Principles and Applications of NMR Spectroscopy
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Module 7
Lecture No 37

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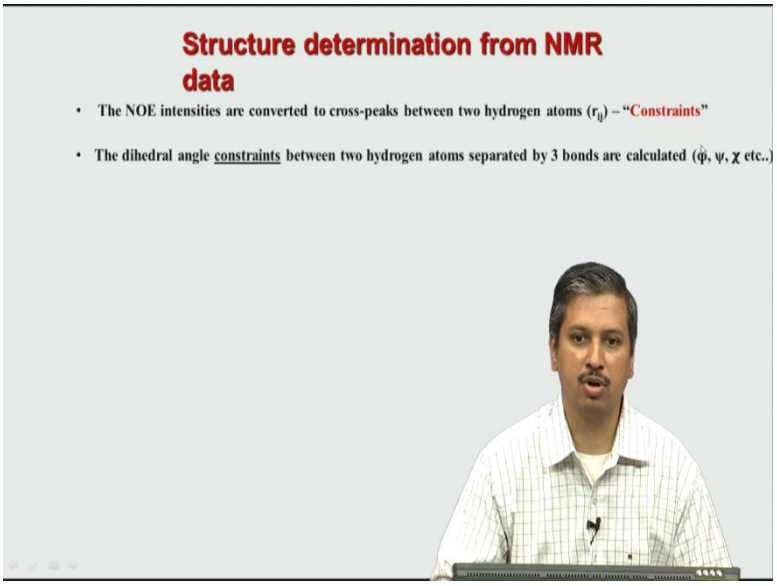

In the last class we looked at different chemical exchange process whether it is slow or fast how it is determine. So let us continue with that in this class, so this is again shown here, that if you have two different molecules or two different states that the hydrogen atoms is going from one state to another state we use the word state A and state B and there is a dynamic equilibrium or exchange going on between these two states. So if it is a slow exchange that means the exchange rate is much slower than the difference in the chemical shift value which is given in Hertz.

So, you have to convert the ppm scale the difference into hertz so that will depend on which spectrometer you are working it and if that this conversion this difference in hertz is much greater then we exchange rate then we use the word slow exchange. So, how does the spectrum look in the slow exchange process, the spectrum very much does not change at all? So let us say this is your state A and this is state B so let me call this as state B, if this is state B then the two hydrogen atoms or the hydrogen atoms in the same hydrogen atoms in the two different states will have two different chemical shift values and they will exhibit or show two separate peaks.

So, that that this is HA and this is HB, so of course we will just seeing two different peaks does not tell you whether the exchange is slow or fast, but we will come to that later we will see how we can find out whether there is any exchange happening between these two molecules or not and if it is happening, whether it is fast or slow all those we will figure out in the next few slides but the point here is that if you have a slow exchange going on, then it manifest is shows up in the form that you will get two separate peaks for A and B, okay.

There will be a small difference change in the line widths, which will depend on the exchange rate but we will ignore that for the time being we are only looking at the overall picture of of what is happening when exchange it is slow or when exchange is fast. So, as shown here, when the exchange is slow you will see two different peaks of the same hydrogen atom and one will correspond to one state A, other will corresponds to another state B, so this is how the spectrum will look like in the case of slow exchange.

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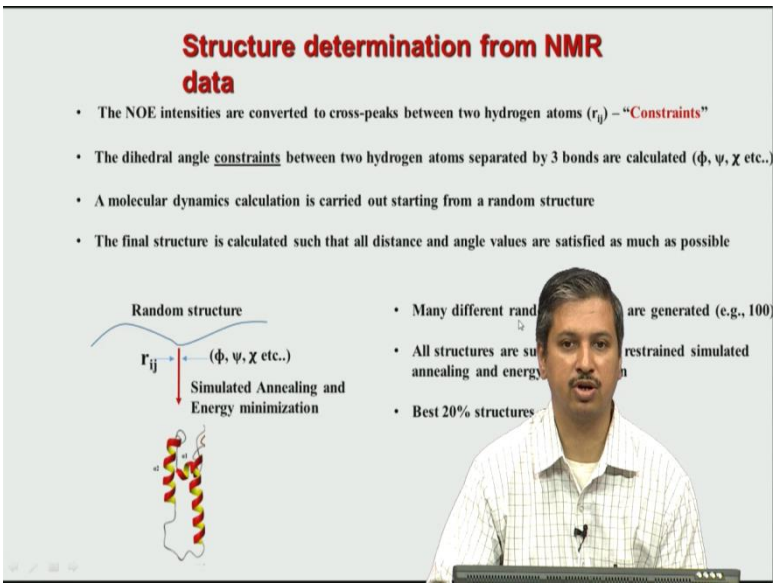

