NMR spectroscopy for Structural Biology
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Lecture: 45
NMR Analysis of Ligand Specific Parameters in a Protein-Ligand Interaction -II

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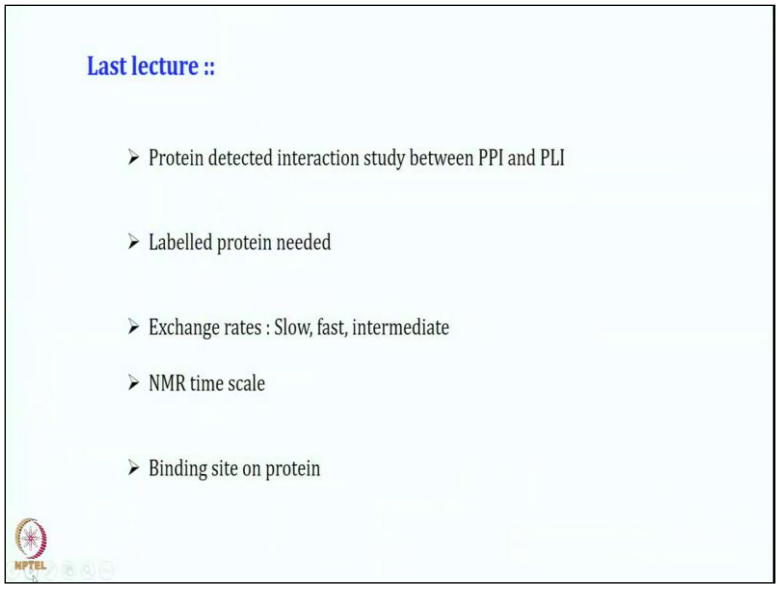

So, good morning. Welcome to today's lecture, which is fifth lecture on protein-ligand protein-protein interactions. So, in last lectures actually we have looked at how we can use protein detected experiments to understand the interaction between protein-protein and protein-ligand. In this case, if we are detecting protein, most commonly used are like a labelled protein, you can isotopically label the protein using N15 or N15-C13.

And then one can titrate with another protein or another molecule – can be ligand – and then look at the perturbation that is happening in each peak. So, that perturbation essentially reports whether interaction is happening; if interaction is happening where actually it is happening. So, not only the strength of interaction can be probed, kinetics can be probed, and also the location. So, you can map those interacting residue on the structure and you can find it out where actually on the structure the ligand is interacting.

Now depending upon the rates with which the interactions happen, they are classified into slow, fast, and intermediate; and I explained this with respect to NMR time scale what is called slow,

what is called fast, and what is intermediate; and we can detect the binding site on protein as I mentioned. So, if something is slow that means you can simultaneously see two peaks. So, one is for free protein, another is for protein plus ligand and simultaneously seeing 2 peaks says that this is happening at slow timescale.

Fast means one average peak, and intermediates generally you see broad peak. So, these are typical thumbs up rule of knowing whether the exchange is happening at slow timescale, fast timescale, or intermediate timescale; and using all these information actually you can map on the protein side. So, here in my protein and suppose here are some residue that are showing exchange or interaction, you can map those, actually you can find it out where the interaction is happening.

So, this is the binding site you can write it – binding site on protein. Now even you can use this information to create a docked model or a complex model of protein-ligand. So, that is what we learned in the last lecture.

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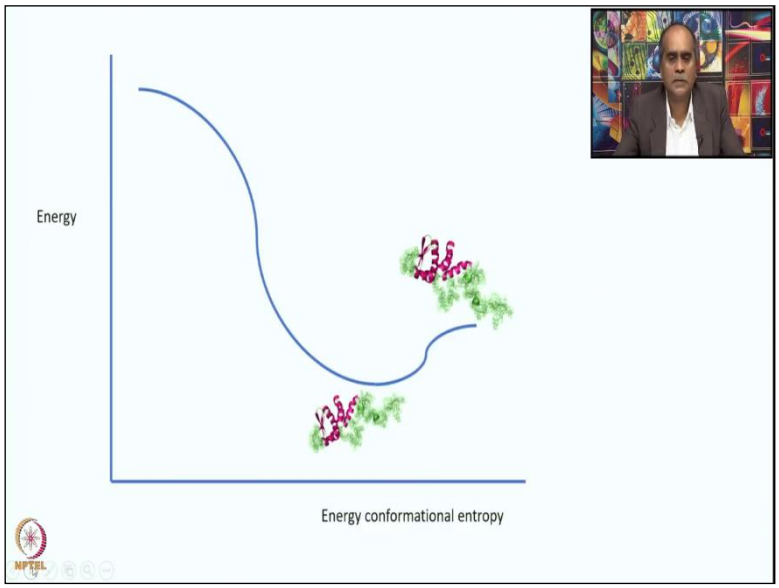

Today I am going to go a little more advanced and discuss two of the techniques that basically proves the exchange that is happening at a microsecond timescale where *K*d is in micromolar range. We call it say typically intermediate timescale. So, what happens, suppose you have studied the folding landscape. Now suppose the protein is binding and populating another state, which is not quite pronounced.

So, like say protein is binding and only populating 5% or 2% or 10% of these states and these are in constant exchange. So, now can we detect this 5 or 10% of bound state. So, actually say these are lowly populated say probably excited state. Lowly populated means like their quantity is less 2%, 5% and excited state because they somehow changed their conformation from one conformation to another conformation and they are populated in low concentration.

