Spectroscopic Techniques for Pharmaceutical & Biopharmaceutical Industries
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Lecture 25
Application of Steady-State Fluorescence

Hello students, welcome back to the lectures. In the last lecture I started discussing about application of steady-state fluorescence. And in this lecture again I will continue with this.

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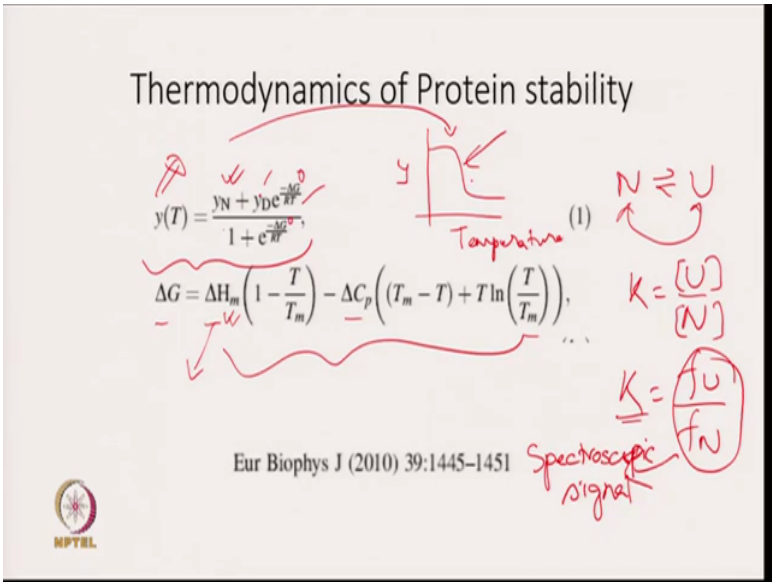

We were discussing in the last lecture, how to know the thermodynamic parameter of conformational change. I started with a protein and I discuss that we can know the delta G for protein stability. Protein stability is basically looked at by looking at a conformational change from native state to denatured state or unfolded state.

So this is the equilibria which we were discussing about and if there is delta G for this process is negative then this process will be spontaneous and protein will be unstable. Because it is going from native to unfolded form. When delta G is positive it means the process is non-spontaneous and that will tell you that N is more stable than U. N form of the protein is more stable than U and thus protein is stable.

Although I am discussing about the native to unfolded state equilibrium but you can think of several different kind of equilibria where one from changes to another form. For example, you can discuss about cis trans isomerism, you can discuss about ring flipping. For example, you are going from one confirmation to another confirmation of cyclo hexane, from chair to

boat or boat chair. Similarly, we can discuss about suppose keto enol tautomerism that is again two forms of a same compound, two different forms of the same compound.

So when you are trying to look at the thermodynamics, you are basically looking at how one confirmation is more prevalent than other confirmation under certain set of conditions. So that is what we do when we are looking at the protein stability. We are looking at the conformational change from native to unfolded, we calculate Delta G under particular condition. And if Delta G is negative, it means a reaction is spontaneous and folded form is more stable whereas if Delta G is positive it means native state is more stable.

Generally, to do this we can write the value of K first and as you know that K is equal to U by N. For this process, K is equal to concentration of U by consideration of N. And that can also be written as fraction of unfolded form by fraction of native form and this is related to your spectroscopic signal. And that is what we discussed in the last lecture spectroscopic signal. So just by measuring the total fluorescence intensity, look here I am not talking about intensity due to unfolded form or native form. We related total fluorescence intensity to the ratio of fraction of unfolded form and fraction of native form.

When we calculated this ratio and related to K we can express a total signal total fluorescence signal or total signal due to any spectroscopic quantity like a absorbance. So it is not only related to fluorescence it is also related to signal from signal total signal from any spectroscopic techniques. So why at certain temperature means signal at certain temperature is equal to y due to native form plus y D multiplied by exponential minus Delta G by RT divided by 1 plus exponential minus Delta G by RT. Here we mean by Delta G naught.

And so your fluorescence intensity can be related to Delta G of the process, Delta G of this process conformational change process. So if you experimentally obtain profile of fluorescence signal with temperature temperature, you can fit this profile with this equation and that will give you value of Delta G naught. You can also get the value of Delta H m, Delta C p and T m.

Since Delta G is related to this whole quantity which I discussed last time. So not only you can calculate Delta G naught, you can also know Delta H m, T m, Delta C p and Delta C p. So all this quantity can be calculated at the particular temperature just by nonlinear fitting of this profile. We know Delta G, we know Delta H m and so we can calculate Delta H at certain temperature because we also know Delta C p.

So by using Kirchhoff equation and putting the value of Delta H m and Delta C p, we can get the value of Delta H at a given temperature. We already know Delta G at certain temperature T, so now we have Delta G at a given temperature, Delta H at given temperature. So we can know Delta S of given temperature and so we can tell whether this process N to U if it is spontaneous. Whether it is driven by entropy or if whether it is driven by Delta H, it is driven by enthalpy. We also need to know that yN also dependent on temperature, yN and yD is also dependent on temperature. So we cannot put simply yN at certain temperature here because yN will change for each value of T.

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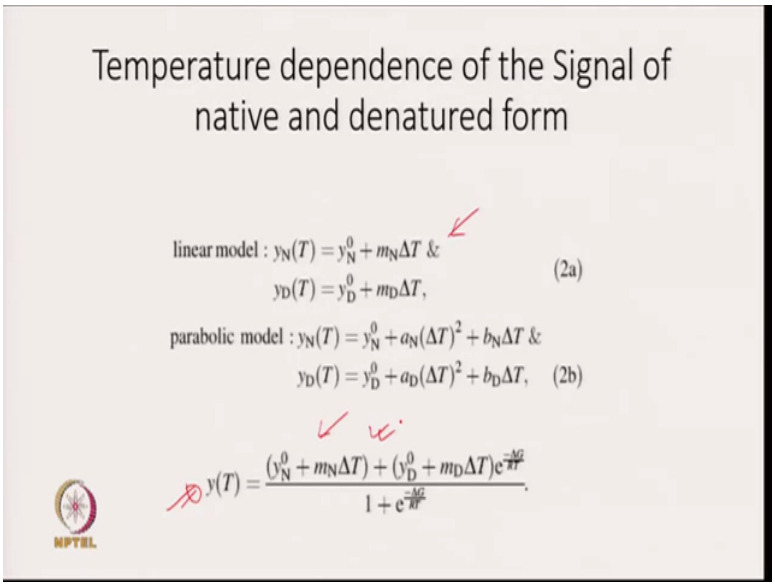

And that is given by this equation, yN at certain temperature is equal to yN naught plus m N Delta T Delta T. And if you put in this equation, you can have the yN naught plus m N plus Delta T plus y D naught m D Delta T exponential minus Delta G by RT one plus exponential minus Delta G by RT. And this is equal to y T and when you are trying to do nonlinear fitting you must keep this thing in mind. And when you do that, you can get the value of different thermodynamic parameter.

