Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

Prof. Hanudatta S. Atreya
Department of Chemistry
Indian Institute of Science, Bangalore

Lecture – 37
Understanding Protein ligand interaction by NMR: Chemical exchange

So, we have started looking at, the concept of chemical shift perturbation. This is a method where we want to study how protein and ligand interact with each other.

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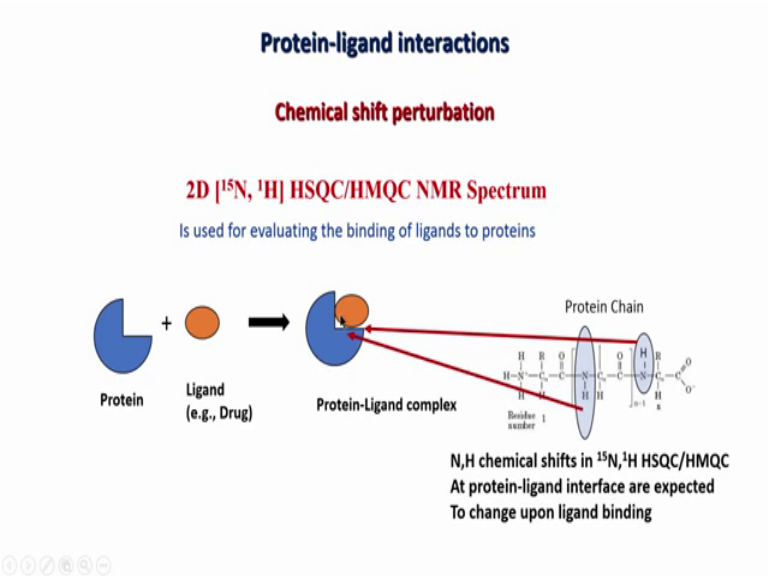

So, we saw briefly this in the last class that if we have a protein and a ligand, you can have a complex where it binds to a particular region of the protein. The ligand binds to a particular region of the protein. And we saw that this interaction can be studied by HSQC NMR spectrum. So, typically we take N 15 proton HSQC not carbon. Carbon also can be used. In case of carbon HSQC normally we look at the methyl signals. But in 915 HSQC we look at this signals coming from the amide protons and nitrogen.

So, for each peak in the NMR spectrum we know in HSQC corresponds to one amino acid. So, what happens is, those amino acids which are at the interface this is called this here. This is called a binding interface where the ligand binds with the protein. So, the amino acids on the binding interface are expected to undergo chemical shift changes because, the chemical environment around this residues change when the ligand binds to the protein. So, this is interface is amino acids are expected to therefore, shift in their

peaks. And that is something which we capture by HSQC and that is what we looked at last time how do we do that?.

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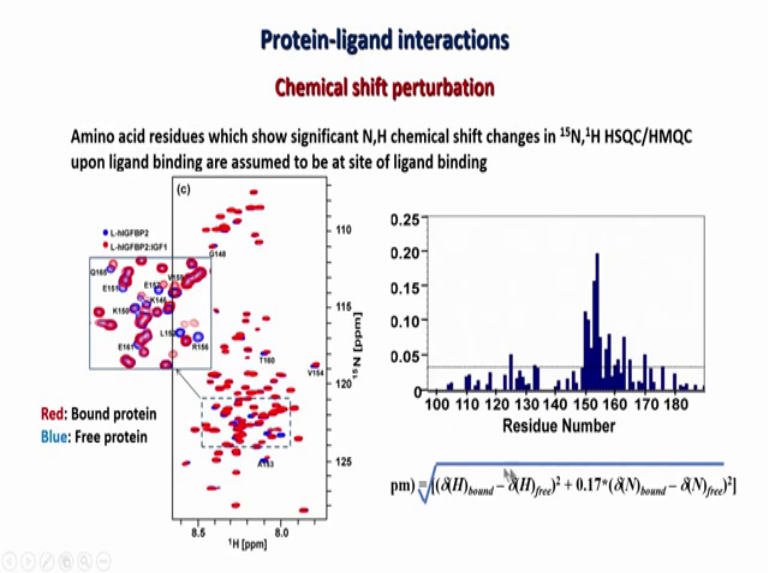

So, we saw that you can have basically interaction with a specific set of residues on the interface. And that is now shown here, this is called a Chemical shift perturbation plot. So, what is plotted on the y axis here is the amount of shift. That means, how much shift has taken place for a given amino acid from the original free protein. Means, what we do is we take a free protein. And we add the ligand, normally we do what is called titration is slowly add ligand in a small amount and as the reaction proceeds the binding takes place, and we can monitor the chemical shift changes the difference here between the red and the blue is plotted on the y axis.