So, can we detect these states, right. So, can we detect the states using NMR experiments? Yes, we can and that is what is done using called CPMG experiments or relaxation dispersion experiment. So, we will be discussing today what actually is relaxation dispersion experiment briefly not very much detail because this requires quite elaborate explanation but quite qualitatively I can tell you that how these experiments can be used to probe this lowly populated excited state, which populates only smaller fraction like 2 to 5% or even maximum up to 10%.

So, how we can detect this now the question is that. Here, we have a ground state or say energy minimized state. So, here is my landscape funnel and here are various states that populates. So, one of this is say one of the state and it is exchanging with another state which is also near native but this is ligand-bound state and this happening in only two-state fashion. So, CPMG mostly deals with a two-state exchange – bound and free states.

So, now not only it detects the k_{ex} rate, it also finds it out population of p_A and p_B, p_A is a state which is say here the unliganded state, p_B can be liganded or excited states. So, the kinetics, the thermodynamics, and also population it detects it. So, how it detects, let us look at little bit more in detail.

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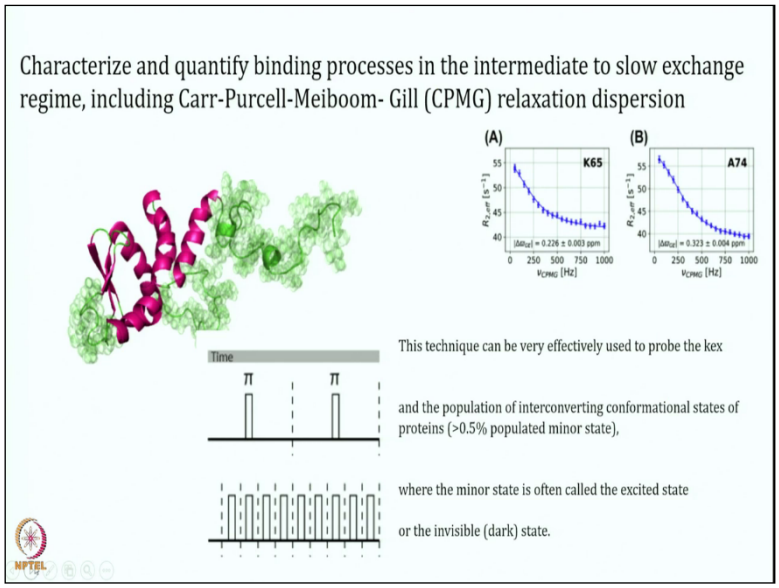

So, this is called CPMG experiment: Carr-Purcell Meiboom Gill experiment, relaxation dispersion experiment and basically this is used to characterize and quantify the binding process in the intermediate to slow exchange regime using relaxation dispersion experiment. So, this is nothing but a T_2 experiment, we have learn the T_2 experiment or R_2. So, R_2 is transverse relaxation.

So, R_2 has two components: R_2 intrinsic plus R_{ex}. This R_{ex} is telling about the exchange process that is happening and R_2 is R_2,intrinsic. So, when we measure R_2, we essentially get both of these things, intrinsic transverse relaxation rate and the exchange rate given by R_{ex}. So, this R_{ex} has this phenomena of exchange. Can we use those experiments like a CPMG experiment to find it out what is the exchange rate happening.

So, this very effectively probe by the k_{ex} rate and as low as like 0.5% of minor populated state, often these minor populated state is called excited estate or many people call it dark state are invisible. Because you can imagine the 99%, 98% is populated by the ground state and some states which is very low populated 0.5%, 1%, 2%. So, that is a dark state because the signal will be dominated by this ground state 98% fraction.

But still NMR enables you to detect this 2% or 1% states which is excited state, but it is invisible state. So, how we can do that by doing R_{ex} experiment. So, quickly I will just try to explain you what essentially we do.

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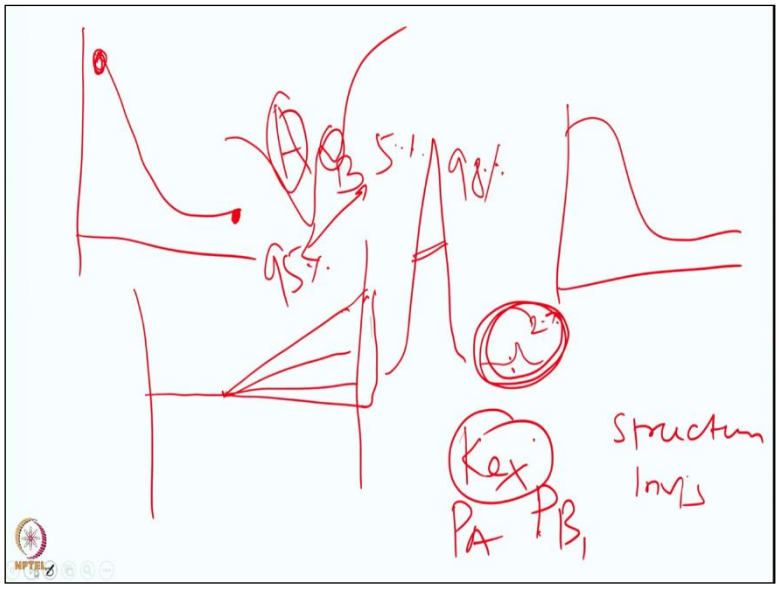

So, here is my state A which in energy landscape here and here is my state B and they are in exchange, right. So, this is say 95%, this 5%, okay. Now you have to record this CPMG experiment at various τ, like where you vary the distance between the π-pulses that I show you in the previous slide. So, here π-pulses if you see is these two, which gives you the $R_{2,\text{effective}}$ here and if you increase this you get $R_{2,\text{effective}}$ here.