When you go to intermediated exchange there it is situation will be slightly different, there you what will happen is that you will see a broad peak covering both the chemical shifts of the state A and the state B. So this is called the intermediate situation when where exchange rate of the 2 system which is exchanging between themselves a rate of exchange is coming close to the chemical shift difference in calculated in hertz. And then when you go to the other extreme that is the fast exchange, where the rate of exchange is much higher than the difference in the chemical shift value then how will the spectrum look, it look something like this where the peaks state A and B are gone, but you have got now an average chemical shift peak, the peak average chemical shift value.

Now what is that average value, it is given like this that the average value is the population weighted average means if population A suppose the molecule A was 70% in population in concentration and the B molecule state B only let us sat 30 percent. So, that comes from here thermodynamics, remember in thermodynamics we use the word the equilibrium constant or we will use the word dissociation constant and so on, where we characterized the population of the two different states and that population if it is you can call as PA and PB or we can say it is a fraction, fraction because the total value should be equal to 1. So the fraction of A the molecule population fraction of A and population fraction of B now determines where the average will come because with this formula we can see it is weighted or based on the population it will decide whether delta C is where it is.

Suppose PA and PB are suppose equal means it is 0.5 and this is also 0.5, in such a scenario your delta c that is a center the peak will be right at the center of these two because it will be half into A + half into delta B, so half of these two value so delta A + delta B divided by 2. But if it is 70% of A and population of A is 70% and population of B is 30 percent, when your delta c that is the average chemical shift will be more located towards A. And if the population of B becomes more, then it will be move towards B so, therefore it depends on the population of the species of the state A molecules I hydrogen in state A and how much have the same hydrogen is present in state B.

So that determines where the average chemical shift peak will come, but this is a scenario it comes on the fast exchange. So, now if you see from all the discussion still now the slow exchange the intermediated exchange and the fast exchange all of this depend on the field strength because look at here we are calculating in ppm. So, the chemical shift difference in ppm will not change, but when we calculate in hertz it will now change completely depending on where you are.

So, if you are doing the experiment on 400 megahertz, it would be some 1 value, but if you go to 800 megahertz it will be completely different value. So, therefore the exchange rate will not change the spectrometer right, the exchange rate is something a constant it is only constant for that process. So by simply changing the spectrometer at a given same temperature of course exchange rate will depend on temperature, PH, but if will not depend on spectrometer frequency, but the exchange rate now it can be now called as slow or fast based on what is the value in hertz.

So, therefore a fast exchange on a low field spectrometer let us say 200 megahertz can become slow exchange on a high field megahertz suppose 800 megahertz, because this will now increase okay. So when this increases you are respect to the K if changes. So, if the K let us say is 4 hertz a 40 hertz and it is coming closed to this value in it becomes intermediate. But if I go to 800 megahertz from 200 to 800 I will increase this number by 4 times, then it will become a slow exchange process.

So therefore, by changing the spectrometer frequency we can make a exchange rate slow or fast on the chemical shift time scale. So, remember here the time scale we are discussing is all based on chemical shift values. But we will see later that it can also be based on some other parameter in NMR. So, let us see now how slow exchange can be identified okay, so we discussed this we crease this question that okay I will see two different peaks, but how do I kbnow that these two peaks are coming from slow exchange or not.