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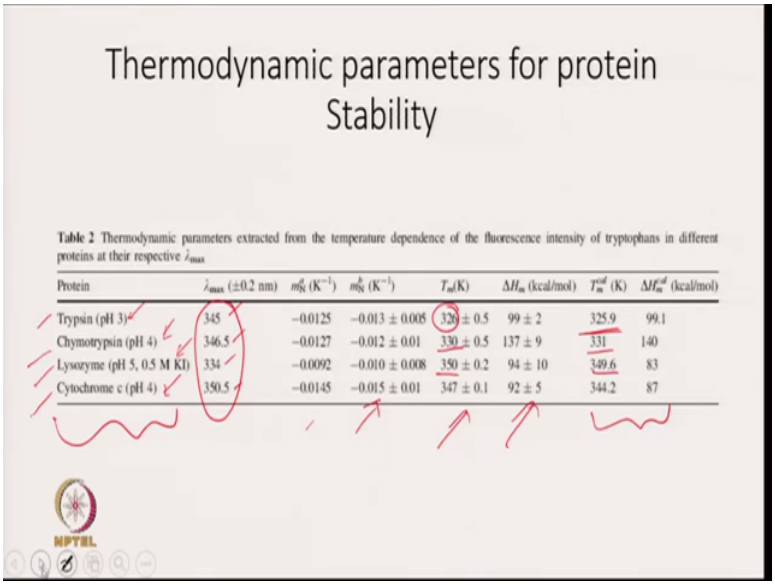

So there are some protein for which thermodynamic parameter has been reported. Our lab has already reported for certain proteins. So these are the few proteins Tripsing, Chymotripsin, Lysozyme, Cytochrome c and the thermodynamic parameter has been obtained for Tripsing at pH 3, for Chymotripsin at pH 4, for Lysozyme at pH 5 and 0.5 molar KI and for Cytochrome c it is taken at pH 4. And the thermodynamic parameter here is obtained using fluorescence spectroscopy, thermodynamic parameter has been obtained using a spectroscopy.

You can also get the thermodynamic parameter using differential scanning calorimeter. But most of the time differential scanning calorimeter is not available in the lab, so people depend on fluorescence spectroscopy. And here what we do is, we excite this protein at different lambda Max value and that you can know by taking simple fluorescence spectra.

And this is the T m value, this is Delta H m value and this is the one obtaining from calorimeter. These are the T m and Delta H m obtained from the calorimeter. So we can know the value of T m and Delta H m using fluorescence spectroscopy. You can see that T m obtained by fluorescence is quite close to the T m obtained by calorimeter DSC technique. Here you can again see Chymotripsin in this case T m is 330, whereas in case of DSC it is 331 Kelvin and this is 350 Kelvin for lysozyme which is 349.6 using calorimeter.

Say can see that we can almost get quite accurate value of T m and Delta H m using fluorescence spectroscopy. We do not have to go to a calorimeter. So one of the important application of fluorescence is to obtain the stability parameter for protein. And similarly can get the confirmation change constant or you can say equilibrium constant for a

conformational change whether you are looking for cis trans isomerism, whether you are looking for keto to enol transformation we can get the value of K which equilibrium constant for that conformational change.

And if we measure the K as a function of temperature, we can get the value of Delta H and once we have Delta G and Delta H we can know Delta S value. So fluorescence in not only used to know the protein stability or Delta G naught value for any conformational change. It can also tell you about effect of different conditions different additives on that conformational change. For example, generally protein need to be stabilized protein need to stabilized for its longer self life.

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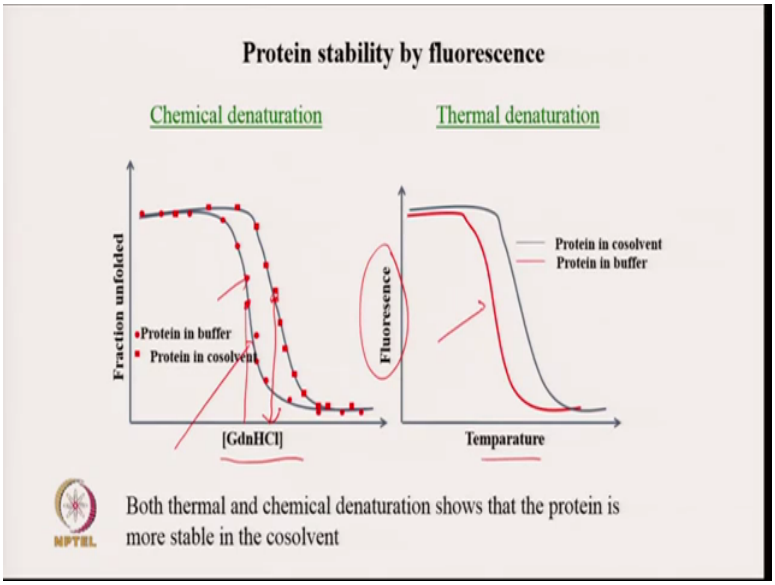

And so people use additive we increase the stability of protein increase the stability of protein and for that they do some studies thermal stability and try to look at how those additive can stabilize the protein. So for that we can either try chemical denaturation and again we are looking at fraction unfolded versus guanidine hydrochloride concentration which is a well-known denaturant. You can also look at fluorescence with respect to temperature and you can see here that this is your protein in buffer.

And this is again protein in buffer, this red one is protein in buffer if I put co-solvent. And we see the shift what right-hand side we know that your protein is more stable under that co solvent condition or on the addition of protein get stabilized on the addition of an additive. So here you can see that C m is almost here, it gets increased to this point and so your protein is more stable. Since more amount of denaturant is needed to unfold the protein.

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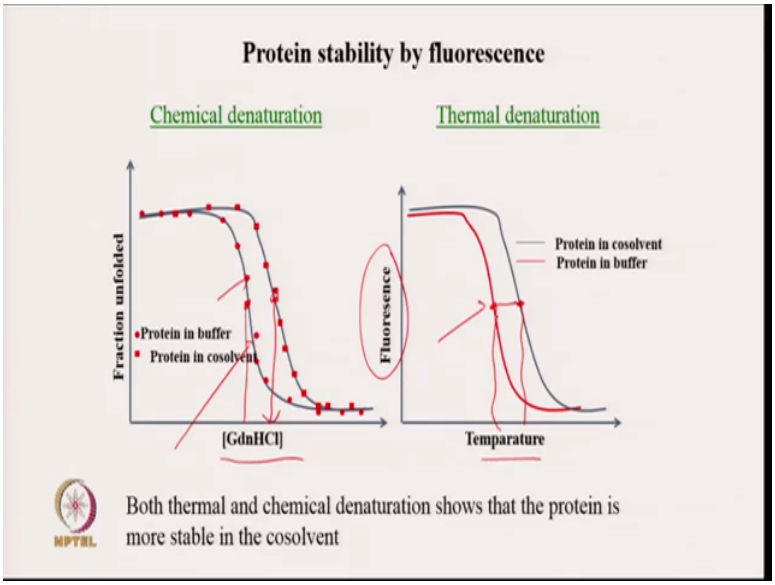

Here also you can this is the T m value and this is the T m value in presence of co solvent. And so there is increase in T m value presence of co solvent and that means you require higher temperature to denature protein or to convert protein from its native form to unfolded from. And that is why we can see that now protein has got a stabilized.

