NMR spectroscopy for Structural Biology
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Lecture: 44
NMR Analysis of Ligand Specific Parameters in a Protein-Ligand Interaction -I

So good morning. This week we are discussing protein-ligand and protein-protein interactions. Today we have a fourth lecture. So, in the last lectures discussed various techniques that mostly detected the small molecule ligand in this protein-protein interactions and some of the technique that we discussed like STD-NMR, saturation transfer difference, the water-LOGSY, looking at the linewidth, and transfer-NOE, how these techniques can probe the ligand side.

And once we find the epitopes, we can even create a docked model or complex model where because we know from where the ligand, like what are the sites on ligand that interacts with the protein. So, those can be used as a restraint to create a complex model. Now let us go into little more detail and today we will be discussing how you can look at the protein-side. So, the other partner which is a bigger molecule protein how we can look at this and that is what last time we summarized.

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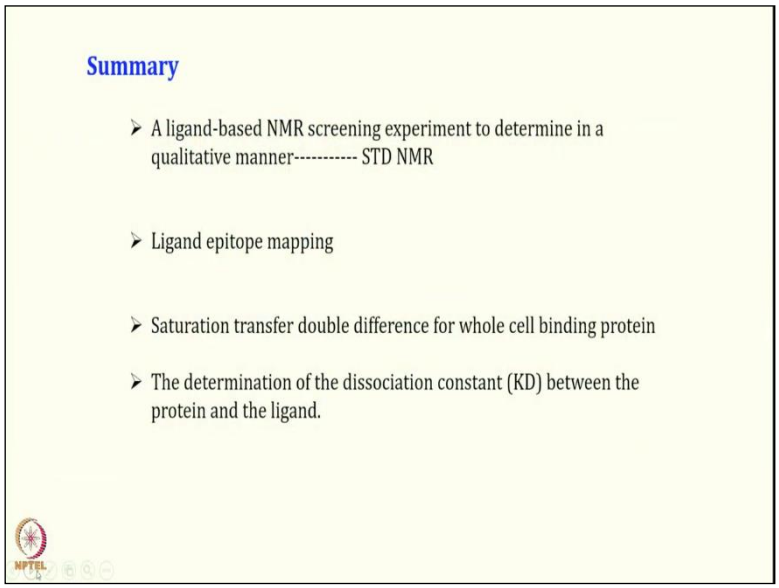

Ligand based NMR methods for screening experiment to determine in a qualitative manner STD-NMR. We can map the ligand like ligand epitope mapping and then we can use even if we do multiple concentration like what we discussed is called STD amplification factor, we

can do that and we can find it out the dissociation constant in a quantitative manner. Then we also discuss saturation transfer double difference for whole cell binding. So, we ended up here.

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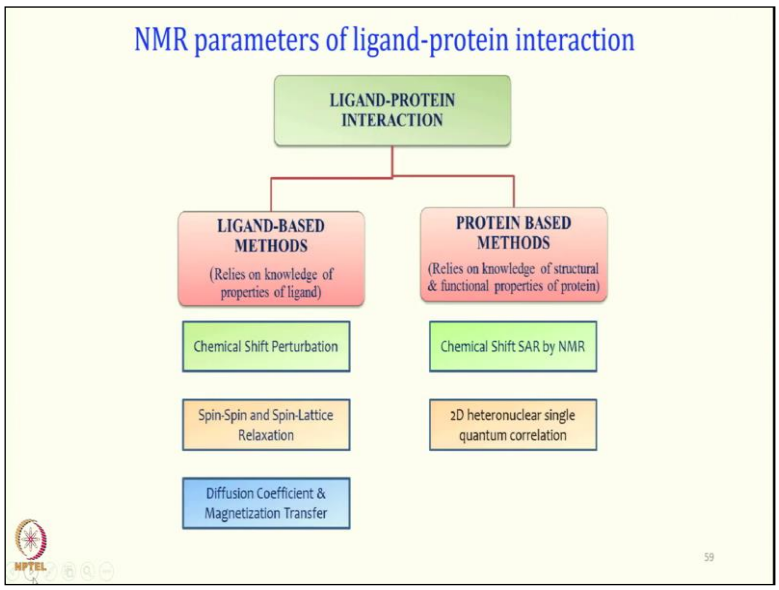

Now today we were going to look at the other side, that is protein-based method. So, we can classify this ligand-protein interaction into two, ligand-based method. Again, it relies on knowledge of property of ligand small molecule typically. Then we can look at the chemical shift perturbation, the relaxation that we already discussed, and change in their resonance position, and also how they are diffusing.

So, diffusion coefficient. Upon binding the small molecule will diffuse differently than whenever it is free. So, all those we had already finished. So, all these are done. Now protein based method, basically we are now looking at a bigger molecule protein right. So, we need to have a knowledge of a protein, what is the structure of this; what is the function of this protein. So, structural functional aspects of protein should be known when we are using this protein-based method.

Now actually the deterministic signal that comes from protein is chemical shift change. So, we can do SAR by NMR, monitoring how chemical shift is changing and typically for doing this 2D Heteronuclear Single Quantum Coherence is used. Where each peak gives an idea of one of the correlation, like if you are putting it 15N and proton correlation. So, that means each peak which is here is reporting about one amino acid.

And the changes that happen in these amino acids essentially can be captured when we do protein based NMR methods.