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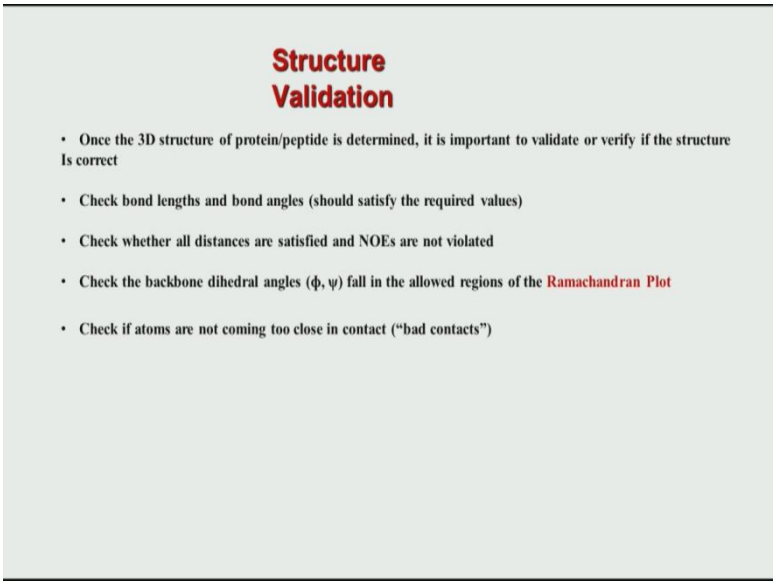

So, very simple idea so idea is suppose let us take an example and let us say we are doing a ligand binding experiment. So, a ligand is binding to a protein we will call it as B and ligand we will call it as A. And when it binds it will form a complex like this okay so there is an a equilibrium between that two that means is a monomer to complex going on it is dynamic exchange and let us say that the chemical shift of A in the free form that is the ligand form free ligand, let us call it as delta A free and when it is bound to the protein let us call that chemical shift of again A we are looking at A as delta A in (())(9:10).

So, now let us see how the exchange will happen if there is a fast or slow what kind of spectrum we should expect to get. So, suppose we start these experiments with only and no B is added so obviously that is a free form there is only 1 peak which will correspond to the free form of the ligand. Now slowly I do this titration by increasing the amount of B. So suppose I add a very small amount of B, then if it is slow exchange this is what you should expect to see.

If it is a slow exchange process then another new peak will appear somewhere, which will be corresponding to the bound form and the old peak will slightly decrease in the intensity. So, because right now we are added only 0.1 so the B population that is B is very less. So, the complex is also less and that complex now shows a peak somewhere away from here and that peak will now appear with a very low same density but it will be new peak and that peak will not be present in the first spectrum.

So, by looking at the appearance of a new peak aware from the old peak, one can tentatively say that this is the bound form and this is the unbound free form. Now suppose, I increase the concentration of B and I add little more than the population of this should go up and population of this should come down. So, they may be equal, why because suppose there is a tight binding strong binding this is what is written here. If it is a strong binding, then 0.5 of B will interact with 0.5 of A and they will form a complex.

So, we are assuming it is a 1 is to 1(())(10:56) geometry means 1 molecule of ligand binds 1 molecule of the protein. So, in that a scenario you will have 50% of population in the free form and 50% of the population now is in the bound form okay. Now suppose I increase this, I add a large amount of B and now B and A are 1 is to 1 in that scenario the all the complex should now go to A to B through the complex.

So the peak of A will be very less, it may be 0, it may not be 0 remember there is an equilibrium so it is possible that this goes back to the free form and therefore there is a some peak of A also free A will come because it is not 100% going here, it is always a dynamic equilibrium. So in that scenario you will get a small peak or you may even get a very little 0 peak, but the idea is that this peak is going down gradually as we increase the amount of B and this peak goes up as we increase the amount of B.

So, this is how we can say that the exchange is now free as is a slow exchange between the two, but remember again this type of exchange depends on the chemical shift values because

they are far away suppose, and compare to the exchange rate we are able to say that these two this is a slow exchange scenario. But the same binding process can become fast exchange if the chemical shifts are not very far. Suppose these two peaks are very close then what happens is then we cannot say that is a slope because the difference in the chemical shift now becomes less compare to exchange rate and that will become a fast exchange scenario so that is what we will see in the next example that how fast exchange.