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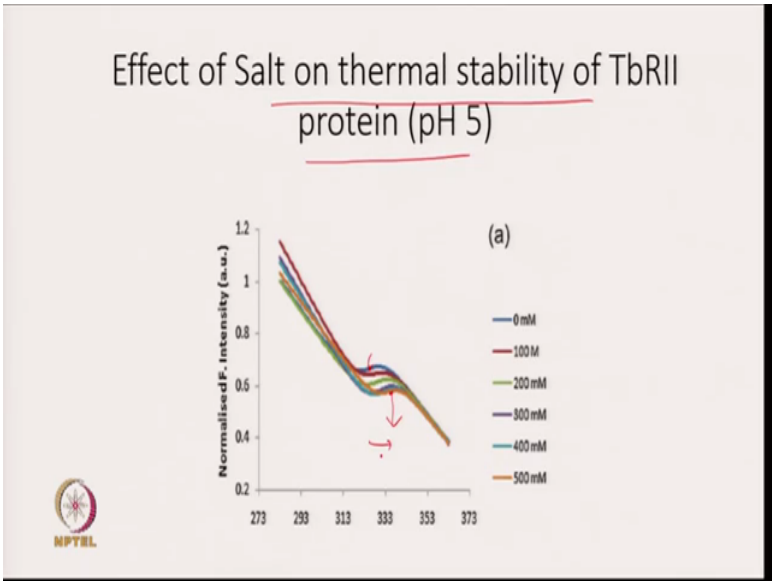

We can also look at the effect of salt, this is one of the experiment done to look at the effect of salt on thermal stability of a protein called transforming role factor. The data is obtained at pH 5, what you can see? That this is your at zero molar and this is at around 500 milli molar. Salt is known to destabilize or a stabilize protein and in this case what you are looking at is T

m is going from this position to this position and so there is a shift of T m towards higher temperature on addition of salt. And what does that mean is salt is stabilizing the protein salt is stabilizing the protein.

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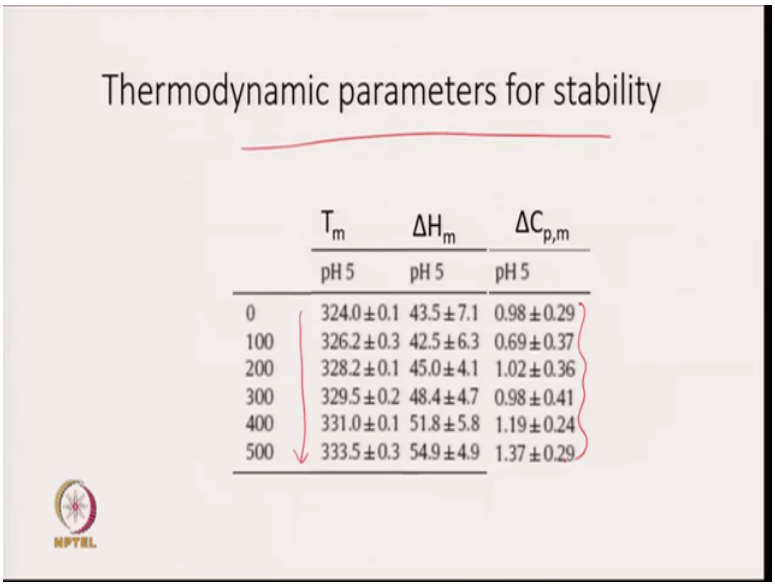

And this is your thermodynamic parameter for stability you can see that once I add salt, the T m value is increasing we can also get Delta H m value. Delta H m is also increasing and Delta C p has changed to a certain extent. So we can know what is the effect of additive on conformational equilibria by using fluorescence spectroscopy.

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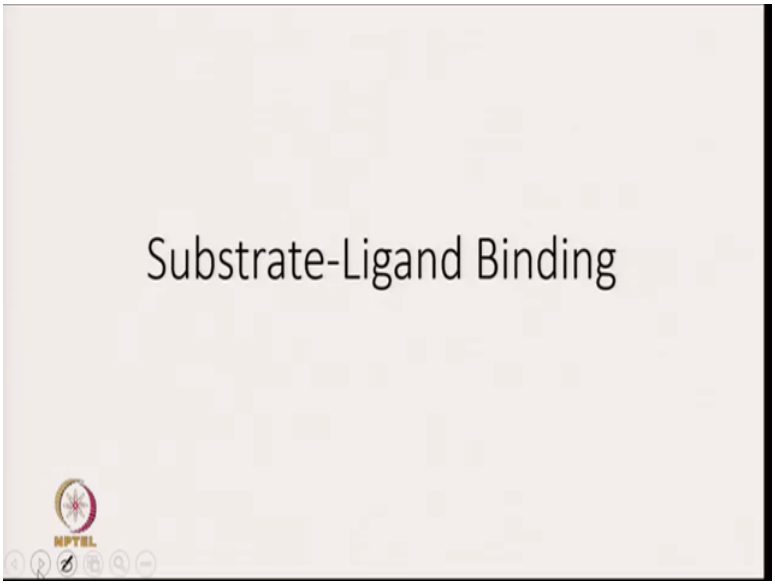

There is a very important application of fluorescence in calculating binding affinity for a substrate ligand binding. And substrate ligand binding is quite important for example, if you are looking for drug you need to look at how does it binds to its target how does it binds to its target? Similarly, in biochemistry we also need to look at protein ligand binding, since protein ligand binding is very important phenomena in our body. No function in our body is possible without protein ligand binding and hence it is important to study substrate ligand binding. So to know how to apply fluorescence to look at protein ligand binding, first we need to understand the basics of binding.

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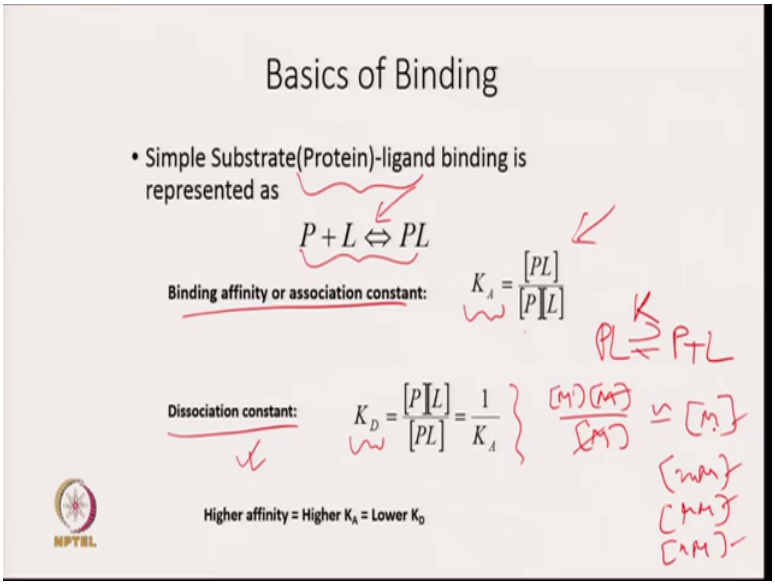

Here I will start with protein as substrate. And we will look at protein ligand binding but it is equally applicable to any substrate binding with a ligand. Now we will look at this simple process, one protein interacts with one ligand to give you protein ligand complex. There are two ways denote its binding substrate binding to ligand. One is by using binding affinity or association constant, K A will be given by PL divided by P into L. So this is nothing but equilibrium constant of this process.

There is a second way to denote this what we look at the opposite of the equilibria PL going to P plus L. And equilibrium constant of this process is known as dissociation constant and K D is given by P into L divided by PL and that is nothing but simply inverse of K A value inverse of K A value what does that mean has higher affinity is equal to higher K A value or lower K D. So if you get lower K D it means substrate at higher affinity for ligand or ligand has higher affinity for substrate.

Generally, if you look at unit of this K D you will say that this is molar multiplied by molar divided by molar and so unit is either molar or millimolar or micro molar or nano molar. So unit of dissociation constant will be either in molar unit, millimolar, micro molar or nano molar. Whereas unit of K A is going to be molar inverse or millimolar inverse, micro molar inverse or nano molar inverse. Since it is easy for us denote the unit as a molar, millimolar, micro molar or nano molar.