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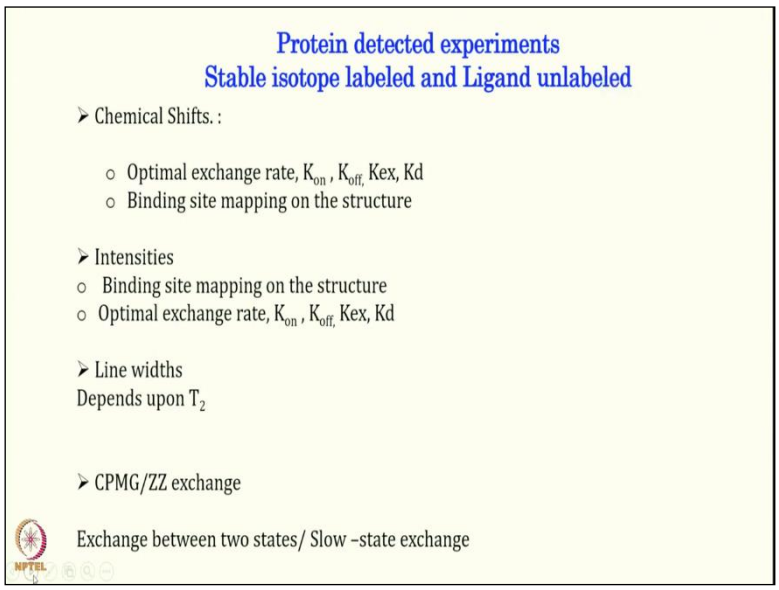

So, what we can look at a protein detected experiments. So, you need to first label this with a stable isotope and here our ligand can be unlabelled. So, protein has to be labelled because now we are looking at the protein and since protein molecules are big. So, to get resolution to understand more in details because now we want to go to get residue a specific information.

So, we need to enhance the resolution by isotopically label these proteins, however our ligand can be unlabeled. So, what we are going to monitor here is a chemical shift. Now chemical shift of a protein actually may change upon binding or intensity may change upon binding. So, one of this parameter we are going to monitor. So, the change in the chemical shift indicates that the chemical environment of that particular nuclei is changing.

And that basically can report about what is the on-rate of binding, what is the off-rate of binding, what is the k-ex, chemical exchange phenomena and using that actually one can get the K_d of the binding, ligand-protein binding. Then we can even find it out the binding site on the structure, where the chemical shift is changing what are the residues that are engaged in this interactions that that is going to tell us about binding site mapping.

We are going to discuss this in detail. Then the other parameter that we can monitor. So, if you remember in the last class I just on the basis of 1D I showed, that here is one parameter called

chemical shift, another parameter called intensity, and third parameter is called lambda, linewidth. These three are easily determined.

So, chemical shift tells about the chemical environment, intensity tells about what is the population that is being participated in these. So, intensity also can tell you about binding site mapping on the structure and using this of course one can get it k_{on}, k_{off}, k_{ex} and K_d. The third parameter is linewidth. So, linewidth depends upon T_2, the transverse relaxation rate.

And if the molecule becomes bigger and bigger, your line width changes and that also tells about this changes. So, for unliganded you have a sharp line, once it binds you go to a broad line. So, that generally; it may go depending upon how binding is happening. So, go and get the broad line and that also tells about the binding phenomena. Then we will be also discussing some of these other techniques like a CPMG techniques that essentially use for determining the lowly populated state in the protein-ligand interaction or even protein conformation change.

And ZZ exchange, we are going to discuss both of these. So, essentially depending upon what we want to probe. So, CPMG actually probes the exchange between two states and if they are slowly exchanging then ZZ exchange can be probed. So, briefly we are going to discuss these two techniques in coming slide.

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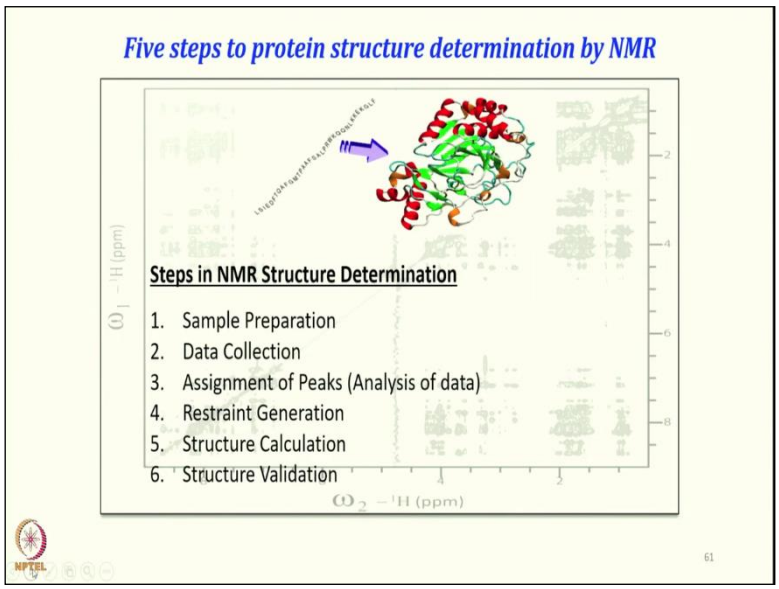

So, let us start from the beginning. So, as we said if we are looking at the protein, we need to have some structure functional information of a protein and just to recapitulate whatever we have done, recap of the steps involved in protein structure determination by NMR. So,

essentially what we are doing, we are going from primary sequence to three dimensional structure and what are the steps involved in doing this.

First you have to prepare the sample. Now our sample has to be isotopically labelled. So, you feed your bacteria with ammonium chloride and carbon-13 glucose and or like you can be singly labelled only ammonium chloride and our ammonium sulphate or for the doubly labelled your glucose also has to be 13C labelled. So, once we prepare the sample, we can go and collect the multi-dimensional data.