And intensity decays, basically this intensity decay as you vary the frequency of CPMG pulses you can pick this and find it out the exchange rate, the population of A state, population of B state. So, for each residue using this HSQC based experiment, you can do this relaxation dispersion experiment, fit the intensity and you can find it out not only the population like what is the fraction, the exchange rate,

but also the probable structure of the alternative state. What I mean, say here is my major state and here is my minor state, which is like say 2% only, this is 98%. So, mostly what we do, we get signal from here but when we do this kind of CPMG experiment you can also get some information from this 2% one. And using this curve, we can fit it out and find it out what is this other state, invisible state chemical shift.

So, if you can figure it out the chemical shift of invisible state, using that information essentially you can find the structure of the invisible states. So, that is what relaxation dispersion experiment enables you to finding it out k_{ex} rate between these 2 population p_A and p_B of these 2 states and also the structure of the invisible states. So, what essentially we are doing is in this constant time we are changing the π-pulses frequency.

And because of this, the intensity decays happen and intensity decay of $R_{2,\text{effective}}$ can be fitted it out to find it out all these parameters, the exchange rate, the populations and also the chemical shift of the invisible states which essentially tells you about the structure of the invisible state. So, how this actually happens? A very beautiful work done by Lewis K and his former colleague called Antonio Mitchell Meyer, he is still working on these areas.

So, essentially they explained it very nicely in a in relatively easy manner giving an example of a runner and a walker. So, suppose a bunch of people are running a cross-country race, okay. Now there are bunch of people who run fast and some people run slow. So say they are running 5 kilometers. So, they starts at T = 0 and at certain time T = 1 hour, they are somewhere and you see there is a large distribution like distribution of distance between them.

What they cover? Who run faster they can quite easily reach to the goal and those who are really slower they will be somewhere 2 kilometer - 3 kilometer. So, you have a large dispersion. So, here is what we are saying. So, here we started and they are running. So, they reach somewhere depending upon on the distance. So, here you have a large dispersion okay. So, here is our $R_{2,\text{effective}}$ large dispersion.

Now you are say a referee and what you are doing. So, they run half an hour and you blow a whistle and ask them to return back. So, when they return back then you see that dispersion between them means distance that they cover between them, it will be slower like a you will be narrow and if you blow whistle many times the difference between the distance that they cover will be minimum.

So, that is what we have at the bottom, the dispersion is very minimal and when we have only one π-pulse, or 2 π-pulse, dispersion between them is very high. So, because of this dispersion measurement that we are doing, 2 π-pulse and many π-pulses, you see the $R_{2,\text{effective}}$ here is high and $R_{2,\text{effective}}$ here is low and essentially you fit this curve to find it out all those parameter that we are discussing.

So, this is the minimalistic experiment explanation that I can give you, how this relaxation dispersion experiment works. It is a really powerful experiment and it is very much used in understanding the enzyme kinetics, understanding the protein-protein protein-ligand

interaction where the other state is populated really low, right, as low as 5% or 0.5%. So, that is all about relaxation dispersion. Let us move to another important experiment called ZZ exchange.

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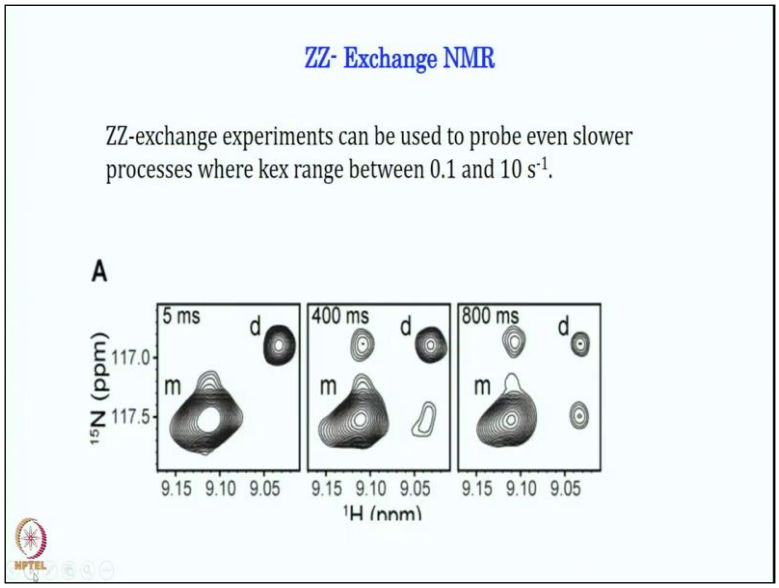

So, ZZ-exchange essentially probes the slower process where the k_{ex} rate ranges from 0.1 to 10 s^{-1}. Suppose 2 states are really exchanging slow okay. So, say suppose protein is going from monomer to dimer. So, it is a self-association, say protein-protein interaction, self-association happening, this is a monomer and this is dimer okay. And this exchange between them is happening really slow, order of say 0.1 s^{-1} to 10 s^{-1}, this is slower at the NMR timescale.

Now what happens, you record this ZZ-exchange experiment and what you do, you put them in Z-magnetization and let them mix, right. Whatever we do in a NOESY experiment, let the magnetization be mixed. So, to start with we are seeing a monomer peak and a dimer peak and if the exchange is happening between them as you increase this time 400 to 800 ms, you start seeing this cross peaks.