So generally we generally we take dissociation constant as a measure of protein ligand affinity. But we need to keep in mind that if there is a lower value of K D then it means there is a higher affinity of substrate for ligand or…

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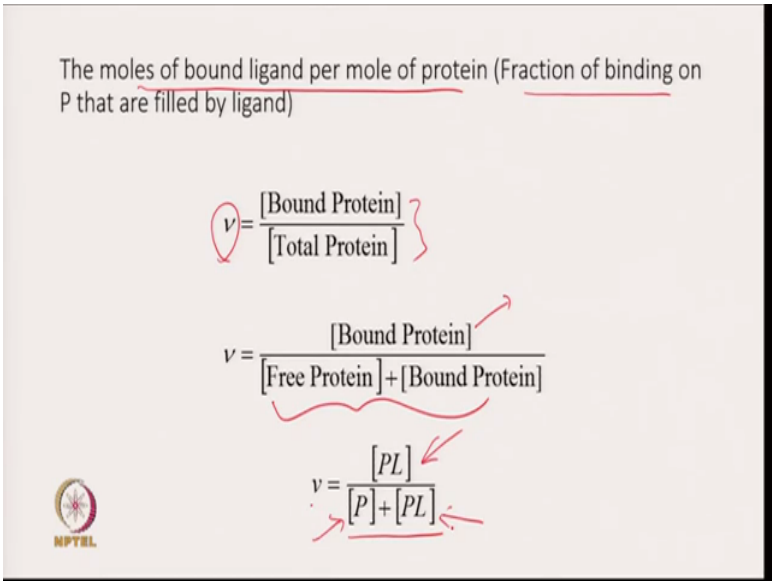

So to know this K D value or K A value, what we need to do? First is to calculate the mole of bound ligand per mole of protein which is given by this nu value which is basically fraction of binding on P that are filled by ligand. So nu is bound protein by total protein, so you see this is a fraction of bound protein and that can be written as fraction is equal to bound protein divided by free protein plus bound protein. So when you add ligand some of the protein will go to bound state, the remaining protein will be in free state. And so total protein is free protein plus bound protein, so nu is given by PL which tells you about the concentration of bound protein divided by P plus PL, P is protein in free state and this PL denotes the protein which is in complex with ligand.

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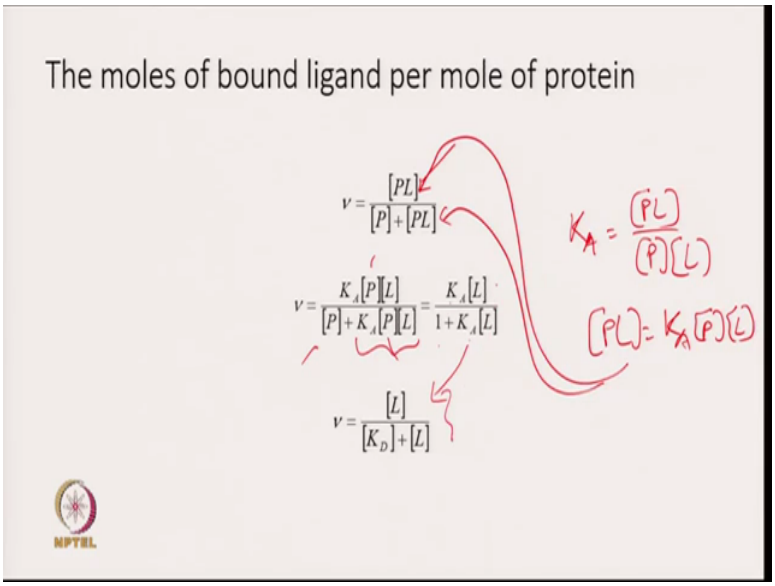

Now if you remember K A is equal to PL divided by P into L and so PL can be written as K A into P into L and that is what we are going to do. So I just plug in this value of PL in this equation and what we will get is? K A into P into L divided by P plus, again if I put it here then I will get K A into P into L. Now you can see P is there in every terms in numerator or denominator. So it will cancel out, so if I remove all three piece I will get K A into L divided by 1 plus K A into L. And if I try to write this same equation in terms of K D what I will get is? Nu is equal to L divided by K D plus L, so this is your equation which tells you how the fraction of bound protein is related to K D.

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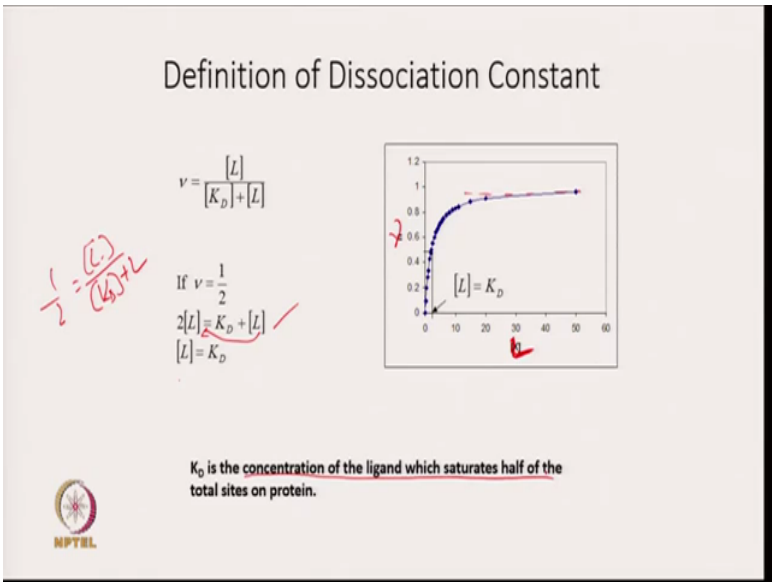

Now if I plot this nu value with the value of L with the value of L what I am going to get is? This kind of equation and that is not quite surprising because at higher concentration of ligand by ligand concentration is in excess, all protein is going to be in the bound state. And so the fraction of bound protein will be equal to one will be equal to one. And if I take nu is equal to one by two so this is for nu is equal to one. If I take nu is equal to one by two, I just put one by two is equal to L divided by K D plus L and so K D to L is equal to K D plus L. And if I take L decide I get L is equal to K D, what does that mean is?

K D is equal to ligand concentration when the fraction of bound protein is 0.5 fraction of bound protein is 0.5. And so K D the concentration of ligand which saturates half of the total site on protein but this is not an easy method. Easy method would be to somehow get a straight line which can give the value of K D.

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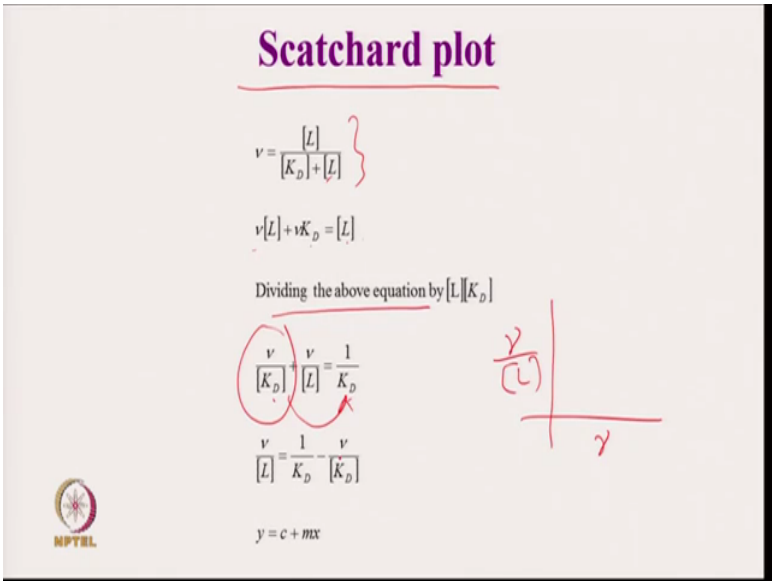

So let us see how to get a straight line to obtain the value of K D and the plot which we use to get the value of K D is known as Scatchard plot. So this equation we already obtained nu is equal to L divided by K D plus L. If you multiply this nu into L plus nu into K D is equal to L. Divide the equation by L into K D, L into K D what we will get is? Here in this L will cancel out, K D will come at denominator. In this K D will cancel out, L will come at denominator at this L will cancel out and K D will come in denominator.