So, multi-dimensional data will be collected. Now since this is our set data, we collected data next step to identify what is this peak, what is this peak, what is this peak. So, all these peak identification is called resonance assignment. So, you have to analyze this data. Now we have a name for these peaks, it can be some L M N, whatever amino acids.

So, and for structure determination you have to generate a restraints. So, distance restraints, angular restraints, and all those you have to generate, which we have already discussed and then you calculate this structure three dimensional structure using these restraints; Angular restraints, the bond-vector restraints and the distance restraints. And finally we incorporate all these restraints for structure calculation, we determine the structure and finally we validate this structure, what is the quality of the structure.

So, once we have a structure, we are all set to understand how this protein is interacting with another protein or another ligand. And that we are going to use this information which we have captured here during the structural determination of a protein.

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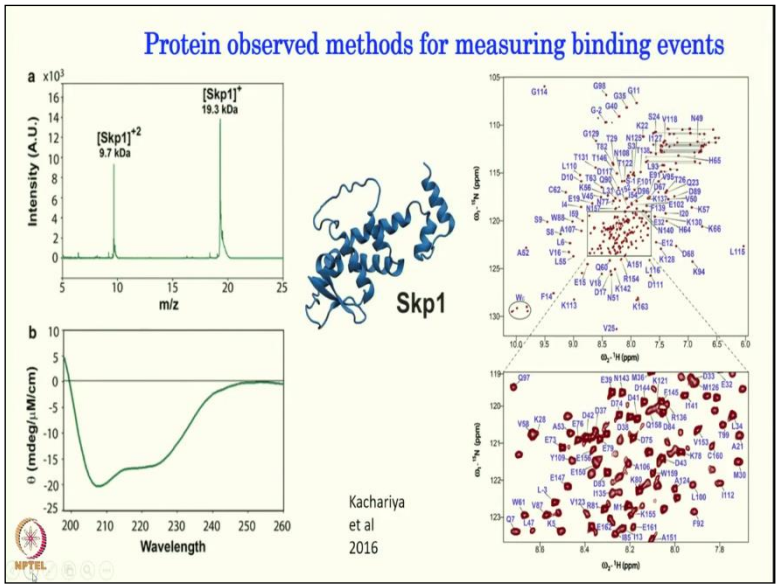

So, just to summarize, suppose I have a protein which we are working in our lab is a Skp1 S-phase kinase protein, some protein which is involved in ubiquitination. So, it is one of the component in E3-ligase. So, don't go in technical details. So, first you have to purify this protein right. So, you have to be getting the exact molecular weight. So, you can do either SDS page you should have a single band of these proteins, then you can validate using MALDI techniques.

So, you are getting the exact molecular weight this is M/2. So, you are you are sure that my protein is absolutely pure and this protein sample that we are preparing is at least N15-label, we also determine the secondary structure using one of the low resolution structural technique like circular dichroism. So, one can see here it is showing helical characteristic and you can see here lots of helices are there.

So, now this corroborates that the structure which should be captured is from helical, then we recorded the HSQC spectrum. Using various sets of 3D experiment, that you have already done one can assign each of these peaks. So, each speaks has a name. So, this is a V25, this is K13, this is say A52. So, now once we do titration experiment or the bindings experiment, any perturbation you see here can be captured and that perturbation is essentially used to understand protein-ligand or protein-protein interactions that is how we are going to do now.

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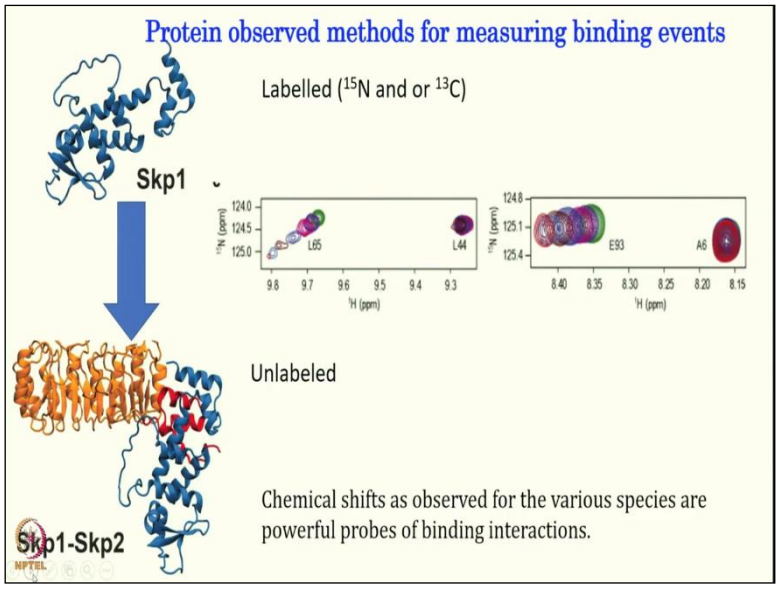

So, suppose this is my favourite protein Skp1 is binding to its partner called Skp2 and binding site is here. So, what do we expect right from theoretical knowledge, this blue is Skp1 and this the orange colour is Skp2 and binding site here is shown in red. So, suppose we are studying this protein right we are doing the titration of experiment. So, we are saying that there will be some change in the chemical shift.

So, to start with what we are doing, we are taking the labelled Skp1 one we are monitoring on Skp1 and then we are taking unlabeled Skp2 we are titrating it and seeing what is changing. So, suppose change is happening. So, I have taken here two residue, here in this plot and here also to residue. If you see one residue is very less changing something is happening that we are going to discuss little more detail.