But as we saw last time that this red and blue are not just changes in the amide proton. This is x axis. It is also shifting in the y axis that is nitrogen. So, you have to combine the shift of nitrogen and proton, and give a single number. So, how do we combine these two shifts or changes? Usually we use this a formula like this, where for a given amino acid we saw that we can actually look at the hydrogen amide and nitrogen shift and combined together and that is a number which is plotted.

Now, here one more thing which we saw in the last class is that region which binds can be identified based on how much significant shift they are showing upon binding. So, in this particular protein, which is a real case example. We see that residues number 150 to

160 or 165, somewhere in this region they are binding sufficiently strongly to the protein because those amino acids are undergoing a significant change in their backbone amide and nitrogen chemical shift. So, this is how we can find out which region of the protein is binding to the ligand.

So, remember we are not; we are trying to characterize a ligand binding in two ways. Number one, we are trying to find out where in the protein on what particular part of the protein does the ligand bind. And the second thing which we are trying to characterize is, what is the strength of the binding means how strong is the binding is it the binding very weak or sufficiently in strong.

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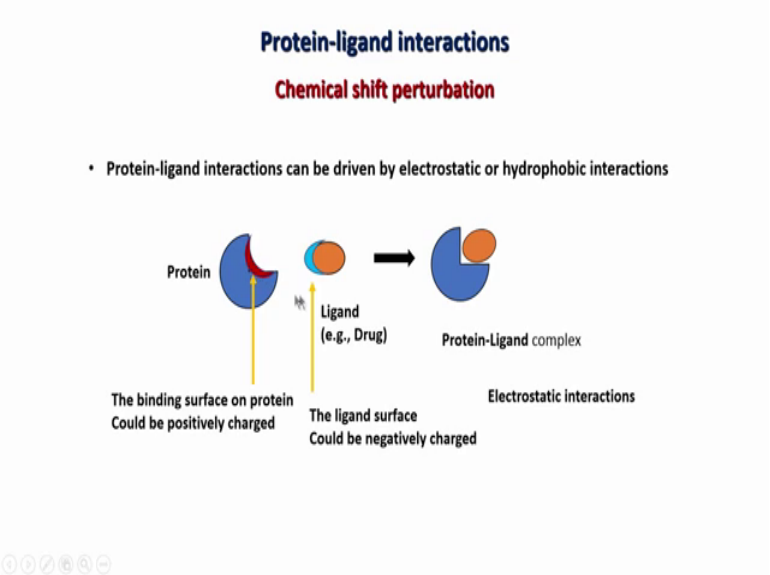

So, that is measured by a number known as dissociation constant. And the dissociation constant K d will give us the information whether it is a strong binding or a weak binding. So, this is something again we saw in the last class, that you can have what is called as Electrostatic interactions, where in the positive charge surface or a negative charge surface of this ligand can interact with the positive or negative charge surface or oppositely charged surface on the protein.

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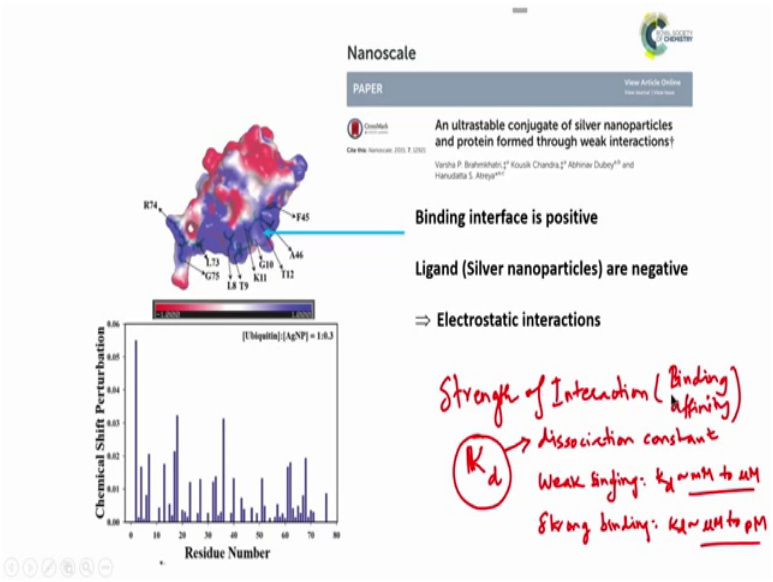

