak can be chosen. Now in this case, ligand will not come, ligand is going to be only in this region. So, we are saturating one with exclusively protein peaks and since they are binding we are going to see the effect here.

So, this is the typical pulse sequence we irradiate selectively. So, there has to be a selective pulse, a pulse terrain that selectively we are irradiating on protein signal and here the ligand signal are absent and then we are detecting like mostly on the ligand signals right. So, this is the difference 1D spectrum from the ligand and now we did STD we irradiated protein this is the STDs spectrum.

So, then we take a difference of this, now we can get some of the peaks that got lower intensity. So, that means STD is happening and if there is no STD happening there will be no signal. So,

now where there is an effect is seen, intensity will reduce for the binder. For non-binders, when you take the difference between the reference and STD NMR the signal will vanish. So, that means those are non binder right. So, that is what you get in STD.

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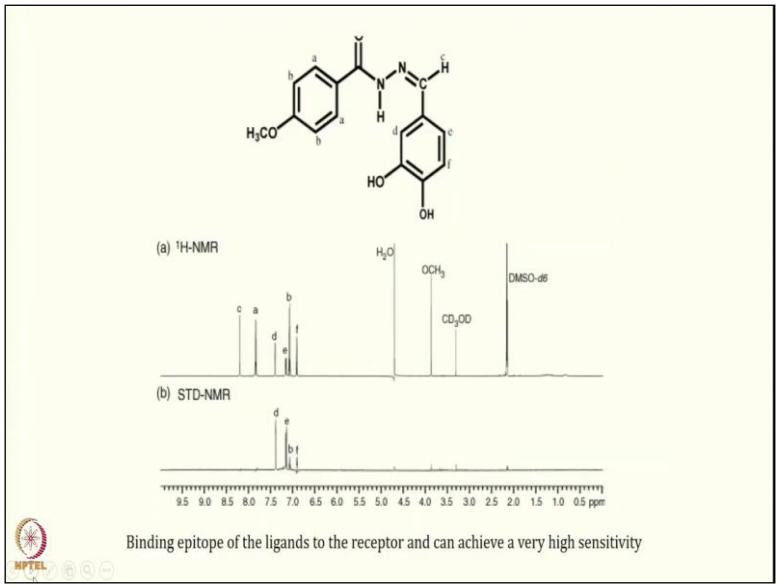

So, let us take one of the example. So, suppose this molecule is binding to a protein, binding to a receptor, we take the proton NMR, you can find it out all the peaks that are there, ABCDE. Then we did STD NMR saturated on the protein and detected the effect of that and then we took the difference of non-saturated versus saturated. How do you do non-saturated you saturate at -20 ppm or +20 ppm where there is no resonances.

And when you are saturating you are saturating at the methyl peaks and you take the difference of these two 1D spectrum. One case we saturated somewhere here -20 ppm, another case we are saturating here. These two 1D subtracted and upon subtraction whatever peak is being illuminated is seen here. Now you can see we are seeing only those resonances, we are seeing only D E B and F.

So, that means here D E F and B. So, these are the atoms that are binding to the receptor or the protein. Now, not only we find binders, but in the binder which are the atoms that are binding. Now this is a great help, when we are doing high throughput drug discovery, we are starting with a 500 compound, just by recording two 1D NMR in few seconds, we find it out binders what are the binders.

So, that gives us lots of clue, now our medicinal chemistry friends can come and start modifying few of these groups and make it a stronger binder or weaker binder depending upon what is the necessity. So, medicinal chemistry can be invoked here to make it better binder or weaker binder. Now epitopes is very important for drug optimization, the binder optimization and that is what STD NMR offers to us.