Now this cross peaks is telling that they are self-associating, there is exchange between monomer and dimer and you can find it out the rates at which it is happening. So, here essentially if you look at we are increasing the mixing time and because of this when they are mixing more, you are getting a cross peak. So, that is measuring the self-association or protein-ligand interaction, even the catalysis that is happening at slow, similar concepts can be used to probe

the phenomena that is happening at the slow exchange rate. ZZ-exchange actually offers this. So, probably I covered you all the range the fast exchange, intermediate exchange, and slow exchange; how it happens and what it happens. So, if we understood this, can we move ahead and just try to understand essentially can we get it more quantitative manner.

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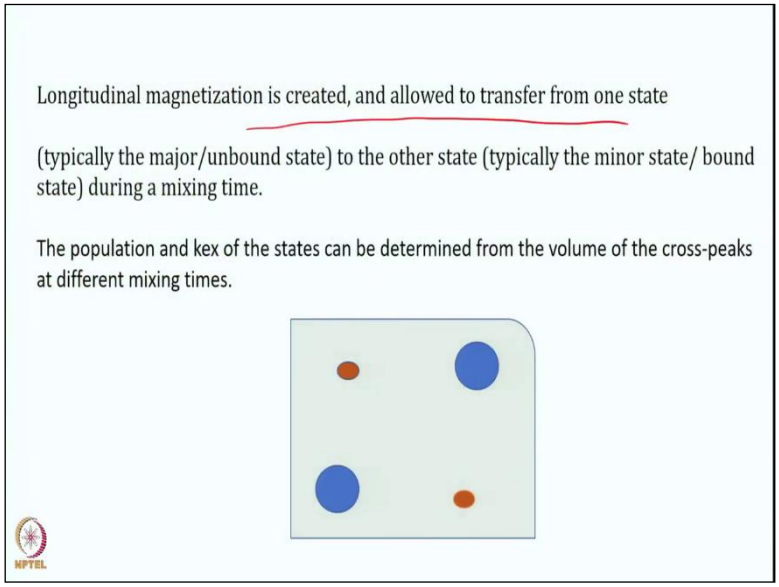

So, in the ZZ-exchange, as I discussed it is a longitudinal magnetization created; allowed to transfer from one state to another state or a major bound-state to unbound states; and here are the cross peaks that are appearing. So, population of the two states can be determined by the intensity of the cross peaks or volume of the cross peak with a different mixing time. So, intensity may change like this and you can find it out the $k_{ex.}$ That is what about the ZZ-exchange.

Now once we have the idea about the slow exchange, the intermediate exchange, the fast exchange, can we get slightly more quantitative, try to fix this parameter and get some parameters that one can do it.

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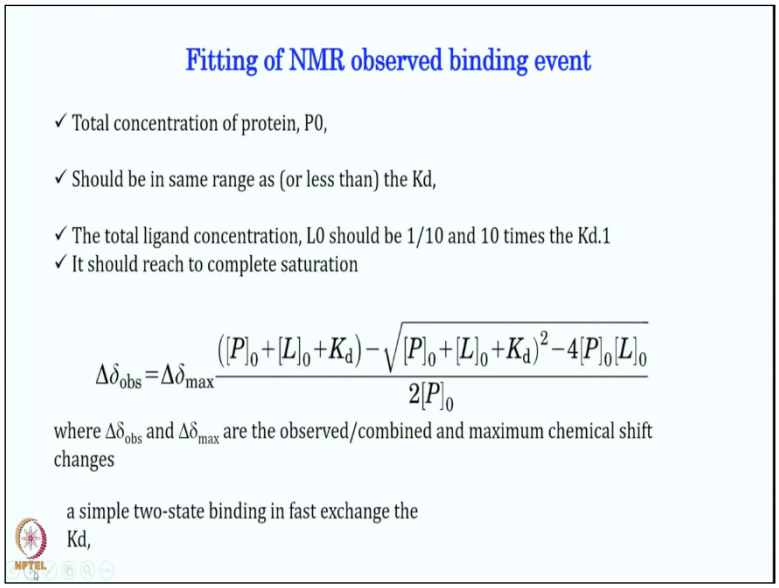

So, how we can fit this NMR observed binding events. So, for getting a quantitative estimate we need to know something like we need to know what is the starting concentration of protein which is P0, in what range this *K*d should come. So, you should have some idea from any orthogonal techniques like we discussed ITC, SPR, or any other technique, fluorescence-based.

So, what is typically order of the magnitude we are getting that will be good idea to have this. So, you should have an idea of the some *K*d and the concentration of proteins should be of that order of *K*d. The total ligand concentration say L0 should be one tenth or ten times of *K*d, depending upon what is there. So, if we are doing a titration and probing this chemical shift change, so it slowly goes and after certain time it saturates.

What is saturating? The perturbation in the chemical shift as you increase the ligand concentration, the CSP changes and saturates. So, you can if you know all these parameter what is the P0, the initial concentration of protein, the initial concentration of ligand, and the *K*d that suppose we want to determine. What we are seeing, $\Delta\delta_{obs}$. You can fit essentially these equations and we can find it out *K*d, right.

Or if we know typically *K*d, we can even predict how my observed chemical shift is going to come. So, essentially here $\Delta\delta_{obs}$ is the chemical shift that we are observing at a particular ligand-protein concentration. This is the maximum, that we are observing here right. So, if we are assuming simple two-state exchange happening between free-form to protein-ligand form essentially we can feed all these parameters and find it out the *K*d of the binding event ok. So, let us see how we can do that I will give you some of the example.