So, intensity is changing but here you can see it a chemical shift is changing. In this case also this guy is not changing A6 and say a some name like A6 it can be from other proteins. So, some amino acid is not changing and here one amino acid is changing very nicely. If you look at if we are titrating it adding more unlabeled Skp2 peaks changes. So, now change in the peak at least it is telling that some interaction is happening.

And now that is what we are going to monitor. So, chemical shift is observed for the various species and those are the powerful probe to understand the binding interaction. So, we have taken the labelled one partner, the unlabeled another partner, we are titrating it, adding and we are maintaining some stoichiometry ratio for each titration. We are monitoring what is happening in each peak.

And a peak wise manner, we are going to assign and understand the point of our interaction on the protein side.

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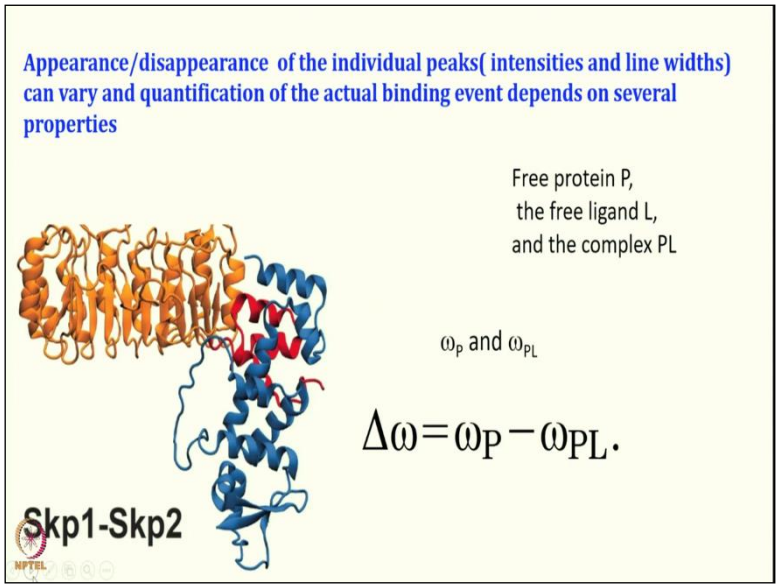

So, as we saw there are certain phenomena happening peak is shifting or peak can appear; appear at a different position or it can disappear. So, now appearance or disappearance of this individual peak that means change in the intensity or linewidth can vary and that needs to be quantified for finding it out actual binding effect and that depends upon several properties of these interactions.

So, let us take it some of the parameter, which will help us in understanding the protein-protein protein-ligand interaction. So, suppose we have a free protein, which we define as a P, it has concentration of P. Now a characteristic P and a free ligand, which is L and a complex, which is forming PL. So, for a free protein, there is a chemical shift, which is ω_P and you remember what we are looking at only protein, ligand is unlabeled.

So, when protein forms a complex, which is PL. Now its chemical shift is changing from ω_P to ω_{PL}. So, the change in the chemical shift that we are monitoring is $\Delta\omega$ that is happening because of these interactions. So, we can say $\omega_P - \omega_L$. So, to make it more understandable, here is my ω_P and it has shifted and made it something like ω_{PL}.

So, this difference that we are seeing $\omega_P - \omega_{PL}$ is $\Delta\omega$. So, change in the chemical shift of this protein which is labelled is $\Delta\omega$. So, upon this interaction we are getting this parameter.

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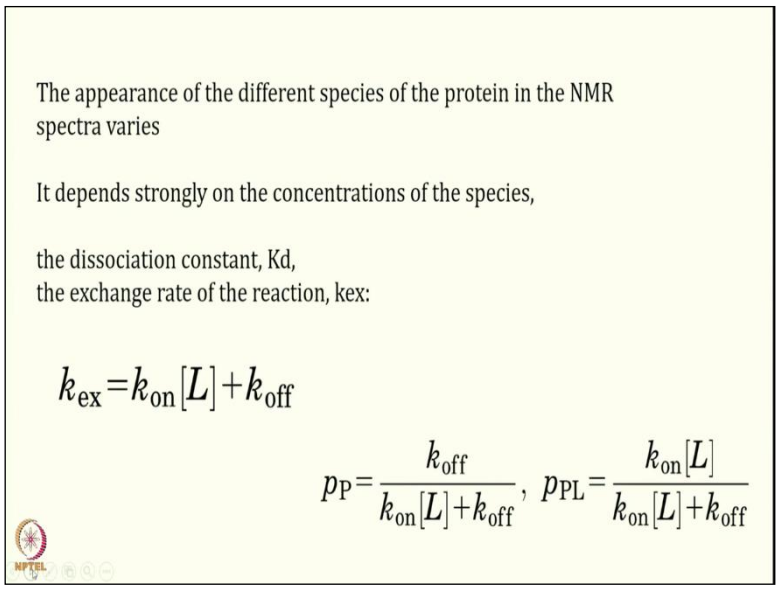

Now the appearance of different species of protein in the NMR actually varies, it also depends upon what is the concentration we are choosing of various species, protein-ligand stoichiometry, it depends upon what is the rate with which they are associating, what is the rate with which we are they are dissociating, and what is the exchange between them. So, how these two, protein-bound and free form are exchanging between them.

So, in next couple of slides I am going to discuss little more details about k_{ex}. So, let us define this k_{ex} parameter:

$$k_{ex} = k_{on}[L] + k_{off}$$

So, one can determine subpopulation of free protein, $p_P = \frac{k_{off}}{k_{on}[L]+k_{off}}$

and bound conformation can be $p_{PL} = \frac{k_{on}[L]}{k_{on}[L]+k_{off}}$.