So, we saw one real case example of a protein ubiquitin, binding to a nano particle. And we and I mentioned that the nanoparticle which is silver nanoparticle, is negative in overall is charged. And therefore, its interacts to the with or with the by positive charge surface on the protein. So, this blue color patch, what you see here is a positive charge surface on the protein. And the red colour means negative charge. So, these are the ways with NMR can help us to identify where is the binding region. But what is not coming out from these studies is, how strong is the interaction.

We want to measure also the strength of the interaction. And strength of the interaction is normally measured. So, when we write the strength, of interaction. Means how strong is this interaction that is measured in terms of a number called K d. So, K d is called dissociation constant ok. So, if it is a weak interaction, weak binding then K d will be in the range of somewhere between millimolar to micromolar values.

And strong binding will be, a K d will be in the range of micromolar to picomolar. So, these are the typical ranges of the binding affinities. So, this is also called binding affinity. So, let me write down binding affinity.

So, NMR can also be used for characterizing the binding affinity, of a protein and ligand interaction. And that as I said is used this number.

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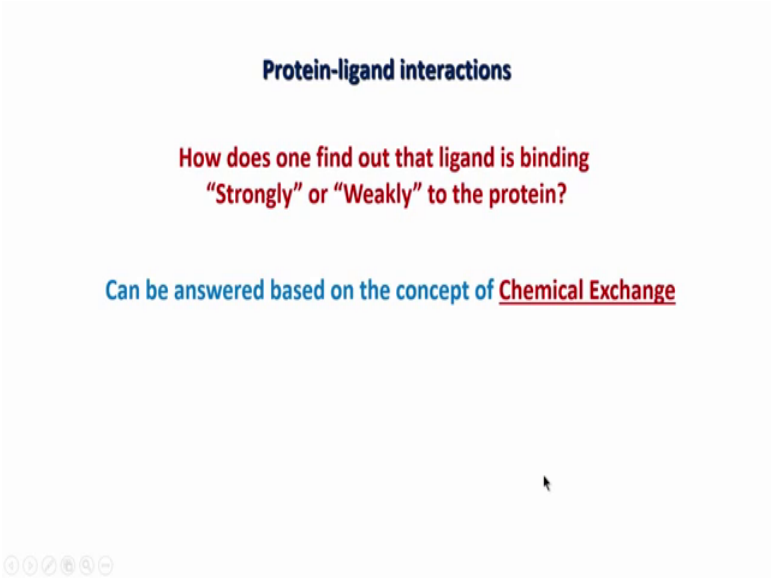

So, let us see what happens in case of whether a ligand is strongly or weekly bind bound. How can we understand that idea? And this lead us to a very important concept called Chemical exchange. So, based on chemical exchange a phenomenon called chemical exchange, we can find out whether there is a strong interaction or a weak. And this is a very important concept because, in most of the drug discovery process where we look at drug molecules it initially the initial screening of the drug molecules are done, based on this interactions and it is normally found that there are not very strongly bound. So, the initial hits what we get in drug discovery are normally weak binders. Then typically it is further improved with the synthesis method and different approaches and slowly it becomes a strong binder. So, the drug molecules are typically supposed to have nanomolar to picomolar affinities so, that they can work very efficiently in our body.