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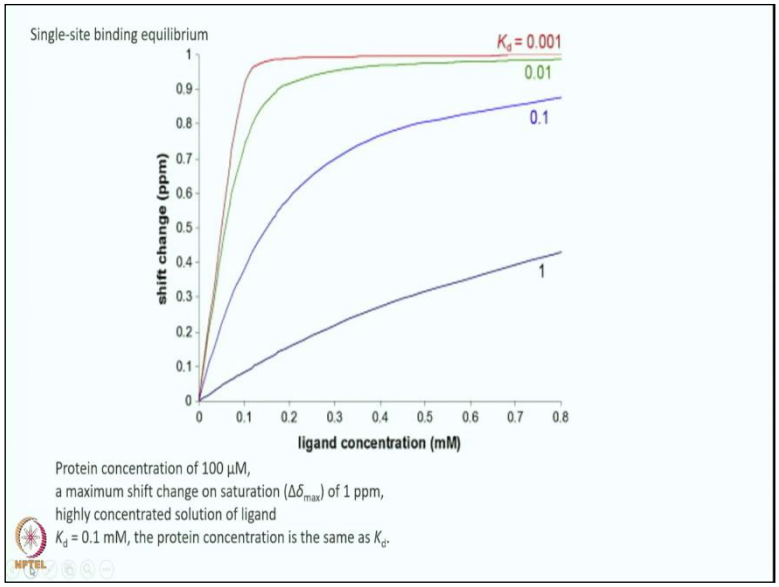

So, here like a binding event happening 2 exchange, single site binding happening between the protein and ligand right. So, typically suppose we take a protein concentration of 100 µM, that is what I said if the *K*d is in micromolar range, you can have the protein concentration in the same range so 100 µM. Now the maximum shift on saturation, δ_{max} can be say one ppm for a highly concentrated ligand and *K*d = 0.1 mM of protein concentration same as the *K*d

So, depending upon how we vary ligand concentration, you can see a different saturation curve. So, here it is going and *K*d is typically of one. Now if we are saturated quite easily so ligand is saturating the protein *K*d is 0.001 and in different case, one can simulate what is the *K*d with these typical parameters. So, that is single binding site the *K*d calculation with the shift change. Let me repeat again.

So, typically we are taking 100 µM of protein and we are titrating with the ligand. So, here you can see we have taken millimolar of ligand, 0.1 mM, 0.2 mM and our protein concentration is 100 µM right. So, that is what we are starting with. So, 0.1 mM right. So, 1000 µM is 1 mM. So, 0.1 mM of protein we are starting.

So, when we put 1, 0.1 millimolar if *K*d is one we are getting a straight line here, ok. It is increasing like this, it will saturate somewhere going here. Now if our *K*d is slightly higher 0.1, we are getting saturation almost now reaching here and if *K*d is very strong like a very minimal in micromolar. So, here 10^{-3} M, so, in mM case you will see that it is saturating very fast.

So, depending upon the strength of the binding, ligand-protein concentration which choose we can find a different kind of curves that can be interpreted to find it out *K*d.

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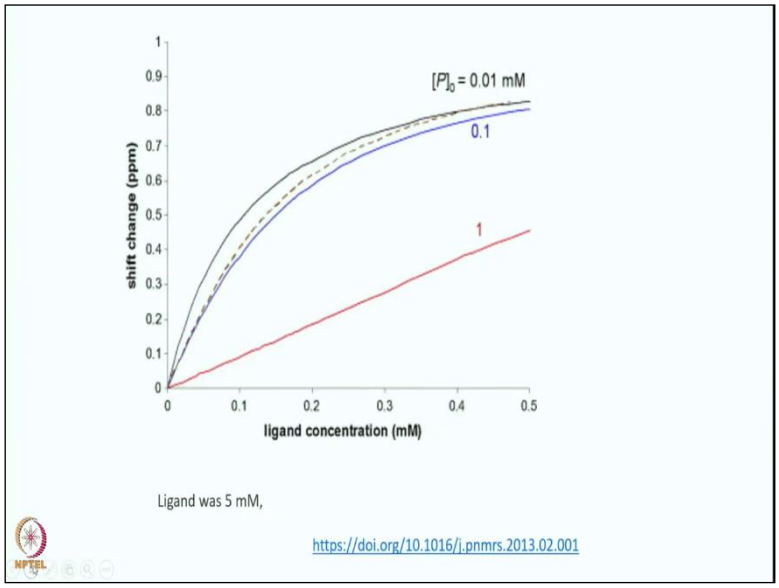

Now another thing one can say here say ligand was fixed at 0.5 mM and protein concentration is being changed. So, depending upon what *K*d we have for different protein concentration, you see a different kind of curve that is coming, right. So, that is what essentially you can find it out with the chemical shift change. So, let us look at some of the example.