So, if we know the ligand concentration, if we know the rate and population, we can find it out what is the free population, what is the bound population. So, by getting these chemical shift and ligand concentration, we can also get the population which is in bound form, which is in free form, and various thermodynamic parameter.

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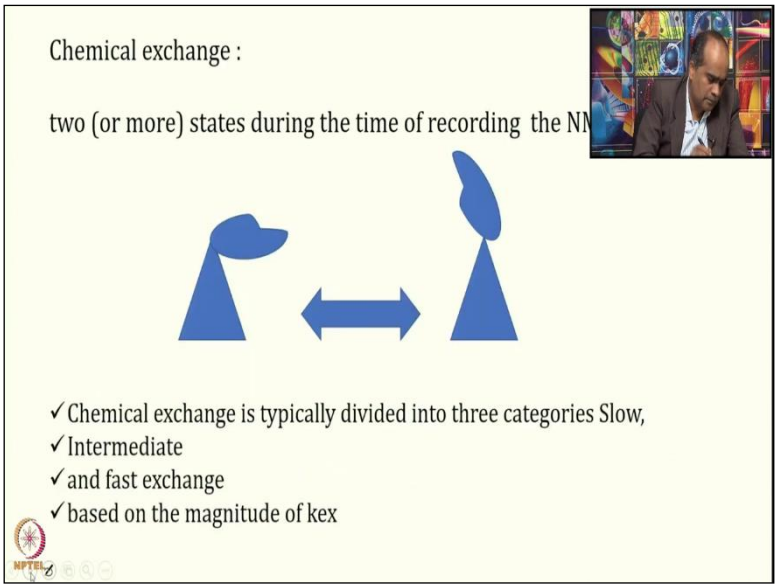

So, you can see now, we are going little more detail in a quantitative manner of binding, in terms of thermodynamics, in terms of kinetics. One of the parameters that we were discussing is k_{ex}. So, this is called chemical exchange. So, two or more state during the time of recording of NMR how they are changing that is called exchange phenomena. Suppose this is my protein, cartoon we have made, and this protein is changing its conformation, here it is a change in the conformation.

So, the rate with which they exchange from one conformation to another conformation when we are recording the NMR Spectrum that is called chemical exchange. So, typically on the NMR timescale, this k_{ex} may vary; it can be fast; it can be slow; or it can be intermediate. So, depending upon what is the magnitude of k_{ex}. So, chemical exchange typically can be slow if the two states are slowly exchanging with each other. What is slow, with respect to NMR time scale, again I am going to explain you what is the NMR time scale. Because we are recording the spectrum,

So, when they are exchanging slowly, we can probably get the two peaks one coming from state A and one coming from State B, if they are exchanging very slowly. The another phenomena can be intermediate timescale, it is neither fast nor slow. So, like you see fan; if fan is rotating very slow you can see all three wings, right. If it is with some speed they are spinning where your eye cannot resolve it, what you see, an average state of these.

So, that we can call is intermediate States. So, where the contribution from A and B are so merged that we are unable to distinguish; and then there is a fast exchange with respect to NMR

timescale. So, then you see an average state, right if the fan is spinning very fast you see an average value of these three wings or average phenomena of these three wings. So, that is a fast exchange.

So, at the NMR timescale the exchange between the two is state can be slow, fast or intermediate and that based on the magnitude of k_{ex} right.

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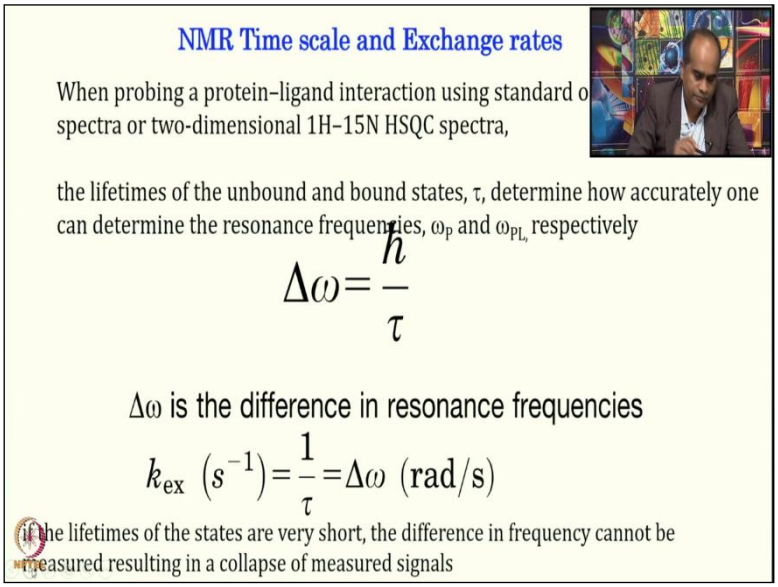

So, what is the NMR time scale and that that we are talking and exchange rates. So, when we are probing and say protein-ligand interaction using a standard either one dimensional proton NMR or 2D NMR Spectrum that is what we are going to do. So, for labelled protein, we typically record this HSQC, but you can also use 1D-NMR that we had discussed earlier right. So, what is the lifetime of say bound state and free state how these say P-state and PL-state how they are exchanging.

And so the lifetime of bound state and free state is τ and then it depends how accurately we can determine the resonance frequency of this P-state and PL-state respectively. So, that is given by the lifetime of these states and that is say Δω.