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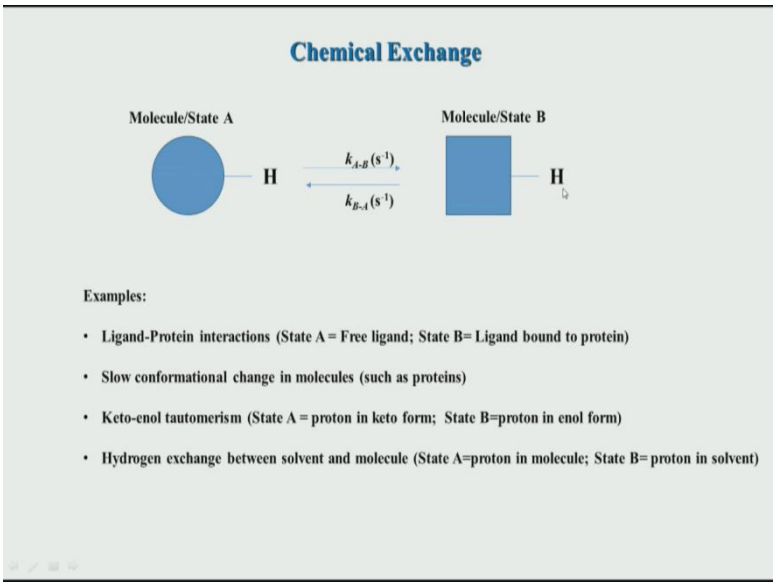

So, before we go to that let us look at suppose we have a slow exchange process like this, is there any other method to find out if this is a slow or not. So, this is one option this is what we saw, another method to find out is known as 2 D exchange spectroscopy is called 2D EXSY okay. So what is 2D EXSY, it says that if the rate of exchange is slow compare to 1 over T 1, so let me correct this, 1 over T 1 because we are looking at exchange rate.

So T 1 is not rate, it is one over T 1 is the rate or relaxation. So if the exchange rate is slow compare to one over T 1 of the nucleus, then the chemical shift this extreme process can be captured also in a 2D experiment. So let us see how that works, so let us say we have the again 2 states now this is not same example as the previous slide, this is just showing that there are two states the hydrogen atom on state A is now going to hydrogen atom on state B and it has two different chemical shift values, we will call it as delta A, delta B.

So, what is experiment which we need to do this is the exchange experiment and this is same exactly same as 2 D NOESY, so 2D EXSY and 2 D NOESY are same, so 2D NOESY itself we can say we are using to characterized this slow exchange process. So now what will

happen when the exchange is slow, let us see mathematically? So, suppose I do a first excitation of a pulse, so then this pulse let us say excites the spin state, the state A that means this RF pulse excites A, then during this T1 evolution period A will evolve with the chemical shift of itself, okay so that is T1 evolution.

Then during this process during with this pulse we bring the magnetization of A to the z axis. If you recollect the NOESY part of this course, we saw then this can either we need to - Z or + Z, so let us say it is sum in z direction during that mixing time now this period the state A will exchange into state B because this is an equilibrium process. So I will have some of them getting converted to B again coming back to A and so on so forth. This is not a one way process, it is a two way.

So, during this time mixing time if the exchange is slow in a then during this time it can exchange multiple times to B and vice a versa. So, when it goes from A to B now when you detect the final the final magnetization it is now B because some population of A has gone and exchanged to B during this time and that B now evolves during the second dimension with chemical shift of B. Same thing will happen with B, suppose there is a population of B will get exchange with to A and A will come here.

So, that would be cosine omega B T1, cosine omega A T2. So, therefore you can have possibilities A going to B or B going A, so now if you do a 2D transform Fourier transform , this is how the spectrum will look. So, these are the two diagonal peaks, as I said it is always that never 100% transfer takes place. So, whatever remains on A during both this periods it is called as a diagonal peak, so A and B will both have a diagonal peak and this exchange peak is called a cross peak.

And that is coming because A is got converted to B or B got converted to A, so both process are happening because in the starting it is not that A is present only. We are looking at an equilibrium reaction where both A and B are present okay and some population. So, A may be 60% population, B may be 40% in the total mixture, but they have reached already a steady state. Now in the steady state the 60% A, which is there we will slowly get converted depending on the exchange rate depending on the mixing time, etc, it will get converted to B similarly whatever was B population will B is getting converted to A but there is equilibrium.