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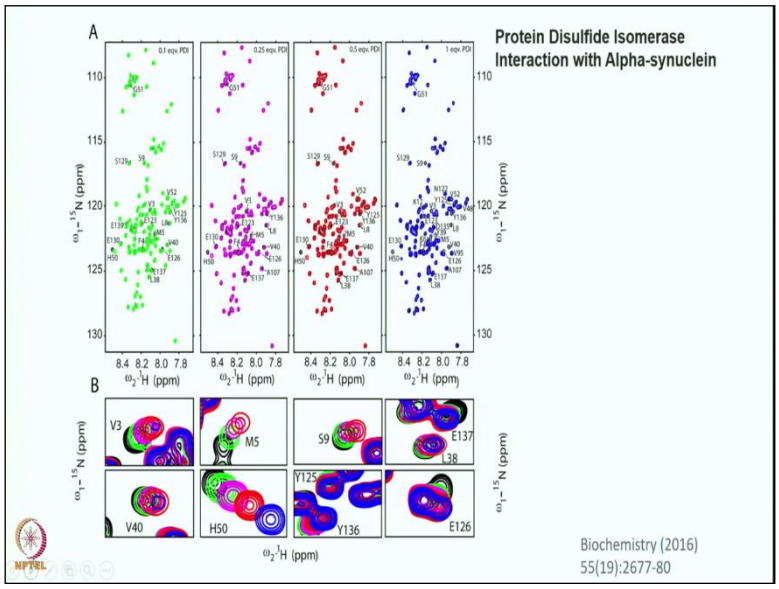

So, here what I am showing you interaction between 2 protein, one is called PDI, protein disulfide isomerase which is interacting with an intrinsically disordered protein called α-synuclein, this protein is involved in neurodegeneration. So, we are titrating this α-synuclein with the PDI. So, first thing what we did, we N15 labelled this protein and PDI was unlabelled,

okay, with no isotopic label. We recorded HSQC spectrum of this protein and then we are titrating with PDI.

So, here we can see the nicely dispersed spectrum of the α-synuclein, you can see. Although it is a narrow range, but the peaks are very sharp and very round shape. And now if you have such a good resolution, you can monitor even peak-wise what is happening. So, once you start titrating your protein here, the other protein is 1 to 0.1 in the first case, 1 α-synuclein, 0.1 PDI. You see some of the peaks already started shifting.

I have blown up picture here. When we increase to 0.25 some more peaks here you can see start shifting; at even more 0.5 you can see lots of peaks now seems to be showing the chemical shift perturbation; and one equivalent you can find lot more are showing the chemical shift perturbation. So, here in the zoom picture, you can see this shift is happening here and M5 again you see S9, you see L38.

So, many of such peaks are showing shift. Now that is fantastic, now you once you titrate it you know how the protein-ligand concentration is changing here. Concentration you have and here $\Delta\delta$ you have, right, change in the chemical shift. So, this is the thing, that if you know the chemical shift change you can probably fit it and find it out how they are binding.

So, that is what one can do. The another thing if you notice what is happening here few of the peaks are showing decrease in the intensity; if you look at closure, 2 things are happening, one shift in the resonance frequency or chemical shift perturbation. The second thing happening is some of the peaks are showing decreasing the intensity.

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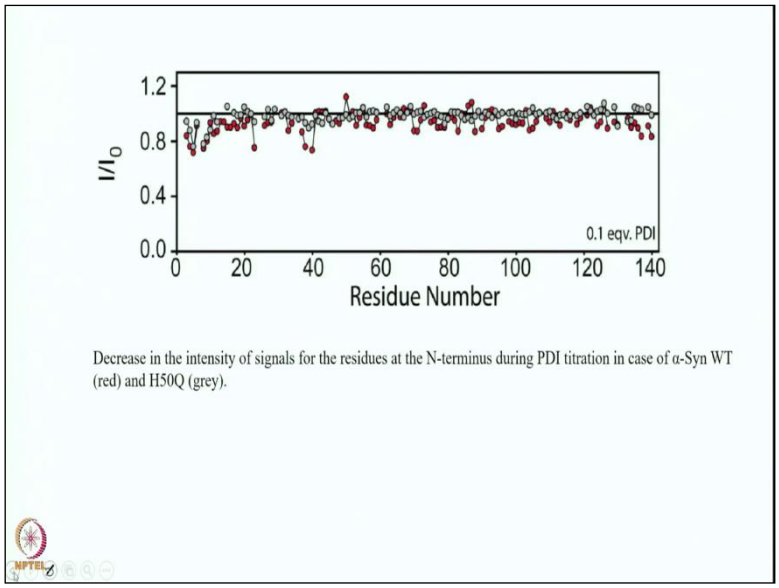

Decrease in the intensity of signals for the residues at the N-terminus during PDI titration in case of α-Syn WT (red) and H50Q (grey).

So, that also can be plotted and what we found that here essentially some of the peaks that we are showing decrease in intensity are coming from the N-terminus of the protein and somewhere from the C-terminus of the protein. So, these from the N-terminus, upon PDI titration of α-synuclein can be measured basically of a mutant and the wild type. You can see there is some variations when you take a mutant. Essentially, in the wild type you see lot more peaks are coming. So, even changing one residue lots of variations you can see.