So, $\omega_P - \omega_{PL} = \Delta\omega$

and that is correlated with this Planck's constant with a lifetime of these two states, how what is the lifetime of bound state or free state.

So, Δω is the difference in the resonance frequency for these two peaks.

$$k_{ex}(s^{-1}) = \frac{1}{\tau} = \Delta\omega\ (rad/s)$$

So, if the lifetime of two state is very short then the difference in the resonance frequency cannot be measured right. So, that is they will collapse to a measured signal.

So, if the lifetime of these two state is very short. So short that, they are exchanging very fast. So, you cannot measure it at the NMR timescale, it will be unmeasurable, you get a collapsed state. But if they are slowly exchanging, the lifetime is quite long, you can measure it, right. So, that is what happens in slow, intermediate and fast exchange.

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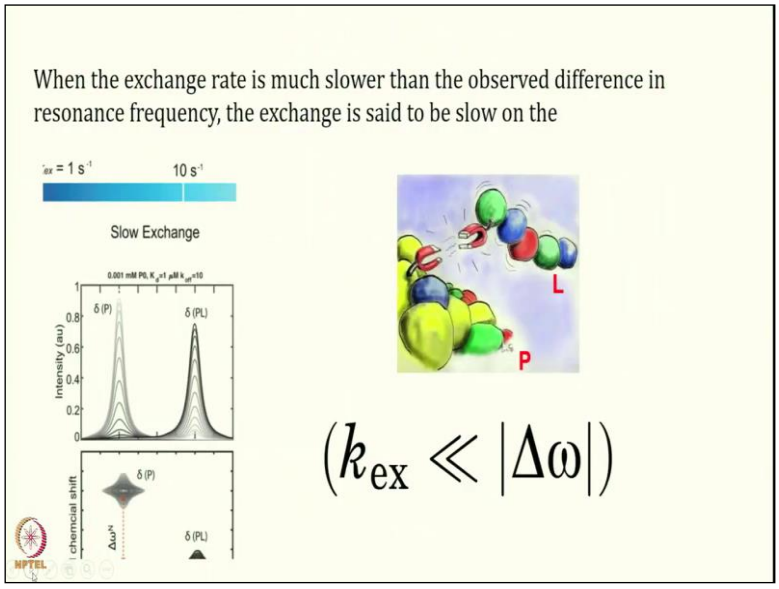

So, when an exchange rate is much slower than the observed difference in the resonance frequency the exchange rate is said to be slow on the NMR timescale. So, here is a protein and here is a ligand right. So, they are slowly exchanging. So, k_{ex} one can have which is 1 s^{-1} to 10 s^{-1} you can see in slow exchange depending upon what is the *K*d, k_{off} rate.

And all those, we are seeing two peaks, one peak coming from the free protein, one peak coming from the protein in complex with a ligand. Two peaks we are getting, our k_{ex} is much slower than the Δω, the difference in the chemical shift of these two, and even in 2D you can measure. So, we are getting clearly to peak one coming from the free protein one coming from the protein complexes, which is here.

So, now this is called this slow exchange, where k_{ex} is much less than Δω, the difference in the chemical shift of these two states.

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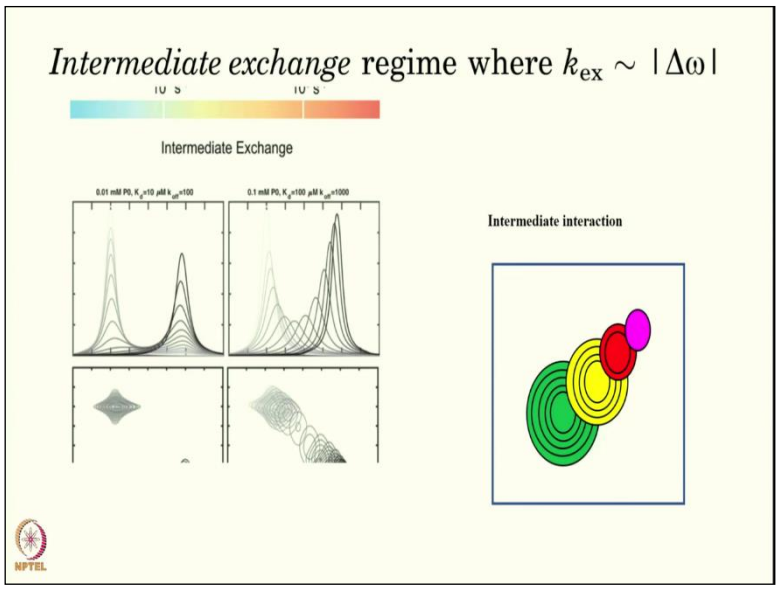

Another one, which is called say intermediate timescale regime where our k_{ex} will be roughly equal to the difference in the chemical shift of P and PL. So, here suppose this is for P and this is for PL. So, you can see. Now lines are getting broader and exchange rate between this. So, here line is going down, here lines are coming up, here also you see the lines are getting broader and line is shifting.

So, depending upon what is in the intermediate regime, what is the exchange between these two, you see peaks is slowly shifting and disappearing, that is the intermediate timescale. So, as we were giving analogy of fan, if it spins so that we cannot differentiate between these two states, our eye cannot resolve it, that is what is the intermediate state k_{ex}. So, here difference in the frequency will be equal to k_{ex} of the of two states.