And if I take nu by K D this side what I am going to get? Nu by L is equal to one by K D minus nu by K D. Now we have a equation where I can plot nu by L versus nu by L versus nu and I can get a K D from the slope because this a equation of a straight line.

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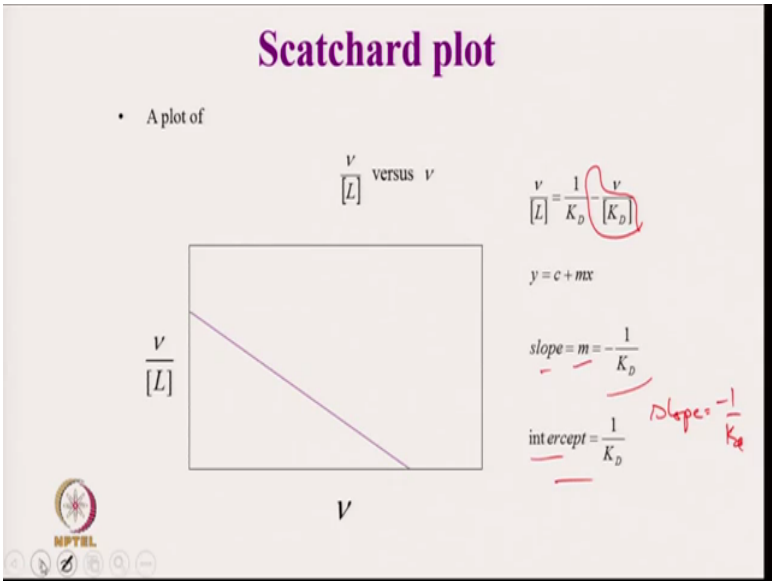

So if I plot nu by L versus nu, I will get a equation like this, the slope will be given by minus one by K D you see here minus one by K D and the intercept will be given by one by K D. Basically slope itself will give you minus one by K D, so if I take inverse of this I can get the a slope. Or from the intercept also I can get the value of K D.

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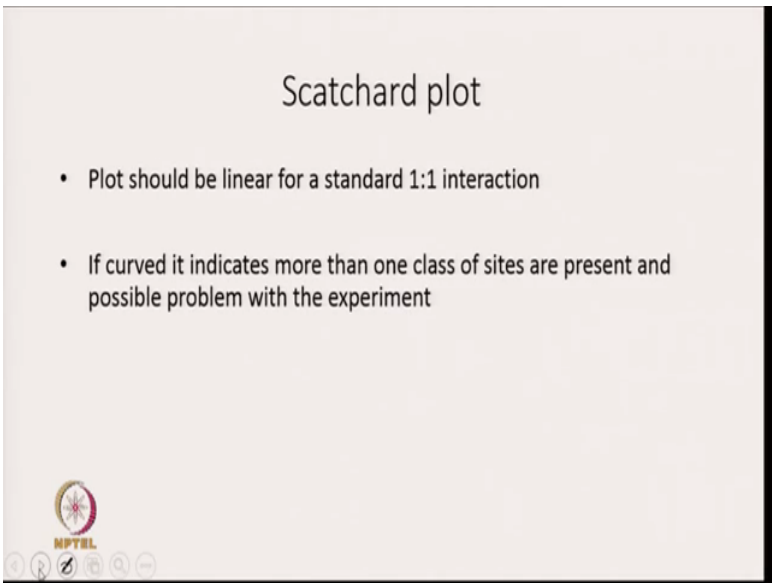

One thing you need to remember that plot must be linear for a standard one is to one interaction. If the plot is not linear it means one is to one interaction is not followed. And it means that the more than one class of sites are present and there can be problem with experiment. So these are few short comings but there are ways to get information for a different kind of model but this is going to be out of scope for this lecture, so I will go ahead

and this is another application. But if you want I have given addisive lectures on the ligand binding, please go to those lectures. You can know if there is more than one side for the ligand then how-to get a Scatchard plot.

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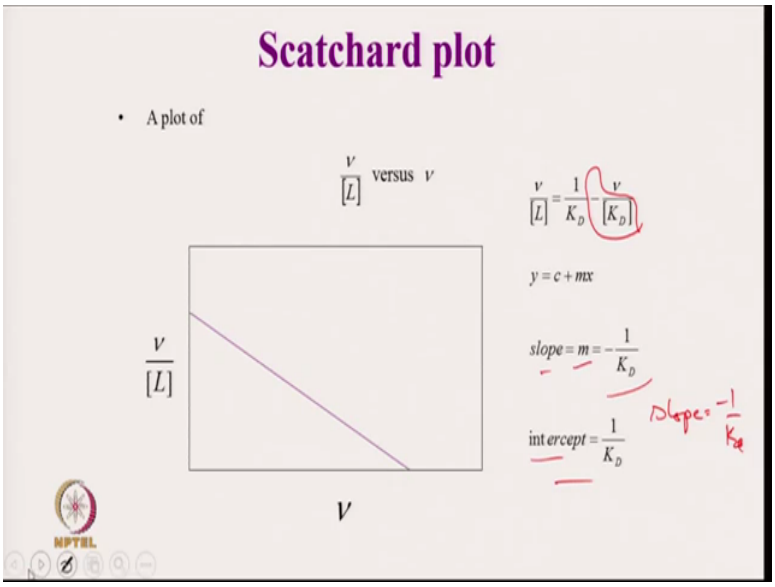

So if you look back what we did? We plot and nu by L versus nu, so one thing we need to know is nu value. And now what I will show is how to get the value of nu from the spectroscopic signal? For example, from fluorescence once I know the nu value I can just plot nu by L versus nu and I can get the value of K D from one is to one interaction. So experimentally what we do is? We take protein and then titrate it with ligand and then measure fraction of protein bound to ligand and then we plot nu by L versus nu.

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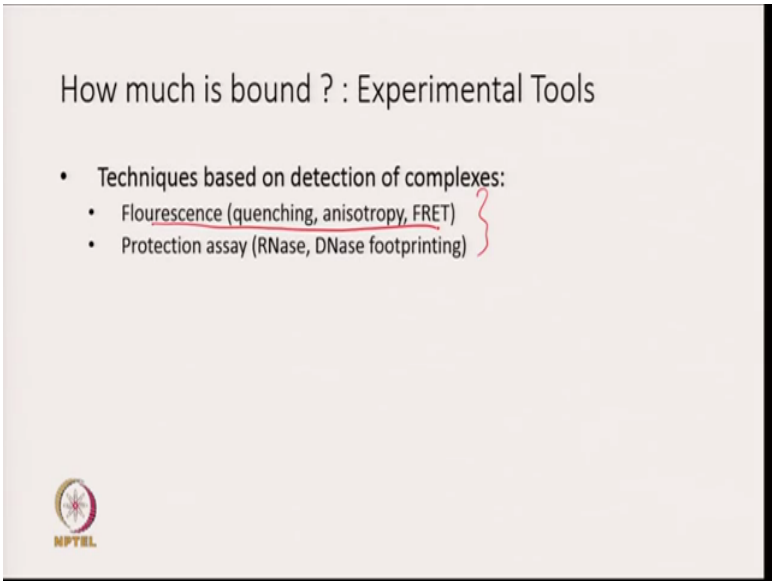

Now how to know the fraction of bound protein that is that is in question which is obtained by experimental tools. The detection of complex can be done either through fluorescence or protection assay. And in this lecture we are interested about how to use fluorescence to get the fraction of bound protein fraction of bound protein?