So, therefore you expect to see intensity from both sides A getting converted to B and G getting converted to A so this is how the exchange peak will look and now remember it is an

exchange during this mixing time. Now if they give a very short mixing time that means I am not allowing the two A and B to exchange, but if I give it a very long mixing time then what happens is the relaxation of A and B will start happening that is T 1 relaxation and therefore I cannot give too long a mixing time neither I can give too short because if I give too short, this peak will be very weak in intensity, if I give too long again it will be weak in intensity because of the T 1 relaxation. So, this whole thing can be now represented by this formula here that the exchange peak intensity depends on these factors.

It depends on the mixing time and it depends on the exchange so here there is a slight error which I will correct this is exponential R1 into T1 okay so, as sorry (())(18:38) which is shown here, so this is shown already here, so the idea is that it depends on the T1.

So, that means if I give a short mixing time a mixing time is very short compare to the exchange rate, then the peaks are very weak in intensity. But if I give very long mixing time which is more than the T 1 than this factor dominates and that factor will now kill the signal because it is an exponential decay and the intensity of the exchange peak will again become very weak okay. So therefore, one has to choose the right mixing time and based on that you should be able to see and if you there is a exchange between two state we should be able to see a cross peak. And this is very important information which helps in a finding out whether any two atoms hydrogen in two states whether they are exchanging with each other or not. So, let us now look further how fast exchange can be identify.

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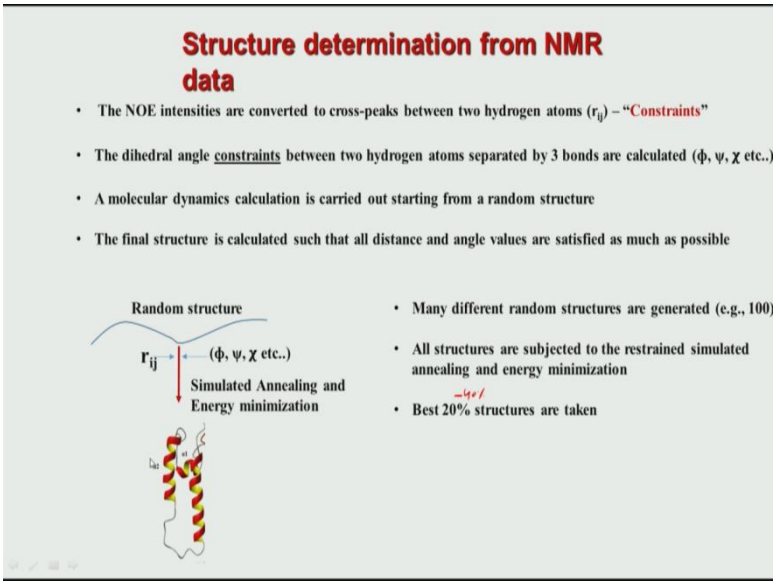

So in the previous examples may have look at slow exchange and they were two methods one is that you look at the a peak appearance of peak when you add A to B, the two ligand and protein and another option was to go to exchange spectroscopy and assuming that the two chemical shifts are well separated A and B and if the relaxation if the exchange is not too slow. If it is too slow that means it is exchange is much slower and the T1 relaxation 1 over T1 then also during the mixing time it will not be possible to see. So, it has to be in the right time scale window, so that is for the exchange. So now let us see what happens if is fast exchange.

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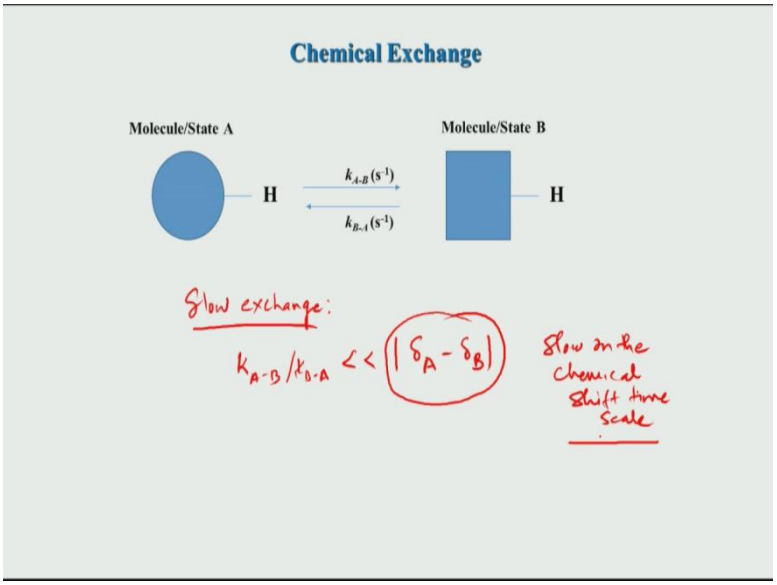