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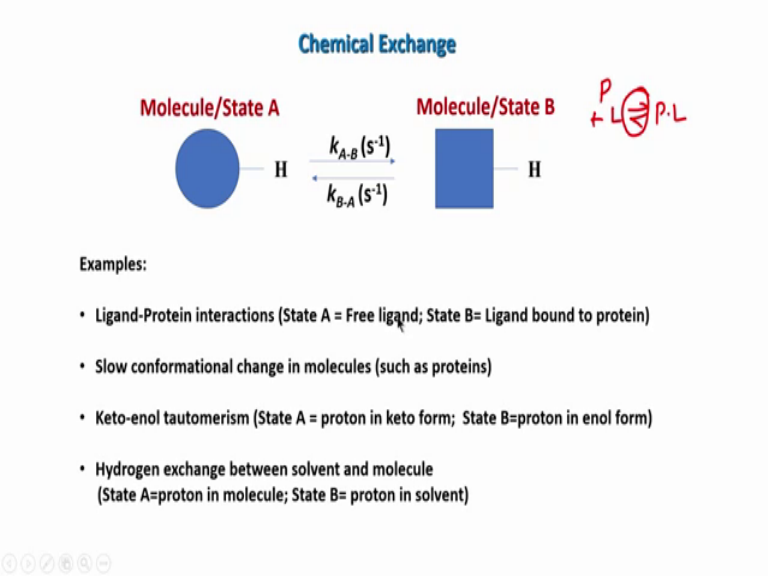

So, let us see how weak interaction shows up in NMR and how does a strong interaction work. So, now, for that we have to look at this concept of chemical exchange. So, we will go here looking at this model. So, imagine that there is a protein molecule and there is hydrogen or a some proton in some this molecule. Let us take any hydrogen, it does not matter this is just a schematic. The point here is imagine that the protein, is an state A means one particular state. Now let us say that because of something, that is interaction with a ligand or change in temperature or anything, it goes into another state B ok. So, this conversion from A to B happens because of some interactions or some reason.

So, suppose this conversion is characterized by this constants of rate constants. So, rate constant means how fast it goes from here to here. And rate constant for how fast it goes back from here to here. So, typically this kind of a change of a protein state from A to B is reversible. Meaning suppose I have a ligand which binds to protein and in the ligand is not very strong binder then, the ligand will not always be bound to protein. So, the bound form can be thought of as a state B and the free state free form of the protein can be thought of as state A.

Similarly, for the ligand we can think of this molecule as a ligand. The ligand in the free form, and ligand in the bound form. So, whenever there is an interaction normally between ligand and protein it will be a dynamic or a equilibrium interaction. So, there will be a conversion always happening from free form to bound form and bound form to

free form ok. So, this can be written more clearly here. Suppose I have a protein plus ligand a protein ligand always as a reversible interaction they are all.

So, if it is very strong binding then of course, this reversibility that is going back is very slow. But if it is a weak interaction going forward and backward is equally fast. So, this is captured by this numbers create constants k A and k B ok. So, let us say that this. So, this is what is shown the different possibilities of state A and state B. So, state A for example, a state A could be a free ligand, and state B could be a ligand bound to protein ok. So, therefore, the ligand has now two possible states and it interconverts, means it goes from one to another and back in a reversible manner.

Or it could be that the protein is slowly undergoing a conformational change. So, many proteins for example, when the in helix, helical or beta sheet. It is possible that the helix is slightly opening up or closing. So, this kind of a motion is also represented in this manner. That means, a protein is going from one confirmation structure to another structure. Similarly, in organic chemistry we know, this concept of Keto-enol tautomerism.

So; that means, in the number of molecule that is a proton is in the kito form in one state. And then it undergoes conversion to an enol form in the state B. So, we can there the enol form can be represented or considered as state B, and the keto form can be considered as state A. Another example of states A and B can be hydrogen exchange. For example, this hydrogen can exchange with solvent and become deuterated. Suppose a solvent is deuterated solvent, solvent proton can change, and go to become deuterated. So, H D exchange.

So, in that is case the protonated form, can be considered as one state and the deuterated form considered as second state. So, essentially in chemical exchange what we are trying to do? We are trying to represent the molecule in two states and then we assume there is a reversibility and then we try to find out what happens in such cases in by NMR spectroscopy.