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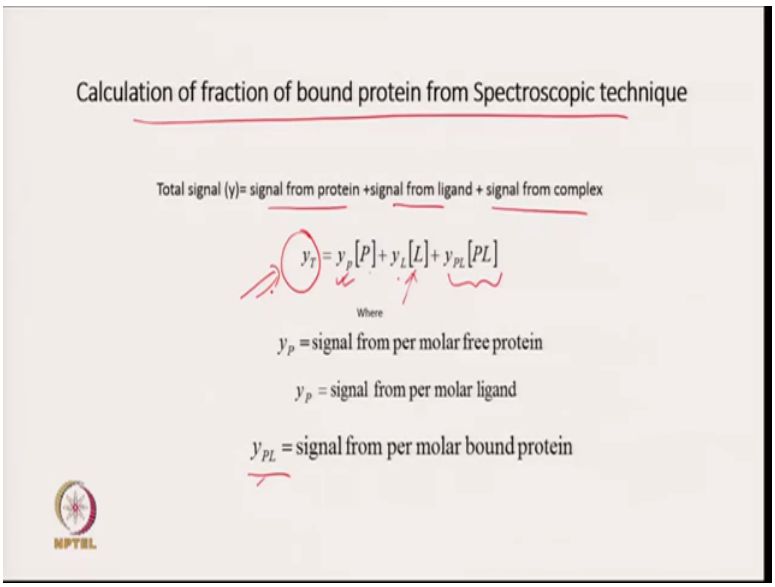

So this is what I am going to discuss in this slide. So you can think of that of in a Q way where there is a protein and I started by titrating with ligand. The total fluorescence signal is going to come from come from three different spaces. One from protein and other from ligand and the last one from the formed complex, complex between protein in ligand. And

again here I will denote y as a fluorescence signal and y t means total fluorescence signal. So it consist of signal from protein, it consist of signal from ligand, it consist of signal from complex.

And y P will give you signal from per molar free protein per molar free protein. So y P into concentration of P plus y L into concentration of L again y L is signal from per molar ligand. So if we take one molar ligand in a solution and then measure fluorescence intensity that will corresponds to y L. And then y PL is signal from per molar bound protein. So one molar complex will give you signal and that is equal to y PL and so if we multiply y P into concentration of P it means we are talking about total contribution from free protein y L into L will give you total concentration from free ligand and y PL into PL will give you total signal from your complex and y T is total signal due to protein ligand and protein ligand complex.

Sometime your signal is only from the bound protein not from the ligand or not from the free protein at particular lambda Max value. It is also important to keep in mind at that particular lambda Max value whether there is a signal from free protein or not. Ligand free ligand or not, so if suppose your complex gives you signal at particular lambda Max whereas other free protein or free ligand gives a signal at signal at a different lambda Max value which is a far apart from the lambda Max at which complex absorbs or complex flourish and then at that lambda Max is no contribution from free or free protein or free ligand.

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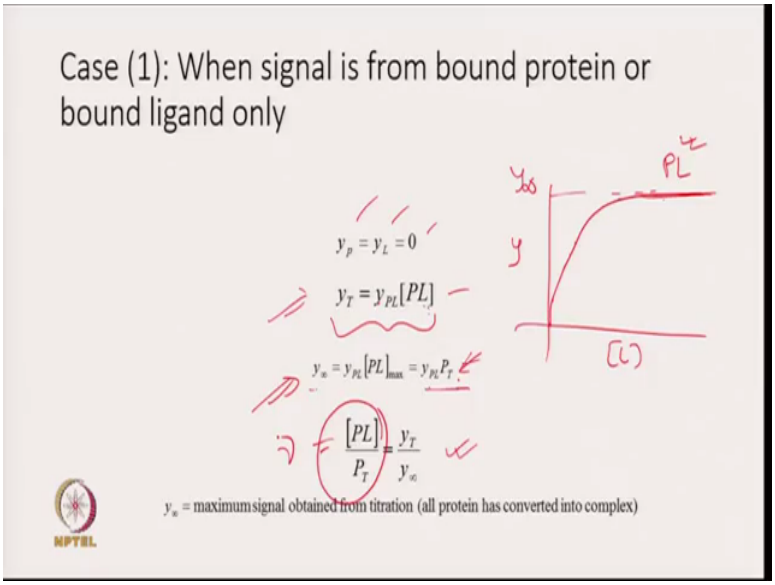

In that case, we can take y P is equal to y L is equal 0. So y T will only be deal to complex, so what will happen? That you will get something like that Spectra. So this is complex started making and this is your signal y and this is your ligand concentration, what will happen? That you go and it will be constant because all of our protein has gone to complex and after that you are just adding only ligand which you does not contribute which does not contribute. And so there will be a constant here and this value is called y infinity and y infinity will be y PL into PL Max.

So this is the maximum amount of PL which can form and PL Max will be equal to total protein concentration because after that formation when P t is equal to PL Max. After that no more complex is getting formed, all the protein has gone to complex form. And so y infinity is equal to y PL into P t and this is your one equation, there is another equation. You just divide these two these two equations, so what I am doing is? I am dividing this equation by this equation, what I will get is? y PL will cancel out, we have PL by P t is equal to y t by y infinity.

So y T by y infinity is equal to y PL, y PL cancels out, so PL by P t and that is what is here. And this is what is your fraction of bound protein and so now you know how to calculate fraction of bound protein. Then you go and plot nu by L versus nu you will get the value K D, this is equal to nu.

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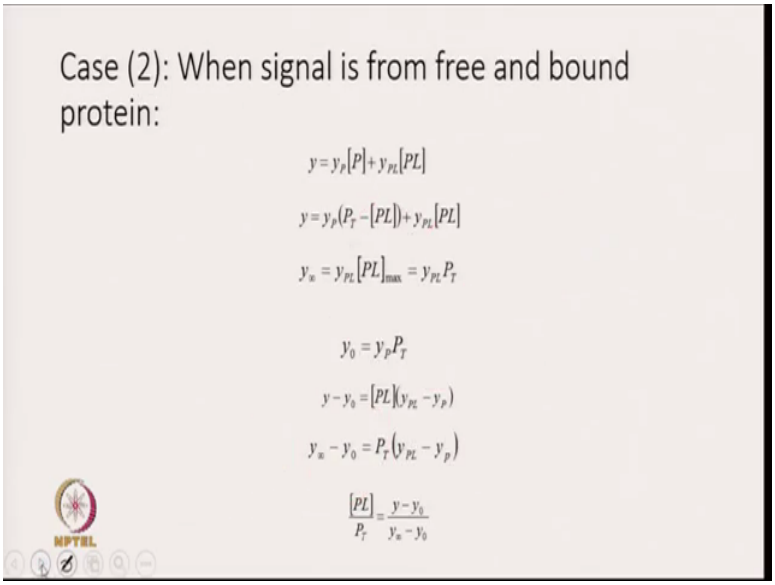

Now sometime signal is from free and bound protein. Ligand does not have any contribution, in that case how does your how to calculate the fraction of bound protein? So y is equal to in

this case, y is equal to y P into P plus y PL into PL. This is from the protein, this is the signal from protein, this is the signal from complex.