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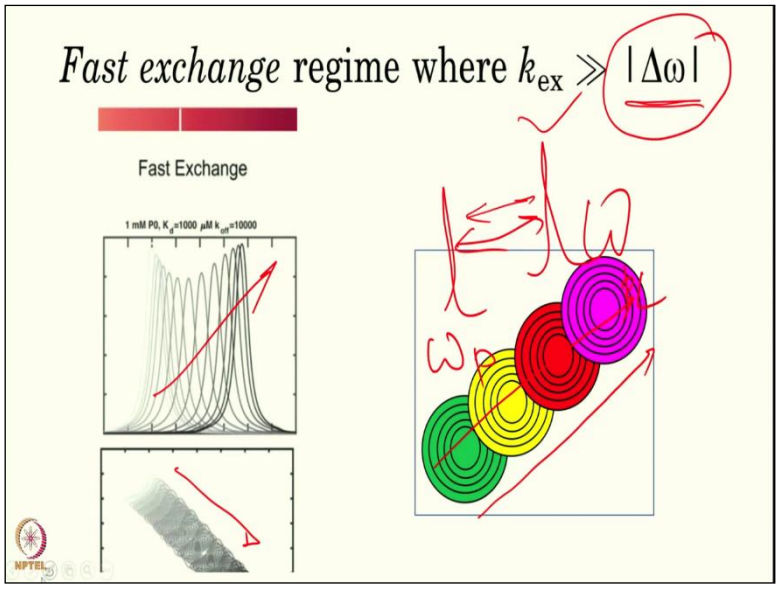

Now coming to the fast exchange. This is the regime where our k_{ex}, the exchange between these two State ω_P and ω_{PL} is happening very fast. So, k_{ex} is much faster than $\Delta\omega$ of these two, so these two states are closer, but peaks gradually shift to a new position and we have an average value that we see. So, this is fast exchange, happening very very fast.

So, these few concepts of slow exchange, intermediate exchange, and fast exchange are measured in terms of what is the difference in the resonance frequency of these two states at the NMR timescale, with respect to NMR timescale and that is basically used in the NMR. So, here one can see. Now in the fast exchange regime, our peak is moving from one position to another position great.

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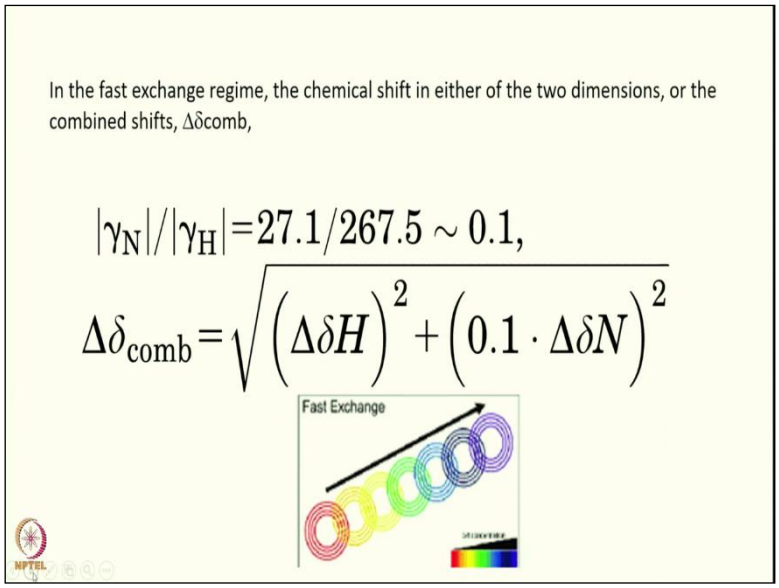

So, now coming back to how we can use this. So, suppose if in fast exchange regime, the chemical shift in either of two dimension, proton and nitrogen, can shift; either proton can shift from one position to another position, nitrogen can shift from one position to another position. So, in that case, one has to take the change in the chemical shift combination. Now, how do you normalize? Because now proton chemical shift varies from 0 to 10 and this can vary about 30 ppm in protein like 100 to 130.

So, you have a normalization factor that comes from the gyromagnetic ratio. So, you can measure it, $\Delta\delta_{comb}$ will be change in the chemical shift frequency of proton square, plus 0.1, which is coming from the ratio of granomagnetic ratio, multiplied with the change in the resonance frequency of nitrogen, square it, take on the root, that is a combination.

You can see fast exchange since here proton and nitrogen both axis are moving. So, in those cases you can find it out how much chemical shift is changing.

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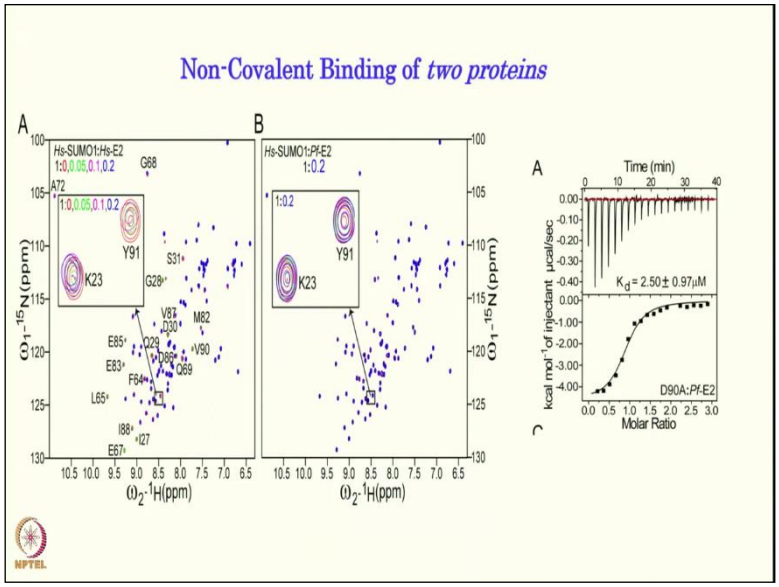

So, we find it out. I will just show you one example of non-covalent binding of two proteins. So, here in my lab, one of my PhD student titrated ^{15}N-labelled SUMO with its binding partner called E2 and he found some of the peaks were shifting little bit, some of the peaks were disappearing. So, he quantified it and looking at these values, since peaks were disappearing more.