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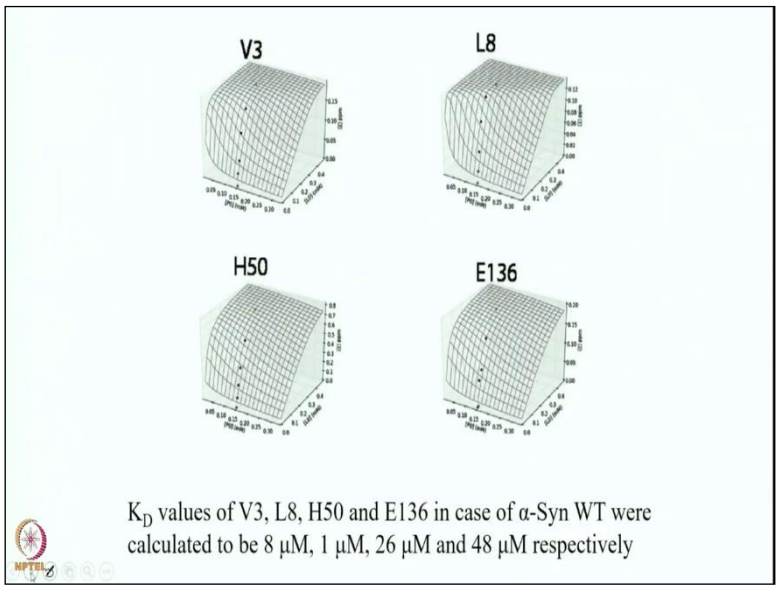

K_D values of V3, L8, H50 and E136 in case of α-Syn WT were calculated to be 8 µM, 1 µM, 26 µM and 48 µM respectively

So, these 2 NMR observable can be used for fitting and getting the *K*d. So, now the chemical shift perturbation we can fix it here is the PDI concentration, here is the protein concentration, here is the ligand concentration, and this is the shift in the chemical shift. So, 3D plot we have made, the peaks are shifting here, and this shift essentially you can plot it. Plot to find it out

*K*d. So, one can find it out the residue specific *K*d of α-synuclein which is found to be in micromolar range.

So, for some it is 8 µM, some for some it was 1, 26 and 48. So, what we can infere here? Now, *K*d you can find it out using the classical thermodynamic technique like ITC or SPR. SPR essentially gives you k_{on} and k_{off} rate which can be used to calculate the *K*d. ITC gives you ΔG, ΔH, TΔS, and stoichiometry; you can even get the thermodynamic parameter.

But what NMR is offering you, not only the value the *Kd,* but also offering the residue specific *K*d. Which side of the protein is contributing more towards the binding which was not possible in ITC or SPR in single experiment, yes you can mutate the protein, create a different mutant and look at the relative importance of the one side C-terminus, N-terminus or middle side. But in a single experiment now NMR is offering you to find it out residue a specific *K*d of these bindings.

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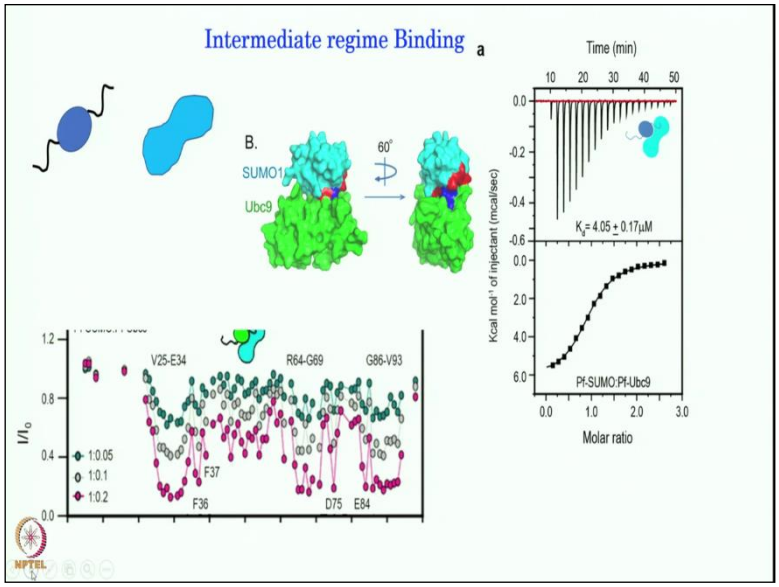

Another example I am giving you where intermediate regime binding was there. So, this is the example coming from a SUMO it is E2 interaction. SUMO is small ubiquitin related modifier. It does the protein modification while binding to various enzymes. So, one of the enzyme is called UBC9 that it binds and it forms a complexes. So, when we did like titration what we see that upon increasing the concentration 1 to 0.5, 1 to 0.1, 1 to 0.1 lots of peaks are changing.

They are showing decrease in the intensity okay. Prominently in this region, in this region, and this region, but other regions were also participating. Now here the chemical shift perturbation

is not happening. So, I cannot fit that data to find it out what is the *K*d. So, but we use again orthogonal techniques and we find it out the *K*d is coming to be 4 µM. So, you saw that here essentially in the previous slide, we found that 8 µM there are reasonable chemical shift perturbation.

But in this case it is happening at intermediate exchange when the 2 protein is binding, 2 globular proteins are binding, the peak starts disappearing which is saying intermediate regime binding and which is substantiated by the *K*d and now intensity is started disappearing okay.