So let us again look at this example, when A ligand is added to the protein B and A forms a complex and that complex now is a weak binding complex means is not tightly binding, so there is a constant operation of free inbound and it is not a small population of free, there is a large population of free because whatever goes comes back in a fast exchange and therefore it is a not strongly binding and it is not a strong complex. So what will happen in such scenario again remember strong and weak and fast or slow all depend on the chemical shift I mean the strong and fast and slow depend on the chemical shift. So, suppose the chemical shift difference between them is not very large and if that is not very large compare to the exchange rate then we call that process as fast exchange.

So, if this is small compare to exchange then what do we expect to see? We will expect to see an average peak, the peak at an average chemical shift position and that average position is determined by the fraction of population of A form and fraction of population of B form weighted by the chemical shifts, okay. So that is what will happened, so let us sat there is a

fast exchange. Now suppose I do not add any B at all, so there is a starting reaction I am not adding any B. So obviously there is no complex, so there is only one peak which is coming at the position of the delta free that is A form A free form.

Now I slowly add the ligand, I add not proteins sorry and it becomes 0.1 is to 1, now we can see that peak has shifted. Why it has shifted because it is based on this formula here there is a fast exchange between these two, and because of the fast exchange we get a peek at the average chemical shift and that is what is shown here. Now suppose we go to little bit more, if I add a little bit more of the B, now what has happened, it has more population of the complex because when I am adding more and more B, more and more complex is being formed and therefore this average value will shift towards the bound form, why because now I am increasing the bound form population, I am increasing the portion or a population fraction of the bound form and therefore my chemical shift total chemical shift is now getting more weighted towards the bound form, so it is shifting peaks are shifting.

So, this is a very interesting thing that you can find out if you this thing happen. Suppose you are doing this reaction when you are adding a ligand to the protein and you start seeing that the ligand peaks instead of showing two different peaks like we saw in the slow exchange case, if it shows like this trend of shifting as we are adding more and more B it immediately indicates that there is a fast exchange on the chemical shift time scale. So, suppose I add some more amount of B, I make it almost 1 is to 1 but because this is not a strong complex it will be a lot of free sum population of A will be there free A, but more of A B will be there because the population of B is very high. So, the bound one is almost coming equal to the free one so the chemical shift is more or less close to the close to the center or little bit toward the bound form.

Only when I go to let us sat B is 10 times or A 5 to 10 times A, then in that scenario this population of this will be very high compare to the free form and at that in that scenario the peaks will be completely shifted to the B form because population of A will be very less not 0 it will be less compare to the B. B will dominate because I have too much of B added and therefore, the complex is now very high population compare to the free form, so in that scenario your population this peak will be completely towards the B form. So therefore, based on this two based on looking at these kind of your peaks structure that is the moving of the peaks as when I am adding B is a very is in the simple and the strong indicator that my fast exchange is going on in my sample and we can say that the ligand is now weakly binding

and because of the weak binding nature it is having a fast exchange compare to the chemical shift difference, so this peaks shifts because the population of B increases.

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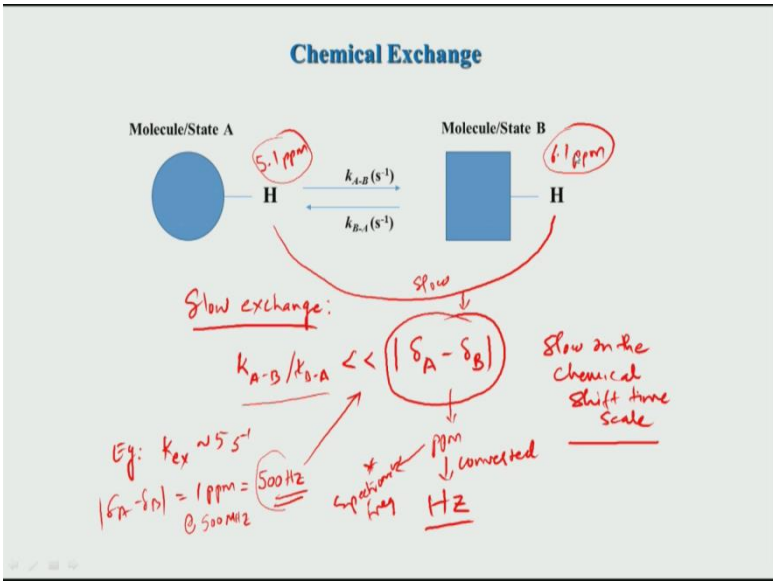