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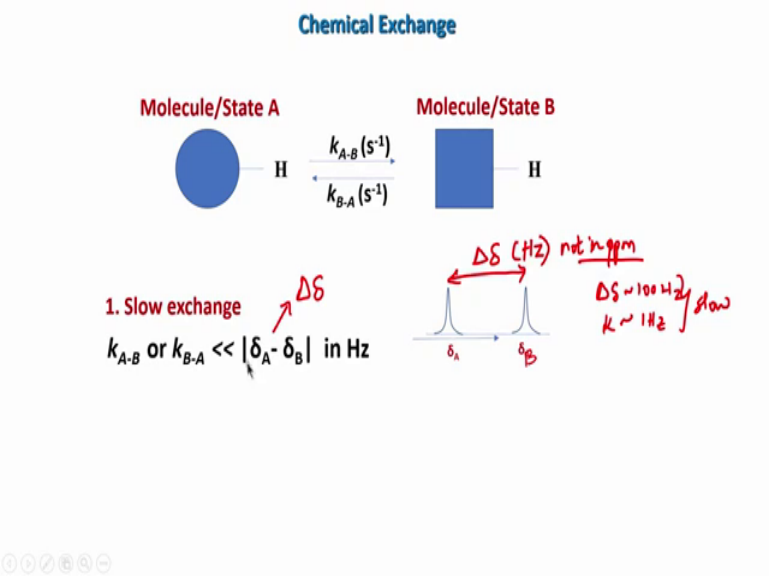

So, let us see from NMR what do we what information we get? So, let us say that this state A, has a chemical shift delta A. And the state B has a chemical shift delta B. So, we are assuming that there is a difference, in the chemical shift between these two states. This is not necessarily true always, if it is not same, then it is difficult to study. But sometime fortunately they are changes in chemical shift because the structure changes slightly. So, whenever there is a change in structure NMR chemical shifts will change. So, let us assume that in our example whatever we are studying now, that there is a two the two different chemical shifts between state A and state B.

Now, how do we characterize the exchange? So, in NMR the exchange is always with respect to the difference in this chemical shifts. So, what matters to us is these difference. We use our delta delta. So, these difference is very very important for us, all the concept of exchange in NMR depends on this. Now remember one thing very crucial here is this difference is not we do not measure in ppm for exchange we measure in Hertz. Not in ppm because in ppm scale it does not change. Whether you go from 600 to 800 or 900 mega Hertz your ppm difference will not change, but the Hertz will change.

So, what really matters in NMR is how much is the difference in the chemical shift between the two states A and B in the scale of hertz ok. So, now, these are the different possibilities. For example, this exchange is very slow meaning the rate of these exchange what is shown on this here, is much much less than the difference absolute difference in

the chemical shift. That means, the K value suppose this is about 100 Hertz. Example let us say, this is delta-delta this is called delta-delta ok. So, if delta-delta is 100 Hertz. and your K is 1 Hertz then, it becomes a slow exchange ok. The example like this. So, this rate constant is much smaller than the difference in the chemical shift, which is measured in Hertz scale ok.

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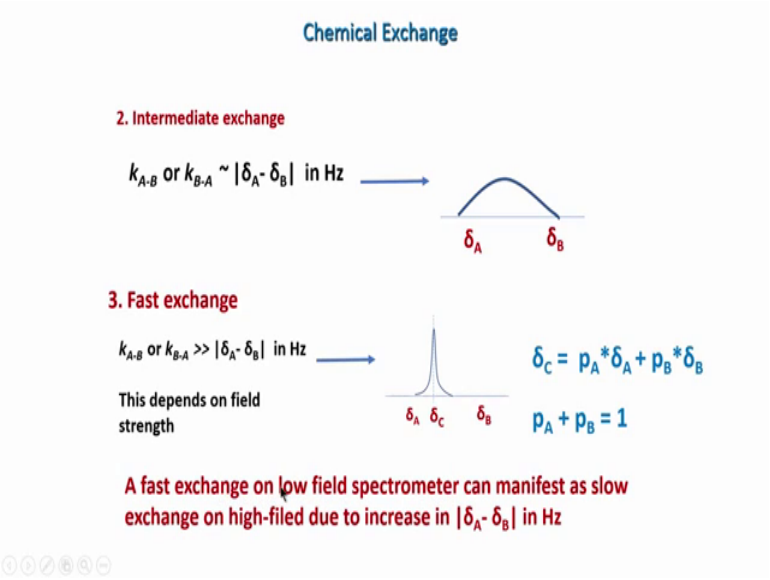

So, let us see how that second possibility. So, that earlier case was the slow exchange case. Now there can be another scenario, another possibility where the rate constants of the conversion from A to B and B to A is almost nearly same as the difference in chemical shift between the two states again measured in Hertz. So, if such if that happens then, the peak of the two peaks which are separated earlier start merging ok. And they become a broad single peak. So, this is called Intermediate exchange.