Now what we can do is? We can express P as P T minus PL or does that mean is you know that total protein concentration is equal to free protein plus bound protein. P is either free or in complex form with L, so this is called free protein and this is called bound protein bound protein. So this free protein is equal to P T minus P L, free protein is equal to P T minus P L that is what we have written here. y p into free protein which is equal to P T minus P L plus y PL into PL.

Now if I take y infinity signal when all protein has gone to complex. Then this is your free protein and so there is since there is no free protein, we are talking about all protein has gone to complex form. It means there is no free protein, it means no contribution from this. So y is simply y PL into PL Max and we already discussed PL Max is nothing but concentration of total protein concentration of total protein. And now you can see that y 0 when there is y 0, we can also calculate the value of y 0, y 0 means we have not added a ligand when ligand concentration is 0.

And in that case, your none of the protein has gone to complex is means all of the protein is in free State and so there will be no contribution from the complex because complex has not been formed. All signals is due to protein itself and protein is totally in the free-form and so you can write y 0 is equal to y P into total protein concentration. So you have three questions, first what we will do is? We calculate y minus y naught when we do that, so y P into P T. This cancels out y P into P T cancels out, what is left is? P L into y P and P L into y P L, so P L can be taken as a common and what is left is? y P, y PL since we are talking about y minus y naught, this cancels out so P L if we take then y PL minus y P.

Similarly, we can get y infinity minus y naught and that is equal to this that is equal to this. If you can see this as a same this term common term, so if I divide y minus y naught by y infinity minus y naught. I will simply get P L by P t, this is nothing but fraction of bound protein fraction of bound protein.

So fraction of bound protein is equal to y minus y naught divided by y infinity minus y naught. So now you can plot nu by nu by L versus nu and you can get the value of K D you can get the value of K D. This is a way K D can be determined or K A can be to determind using fluorescence spectroscopy. In fact, you can use signal from you can use any signal from

any spectroscopic technique which gives you which is basically proportional to the concentration of the fluorophore.

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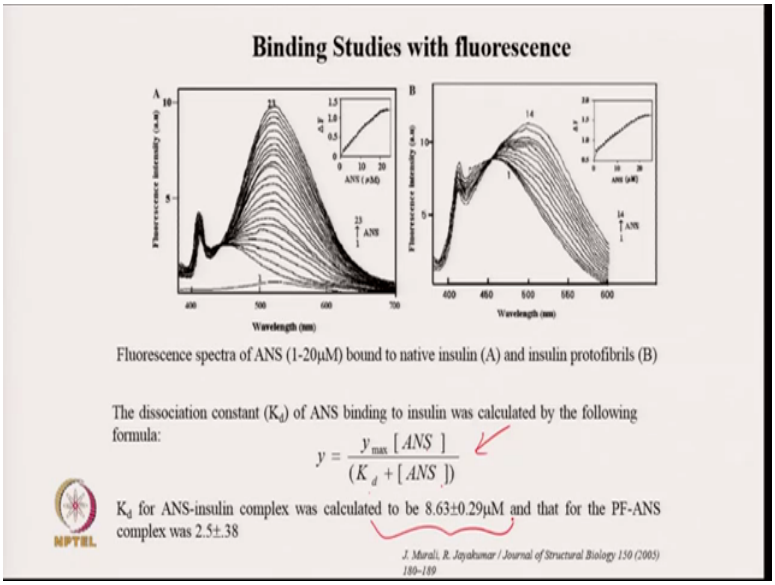

Here, I will show you one result where people have looked at the binding of ANS with bovine serum albumine. They your same equation y is equal to y Max into ANS divided by K D plus ANS and by just fitting this curve they obtained the value of K D which is around 8.63 plus minus 0.29 micro molar. So we can get the K D value using fluorescence.

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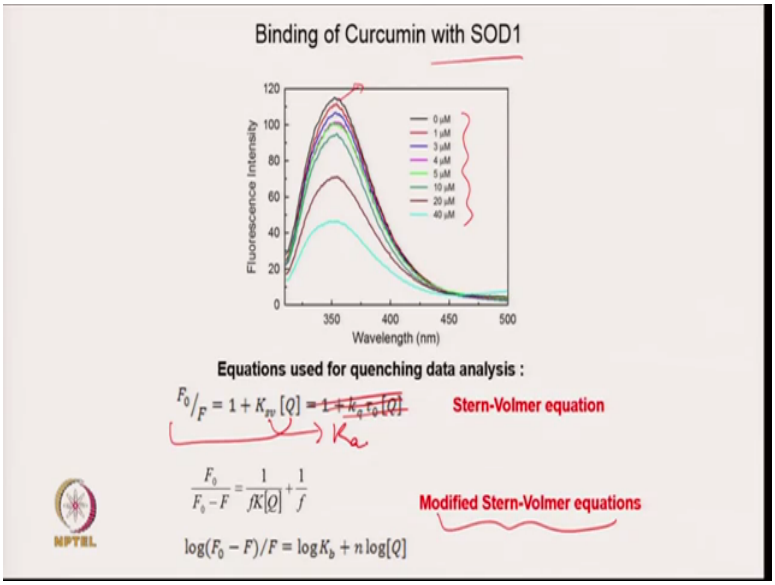

There is another example where we looked at that how curcumin binds with a protein called super oxide dismutase one. So this is your spectra, so here is a curcumin curve curcumin and

it is now titrated with SOD ones, so what we are looking at how fluorescence signal from curcumin changes when we add SOD 1. And here what we did is? We applied the equation which we derived when we are discussing about static quenching. And in case of static quenching, we can use this Stern Volmer equation, so F naught by F is equal to one plus K SV into Q this is for collisional quenching so I just removed this.

And there is one modified Stern Volmer equation, I am not going to discuss that but that can also be used to get your K SV. Here K SV is basically association constant if we are talking about static quenching. So quenching can be due to simple collision of SOD one with curcumin and the second can be due to your complex formation of (())(40:21) state curcumin with SOD one. So first we have to look at whether it is a static quenching or dynamic quenching. If it is at static quenching then we can get the value of K A.

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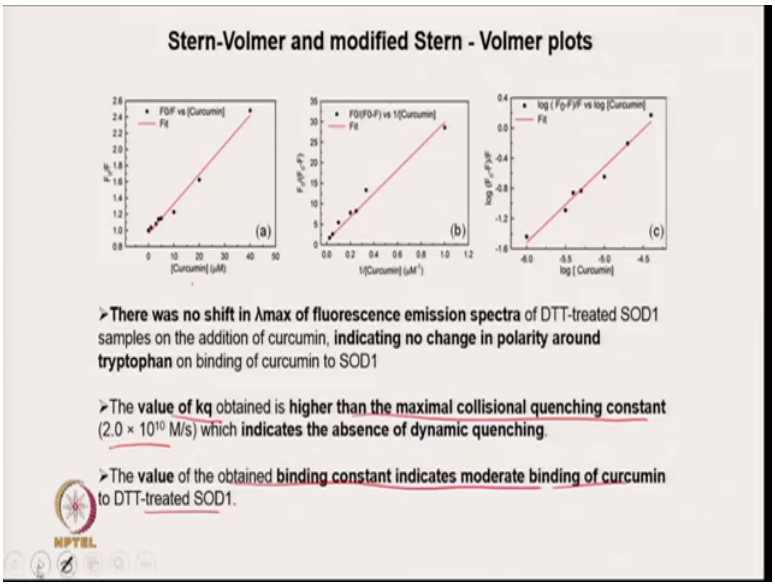

So this is the plot of F naught by F versus curcumin concentration if you remember Stern Volmer plot. So F naught by F versus curcumin and if I assume that this is a collisional quenching then we can get the value of kq which is higher than maximal collisional quenching constant. So we get a very higher value of kq and it means that it is not a case of collisional quenching. The value of obtained binding constant indicates moderate binding of curcumin to DTT treated SOD1.