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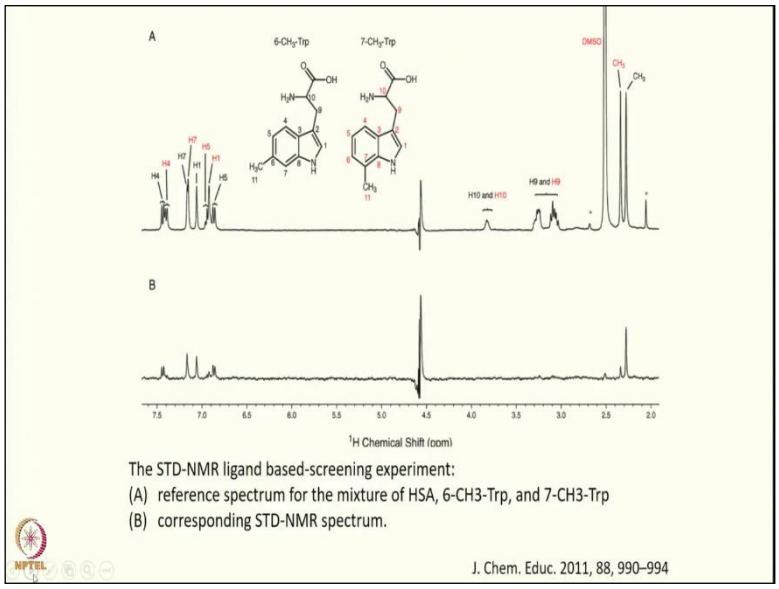

The STD-NMR ligand based-screening experiment:
(A) reference spectrum for the mixture of HSA, 6-CH3-Trp, and 7-CH3-Trp
(B) corresponding STD-NMR spectrum.

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So, like even there are a stereo-specific questions comes or isomers comes, in that case also you can find it out which is the binder. So, for an example, let us take two of these compound 6-methyl-tryptophan and 7-methyl-tryptophan, they are binding to a protein molecule. So, we have a mixture of this and we recorded a 1D spectrum, we can assign these.

So, red peaks are coming from 7-methyl-tryptophan and blacks are coming from 6-methyl-tryptophan. We put the protein receptor for these, we did STD NMR like we saturated on protein at where there is a signal of protein and where there was no signal of protein. We subtracted this to a spectrum and here we got an STD spectrum. Now you can find it out like just by comparing that the black one peaks are available, that means 6-methyl-tryptophan out of these two was a binder and 7-methyl-tryptophan does not bind.

So, just a simple experiment, where the human serum albumin as a protein were chosen and this 6-methyl-tryptophan and 7-methyl-tryptophan, out of these two were able to differentiate the binders. So, corresponding STD-NMR you can find it out, which is the binder, which is the non-binder.

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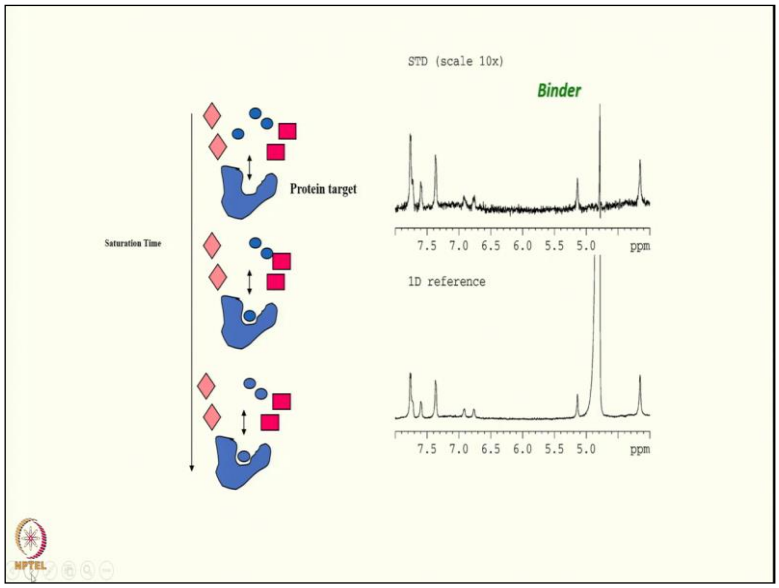

Great so that is a great tool for drug discovery. So, just as a schematic here I am showing we have taken a protein target and a mixture of ligand just for cartoon-stick representation I have taken here, the square shape, rectangle shape, the oval shape, and the diamond shape ligands. And we are doing now drug discovery, which is binding. Finally, we recorded the STD-NMR like here is a reference NMR, here is the binders NMR.

And we figure it out that these oval shapes have binding to the receptor or protein target not the others. So, you can even change the saturation time and you can find it out by doing couple of more 1D spectrum how better they bind. So that we will be discussing little more in detail right.