So, there are different regimes or different types or different categories of exchange in NMR. So, there are three as mentioned here the second is the first one was slow exchange and second is called Intermediate. The third scenario or possibility is that, the rate of inter conversion between A to B and B to B is much higher than the difference absolute. Again remember, we are looking at the absolute difference in Hertz scale between the two states.

So, what happens in such a case? We again see a peak. So, here also we are getting one peak, but it was very broad here it is a very sharp peak. But where does it appear? It

appear somewhere between A and B. So, it is neither A nor B. It has come in between and what is this value? This value is obtained or is based on this formula. So, here it says, that delta C where the final that merged peak comes, this peak comes, is equal to population. Means how much percentage of a is present in the sample multiply by that chemical shift, plus percentage of B population that is how much of B is present multiply with the chemical shift of B.

So, you see here what is happening is we are dividing the population into two parts. Because remember we are looking at either A or B. That means, the total population we will assume to be constant. If A going to B, A will reduced and B will increase. So, therefore, total population should remain constant. So, we say partial populations, fractional population. So, its amount is 1, fraction adds up to 1. Say this is 50-50 percent and this will be 0.5 this will be 0.5.

So, if its 50-50 then, the delta C will come at the half midway between this and this. Because it will be half of that plus half of that ok. But if the population of a is very high, then it will be close to the A. So, we can see this line here seems to be not at the centre, it seems to be towards A because in this example it is possible that A is P A is more than P B. So, it get shifted or dominated by the chemical shift of A. So, one thing you will notice from this equation is that if I keep changing the populations, I will keep moving this peak this peak will shift here here based on how much of A or B I add.

So, this is something which is the interesting thing which we will be using later on I will show you for characterizing fast or slow exchange. Now, again which I mentioned earlier this whole concept of chemical exchange whether it is fast or slow, depends on the field strength. So, if I go from 400 megahertz to 800 megahertz, my field will double. Means my delta will double this delta- delta will be doubled. Because, this let us say this is 3 ppm difference, 3 ppm on a 400 megahertz in proton, is 1200 Hertz.

But the same 3 ppm if I go to 800 megahertz will be 2400 Hertz ok. So, this changes. But this number this rate constant does not change with the field strength. Means it is not different whether it is 400 or 800 that remains the same because, this is not in NMR parameter. This is a parameter which is inherent to the reaction or change of A to B. It does not depend on what magnetic field under what strength of magnetic field I am using or I am doing the experiment. So, this is not an NMR related parameter. So, therefore,

this rate constant is constant. So that means, whether I call it as a fast or slow all depends on what is the field strength I am using.

So, it may happen that at 200 megahertz or 300 megahertz, what I call it as slow or what I called as fast, may become slow in terms of in 800 megahertz. So, this is very important concept chemicals exchange regimes are based on the field strength. So, this is what is mentioned here a fast exchange on a low spectro field spectrometer, means suppose it is 200 or 300 megahertz there this k value may be bigger than the difference. But when I go to a high field spectrometer, high field sorry this is a typo here. So, high field spectrometer it may become reverse. This k may become less than this, and then it becomes a slow exchange. So, we can change from slow to fast based on the NMR spectrometer.