So now based on these 2 processes, we have looked at slow and fast exchange, how do I calculate the constant, so remember our focus is to find out this dissociation constant. This is the binding affinity so if you are working on a drug on a drugs protein interaction or a ligand protein interaction, one of the major goal in this exercise is to find out what is the affinity of the drug or substrate to the enzyme or the protein. So, we usually use the word K D dissociation constant which is basically measured like this, it is a population of A B divided by the population of the complex or concentration okay. So, can you determine this by NMR, so let us sat you are doing as appose you are having a slow exchange process that is a strong binding of A with B then you may except 3 peaks, why? Because one comes from the free A and B is also a proton in have a proton that is free B is shown here and a complex which will have a bound form.

So based on the intensity the concentration of A and B can be obtained, because a intensities of the peak remember in NMR is directly proportional to concentration, we have seen this in the theory part of the course, so the intensities or area under these peaks are directly proportional to their concentration. So, if I plug in those values here at different points in the titration, I can then calculate KD and then take an average, okay. So the relative ligand concentration can be varied at each concentration step you calculate the populations and the KD should not change with the population, KD is a constant it depends on the ratio but accordingly the ratio will change and it should keep getting a series of KD with this, then if

you take an average that will be a KD value for this reaction. How do we do it in the case of slow exchange, it is shown here.

So, suppose we have the same reaction now, but this time it is a weak binding so ligand binds not very strongly and based on the exchange rate it is faster compare to the chemical shift difference. So, remember again all depends on the chemical shift between the two states and if it is the rate of exchange is fast compare to these two states, then the scenario will be that the as you increase the B your peaks will start shifting because it will start going move towards the bound form, so when you are increasing the B value so concentration of B that is what we saw and of course after a long concentration of B that means suppose B is 10 times A, then this peaks will now tens and come close to bound value here.

So, now from this bound value, suppose the peaks comes here after the long if large addition of B that value will correspond to the delta A bound. So, then you can get the delta A bound you have the free form because you start with only A and these 2 extreme values will be used for calculating as fallows. So, this is how the calculation goes this is a formula which we will not derive but it can be very simply derived with based on simple equation like this.

Now the derivation in the finally it says that, at any given time you have to calculate any given time means I mean you have titrating you will titrate several times. So, you will have with free forms you are little B more little more B and so on. So at any given time you will observe the shift of the peak and that shift from the original peak which is free form the difference is called delta observed at any given time and delta max is after adding a long large value of D B let us say, 10 is to 1 at that point the peak will be completely here because of the population weighted average.

So when it is completely here then this distance from here to here is called the max why you maximum because that is the final value. So it is the maximum the peak can shift so that difference is what is called as delta max, so once you know delta observed at different point in your titration and you know the delta max for the last point in your titration after adding a large amount of B you can that at every point calculate and fit it to this equation. So, what is the fitting parameter, KD is the fitting parameter.

This is what is known not known this is unknown. So, that is unknown but your known are this value at any given time and this is the max which you get after adding lot a large amount of B, and based on these values this is nothing but total ligand concentration which you know

because you know how much A you have added and total protein you know because that is how much B you have added, so based on these two numbers you can calculate the KD value.

So, this is how we calculate KD values in the case of fast exchange scenario and in the case of slow exchange scenario. But remember this is not restricted only to chemical shift, fast and slow can be with respect to any other parameter, so in the next class we will see how we can characterize KD value based on we can also look at different parameters like diffusion or we can look at the T 2 values based on that also we can say whether that exchange is fast or slow and this will have a use in the exchange chemical exchange one of the useful application is hydrogen deuterium exchange which we will take up next.