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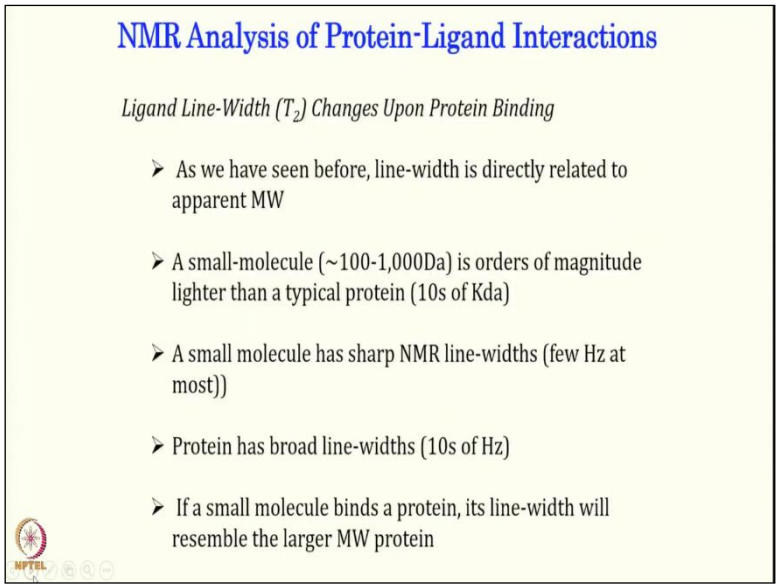

So, now that was the critical NMR experiment that can be used for analysis of the binder. So, now NMR analysis of protein-ligand interaction like a ligand linewidth, as I said when they are in free form they will have sharper line and when they binds since they are tumbling slow, the line will be broadened. So, as we have seen before linewidth is directly related to molecular weight.

So, upon binding the linewidth will be broad. So, a small molecule is order of magnitude lighter than a typical kDa and therefore they have sharper line, these guys will have broader line and small molecule has generally sharp line, protein has a long broad line. So, small molecule binding up on a protein, now lines become really broader and just looking at the linewidth which is which is given by T_2, one can find it out.

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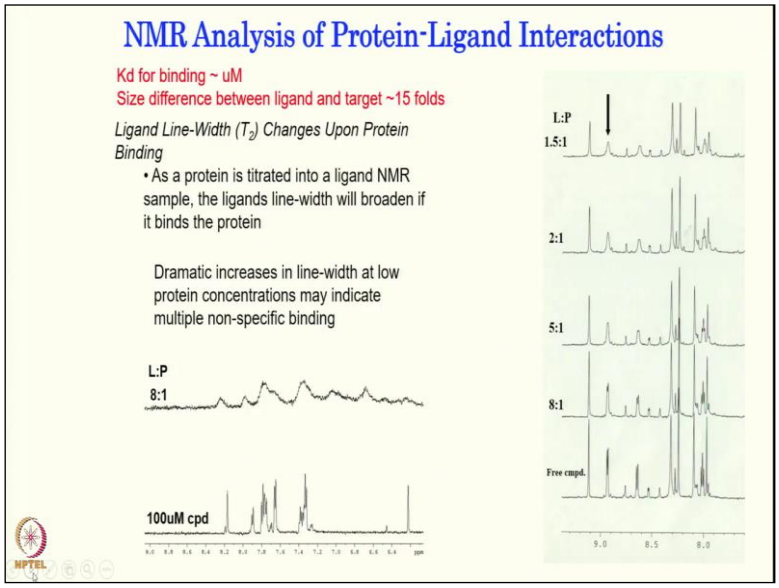

So, these are the few examples of that. The size difference should be 15-fold and you can see the linewidth are becoming broader. So, as you can see we can monitor this ligand to protein ratio, as we are increasing the ligand here free ligand it is a sharp line but like when binding becomes tighter and tighter you can see line becomes broader. So, just looking at the linewidth even one can find it out. STD is another experiment which tells about the binding property of the molecule right.