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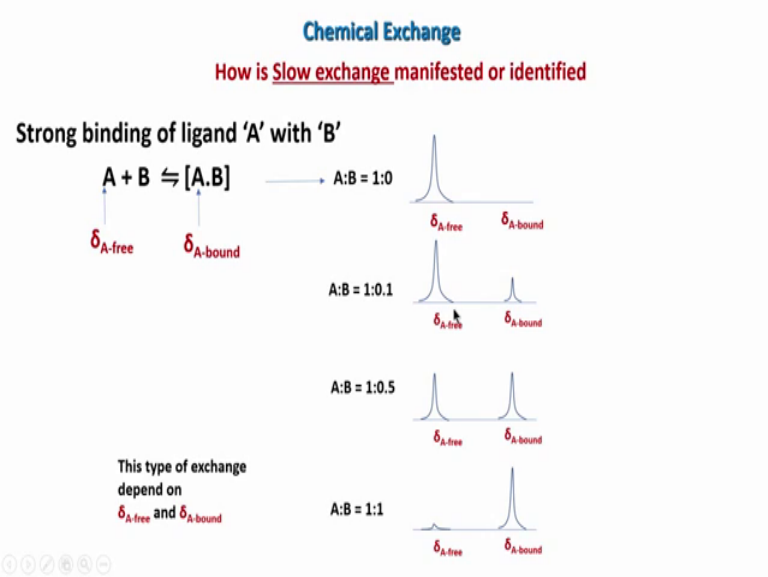

So, now let us see how slow exchange is manifested or identified? How do we find out whether particular ligand interaction or a change of protein from A to B is it slow or is it undergoing a fast exchange? So, typically when a ligand binds strongly with protein. So, protein ligand A is our ligand B is our protein ok. So, if a ligand binds to a protein strongly than, slow exchange is the scenario possible. So, this is typically happens when there is a strong interaction. Again remember what is strong and weak it is based on the K d value.

So, typically if your K d of interaction is somewhere from micromolar to nanomolar. We can say that it is a strong interaction. And in such a case your delta free that is the ligand, free ligand and bound ligand, we will have different chemical shifts and as we increase the population of A means when you add slowly A to B let me let us say initially that there is no protein added; ligand is only present in that case, your peak off only ligand will come there is no bound form, because there is no protein been added such a case the ligand will have a particular free volume.

Now, when we slowly add the protein, we add where is 10 percent 0.1 is to 1 ration. Then the bound population will start coming now. Because this complex is getting formed and this A is decreasing now. So, this A will decrease in intensity, but the A to B the A B complex is increasing in intensity. Now because there is a slow exchange, these two peaks are well separated. So, there is no question of a broad peak in centre or a population weighted average which I mentioned in the previous slide for fast exchange. So, this is the slow exchange scenario, which means strong binding. So, now suppose I increase further the I mean, I add more of B. So, I increase the population of A B complex.

So, you can see here A to B ratio is 1 is to 0.5. Meaning 0.5 of A has reacted with 0.5 of B and formed 0.5 of A B. So, the A B population now is 0.5 in some units whatever be the units But a also is 0.5 because half of A has reacted with the B. And half of A is free. So, now, the intensities of these two peaks become equal because they have same population and NMR basically reflects the population and therefore, that is equal in intensity. Now, I can continue this further and I add equal amount of A and B. So, the entire A will get reacted with B and you will end up with A B as 1.

If let us say I add 1 of B and 1 of A. Then complete A B complex is formed and A 0. So, A will almost be 0 again remember, it will not be fully 0 because this is a reversible reaction. So, there will be always a small population of A present in the solution. So, that is what is depicted here you may have 0 or very small intensity. But what has happened is the bound form is come to full intensity and strong peak ok. So, this is the typical scenario, which can be noticed when there is a slow exchange in NMR spectrum.

And that is what is basically that this type of exchange depends on the chemical shift. This is something I have been mentioning that the exchange is fast or slow, all depends on the chemical shift value of A and B.