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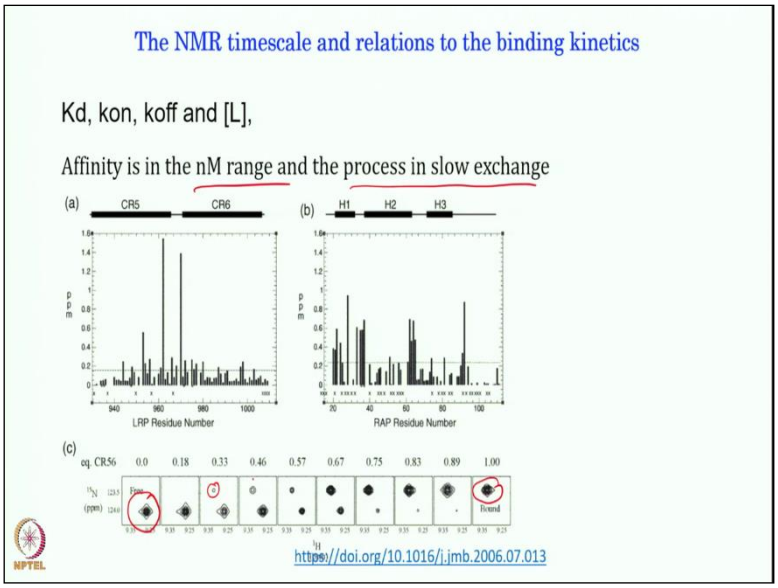

So, the NMR timescale and its relation to binding is very crucial to understand that depends upon what is the *K*d, what is the k_{on} rate, what is the k_{off} rate, what is the ligand concentration. So, sometimes it is possible that affinity can be in nM range, but the process happens in slow exchange. It is exchanging between the 2 peaks. Like for an example in this case you see here is a free-ligand, only one peak.

But in the bound form it is shifting completely towards other state, but in between you see the other peaks starts appearing and one of the peaks start disappearing. So, slowly these peaks disappear and this peaks started to build. This is a slow exchange process but the binding happens in the nM range, okay.

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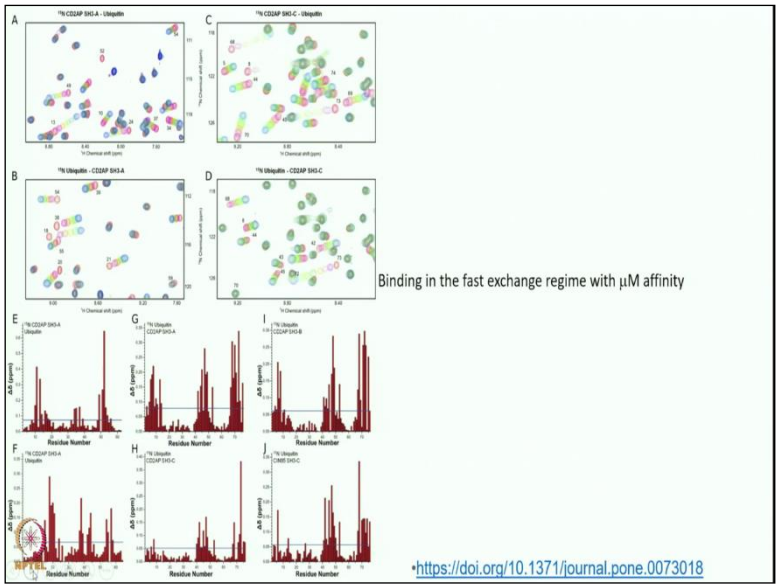

The another case one can see that here binding happens in fast exchange because you see continuously the peaks are shifting here binding happening in fast exchange but the affinity is in the μM range. So, the protein-protein interaction which observe that protein comes in various forms depending upon what is the exchange rate and what is the affinity. So, here fast exchange happening at a μM range, the another case it was slow exchange happening in nM range, okay.

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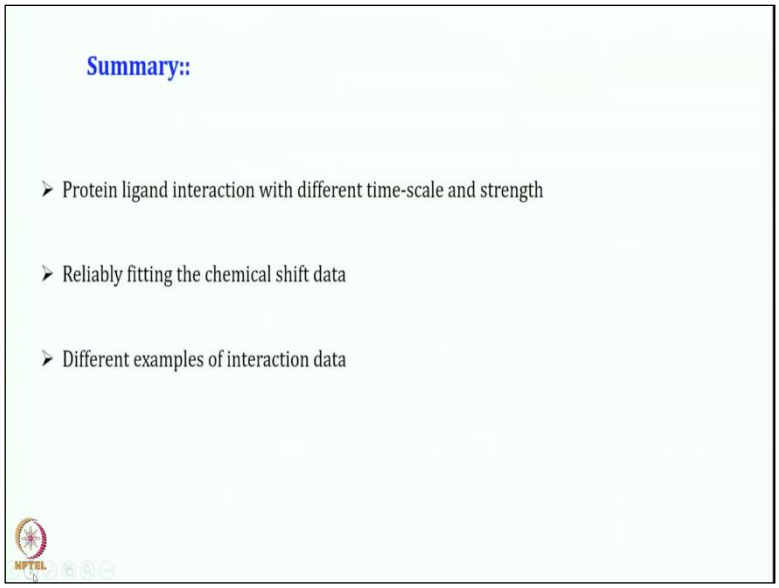

So, that is what is protein-protein interactions. Let us summarize what we saw today; protein-ligand interaction happens with different timescale and different strength, strength in terms of *K*d and timescale in terms of the exchange rate with respect to NMR time. And one can reliably fit the chemical shift data to find it out the *K*d. I show you some of the examples of interactions where you can look at the chemical perturbation or look at the disappearance of the peak to get the idea where basically this protein interacts.

And you can using this information you can create a complex model. So, here with this I am going to end now protein-protein and protein-ligand interactions. And in next week, we are going to now see how this information can be exploited to understand the drug design and drug development using this protein-protein protein-ligand interaction. Can we come up with a molecule and then grow this molecule like in terms of chemical synthesis to make it effective or efficient drugs.

So, that is it going to be discussed in the next week. Thank you very much and looking forward to see you in the next class. Thank you.