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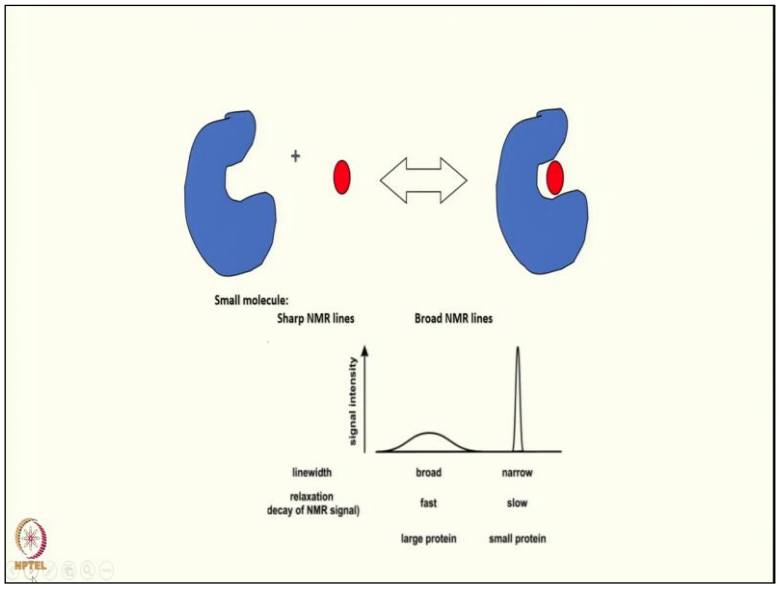

So, to summarize this linewidth based analysis, sharp line for ligand, small molecule binding to protein it's a broad line. So, narrow line – slow relaxation or fast stumbling and broad line – because of the fast relaxation or slow tumbling. For large protein, looking at the linewidth, one can find it out the binding probability or the binding capacity for a binder. So, two of the ligand-based methods I discussed today.

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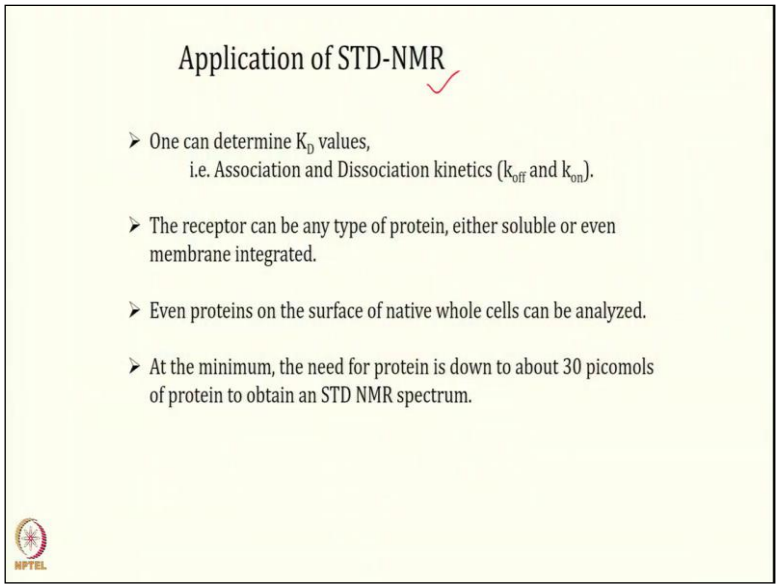

To just give you an overview, application of STD one can determine the Kd that little bit more detail I am going to discuss in the coming class. So, not only binders you can determine the Kd, the association and dissociation constant, k_{on} and k_{off} rate. Receptor can be any type of protein either soluble or even membrane integral. So, I am going to discuss this how you can use this for even the membrane integral protein to find it out the Kd, the binders using STD-NMR.

And yeah the whole cell can be used and at the minimum the need of protein is down like a 30 picomole. You need really tiny amount of protein, your ligand should be high, but proteins should be very low to do STD-NMR. So, this has emerged as one of the powerful high throughput technique for drug discovery STD-NMR. Of course, I explained to you linewidths.

So, in the next class we are going to use the advanced concept of STD-NMR for determining the Kd using like a whole cell lysate, we can take it and how we can use this for say drug discovery, the elucidation of binders. Another technique that we discussed briefly at the beginning of this lecture was Water-LOGSY. So, we are going to also look at the Water-LOGSY in the coming lecture.

And these are the ligand-detected NMR techniques for understanding the protein-ligand interaction. Then we can move to protein-protein interactions; protein-ligand interaction where we are going to look at the protein side not the ligand-side. So, with this I would like to close today, thank you very much, see you in the next class, thank you.