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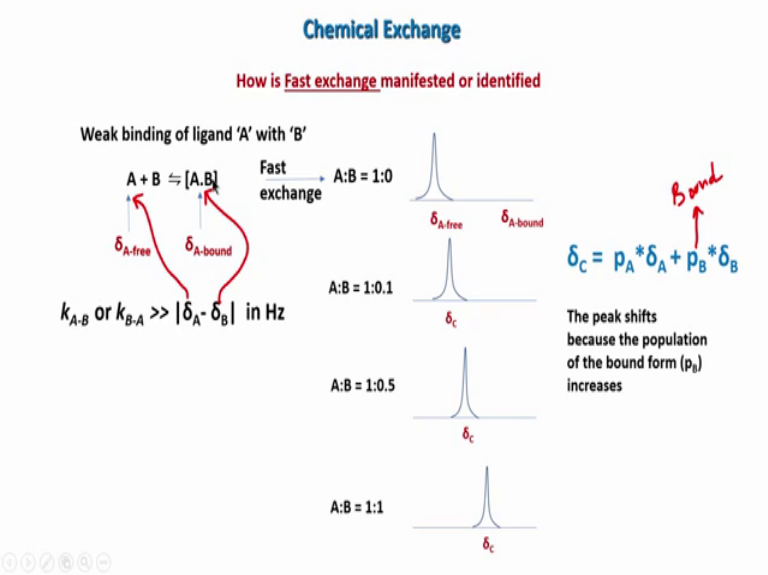

So, now let us see how does a weak or a fast exchange manifest. So, in a fast exchange it will be a slightly different scenario, let us look at that. So, again imagine this free ligand, which is binding to protein. So, B is our protein and forming a complex. Now here this cause we are assuming a fast exchange condition, it means the rate constant between conversion from A B to A this complex. And this back is very much larger than the difference in the chemical shift between the A in the free form and A in the bound.

So, delta B is not B. So, please remember this, this actually is this chemical shift, and this is this chemical shift ok. So, I should mention this in the previous slide as well. It should be free is this chemical shift delta A and delta B is the bound as mentioned here ok. So, in this case, in this first exchange scenario let us start from assuming that no protein is there. So, the ligand is only present. So, it is the free form. Now the whole thing can also be assumed from the protein point of view.

So, I can assume that A is a protein, and B is a ligand. So, it does not matter because I will then be monitoring the peaks of protein and not ligand. So, remember it does not matter I consider A or B, I just have to consider one species and I have to considered the

chemical shift of that in the bound form. So, I can considered A as a ligand molecule or I can consider A as a protein molecule and the B becomes a ligand.

So, for time being let us say that A is the ligand which we thought saw in the flow exchange also. So, then now is no protein added. So, it is 0 the bound form is not present. But now as I increase the population of B that means, I am increasing p B. Because remember p A plus p B is 1. So, if p A was earlier 1, p B was 0 Now I am adding p A as 0.9, I am I mean the reaction population of P A is decreasing because it is decreasing reacted with B but p B is now increasing.

So, but what happens in a fast exchange, in a fast exchange you do not expect this peak to come. Because remember I showed in the earlier case, we get a average chemical shift which is somewhere in between A and B and it depends on the population of A and B. So, therefore, we can see this peak is come in somewhere close to A, because right now A population is more than B. Now if I increase the population or concentration of B, then peaks starts moving even more it has come to the centre.

Now if I go to very high 1 is to 1 where a large fraction of A B is expected to be present. So, the p B. So, p B again remember is a bound form p B is not the population of B it is the population of the bound form. So, I should make it clear again, This is bound form ok. So, that has increased. So, therefore, the peak has shifted towards the bound form. So, this is how we can find out if there is a fast exchange because that will slowly gradually shift the peaks ok. So, the peak shifts because the population of the bound form increases.

This is exactly what I was saying. So, one thing is I again remember this depends on the field strength. What I call as fast, can become slow if I go to a higher field. So, in a higher field suppose this is done at 300 megahertz, and if I go to 900 megahertz, three times bigger in field strength. Than the this may not appear like this it will become like the previous case of slow exchange this is 0.1. And second is again repeat if that A and B it does not matter what you consider I can considered a as A protein and B as a ligand, in such cases I will monitor the protein spectrum.

If I look at A as a ligand then, I will be monitoring the ligand spectrum. So, both ligand and protein will show fast exchange because both are fast with respect to the chemical

shift. So, here it does not matter whether you consider A or B either 1, but you have to look at the bound form.

So, if I am in the bound form of A and I should look at the free form of A. Similarly if I am looking at B in the free form then I should look at the B in the bound form. So, in the next class we will take up a protein scenario means what happens when I look from the protein point of view and we will see how the chemical shifts of protein change when a ligand is added.