So, we are knowing that this is happening at the intermediate timescale, it is not a fast timescale. To substantiate that, he also did ITC experiment that I explained you in the previous class. This

is a thermodynamic parameter experiment that measures the various thermodynamic parameter and one can see here when we titrate SUMO with its binding partner called E2, we are getting a typical *K*d of 2.5 micromolar and looking at this peak disappearance few of the peaks you can see here are either shifting or disappearing.

But knowing that this is happening at intermediate time scale, so, these two techniques are corroborating very well. *K*d at a lower micromolar range and peak disappearance happening in the NMR, one can say that the exchange between the bound form and a complex form of ^{15}N-labelled SUMO was at intermediate timescale. So, we found it out not only the position where it interacts, the order of magnitude of the interaction also one can find it out.

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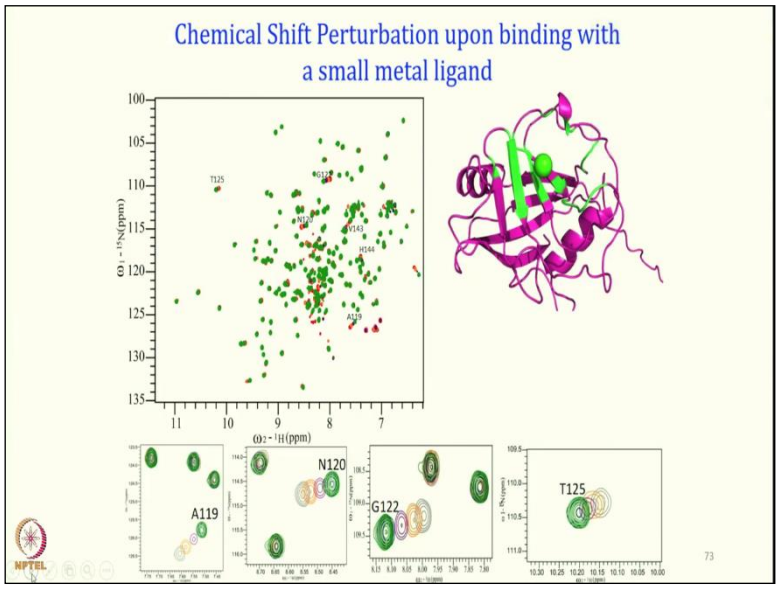

Now another example I am showing you here is another protein, which interacts with one of the metal ions. So, we titrated it with metal ion and what you are seeing here are few of the peaks. Just zoom it here, we have A119, you can see nicely the peaks are shifting here, N120 Peaks are shifting here. So, these Peaks were shifting and they are telling that these are the residues that are involved in the interactions.

So, you can map those interacting site on the structure. Now you know where this metal is binding. So, not only you got the thermodynamic parameter by fitting it which I am going to explain in the next class, how you fit this change in the chemical shift to get an idea of exact binding constant, but also we got the location where actually it binds. You can map those on the structure and find it out exactly the binding site. That is what NMR offers.

You not only know the position of the binding but also it offers the thermodynamic parameter. Here qualitatively, I told you what will the order of magnitude. Next class I am going to explain in a more quantitative manner how we can use these to find it out what is happening.

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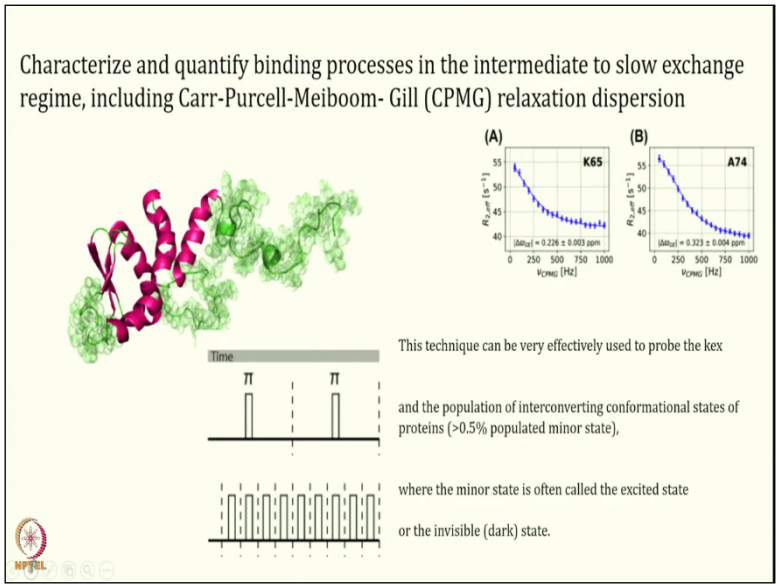

So, let us stop it here and in the next class we are going to discuss two advanced technique which is used for understanding the protein-protein interaction is called CPMG and ZZ exchange that captures essentially the intermediate scale exchange, like a micromolar exchange, and a slow exchange that we are going to discuss it. And then looking at some of the quantitative aspects of interactions how we can determine the parameter. So, hope to see you in the next class, thank you very much for your attention. Thank you.