What we did is? In this experiment we first try to look at whether it is a static quenching or dynamic quenching. And one thing I told you to differentiate between a static and dynamic quenching is measure lifetime in presence of quencher. If life time does not change in

presence of quencher it is a case of static quenching and that is what we did and what we found is your lifetime of curcumin does not change in the presence of SOD one. It means it is a case of your static quenching of this plot and slope I can get the value of association constant or dissociation constant. And what we got in this case, is there is a moderate binding of curcumin, the K D value is in micro molar range which means it is a moderate kind of binding.

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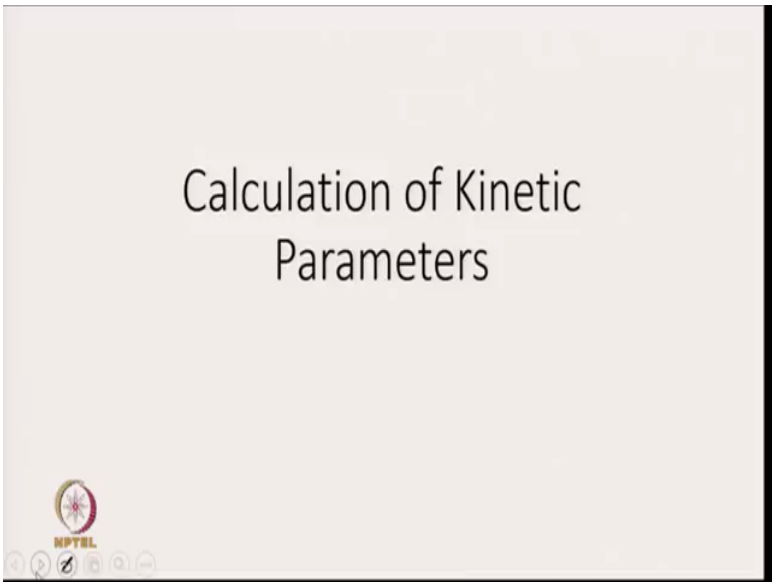

Fluorescence can also be used for calculation of kinetic parameters kinetic parameters. If you remember kinetic parameter for measuring rate constant we used to get we used to we utilize the integrated rate law.

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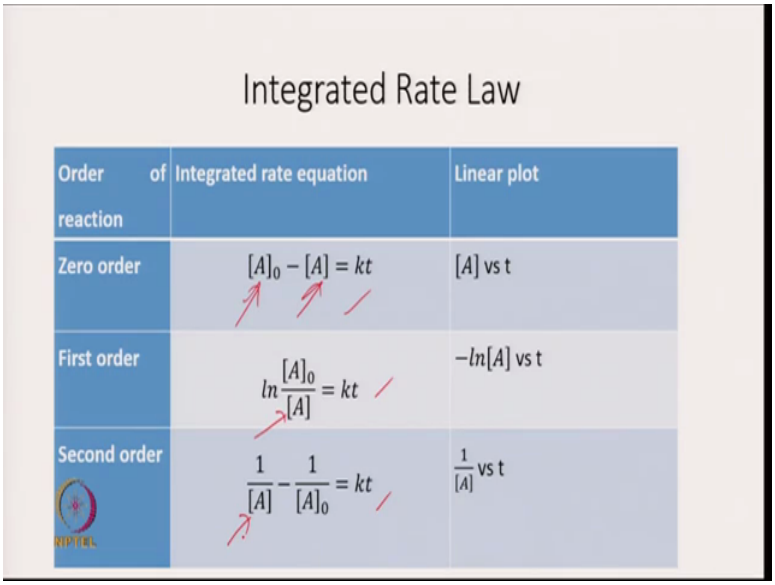

And this is the rate law for zero order. This is the rate low of first order, this is the rate low for second-order. And if you look at we need to measure A value in all these, so concentration at a given point we need to measure. But it is very difficult to measure A concentration of A at a particular point in the reaction or during if you are looking at reaction with temperature it is very difficult to measure concentration of a particular spaces at a given point. So what we generally do? Again we take help of spectroscopy to measure concentration or particularly measure concentration ratio. And I will show you how by measuring total spectroscopic signal you can get a ratio of concentration and that can be used to calculate the value of rate constant.

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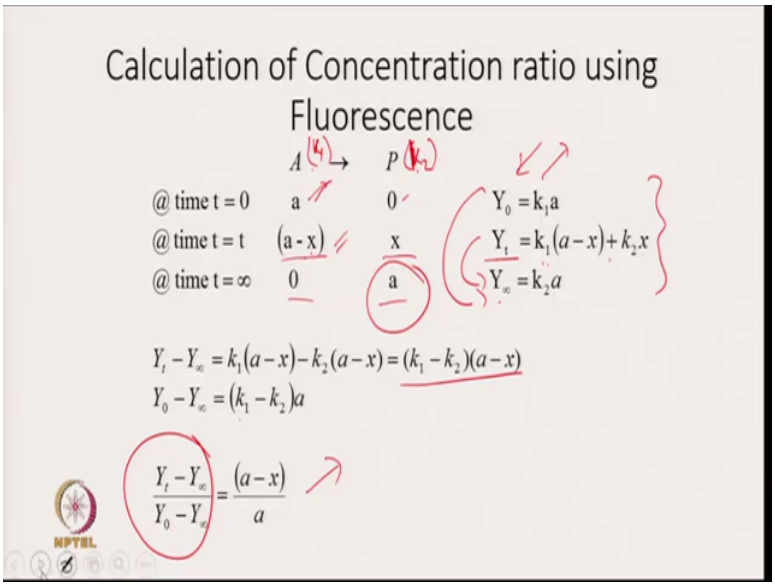

For example, take a simple case A going to P, A is a reactant, P is a product at time t. We started with A mole and P is 0. At time t it is a minus x so x will be the product and t is equal to infinity. A goes to 0 and product will be A. Now think of this spectroscopic signal, spectroscopic signal will be given by ka into a where ka is spectroscopic signal due to one mole of A. So Y t will be k 1 into a minus x plus k 2 into x and k 2 is your small k 2 is your spectroscopic signal due to one mole of one mole of P.

And this is a spectroscopic signal per mole of A. So total spectroscopic signal at time t will be k 1 multiplied by a minus x plus k 2 multiplied x and when t is equal to infinity your signal will be only due to product and so y infinity will be k 2 into a. So now we have three signals at three different point, so now let us do Y t minus Y infinity. If you do that you will get k 1 minus k 2 into a minus x and if you a y 0 minus infinity you can get k 1 minus k 2 into a. And so if I divide Y t minus Y infinity by Y zero minus Y infinity I will get a minus x by a.

So the concentration ratio of A at two different time, at time t is equal to 0 and time t can be obtained by measuring fluorescence intensity at different point. You can try of different reaction for example A going to V plus C. Same thing will be obtained same thing will be obtained you can try yourself at home. But what we need to know that a minus x by a is equal to Y t minus Y infinity by Y 0 minus Y infinity.

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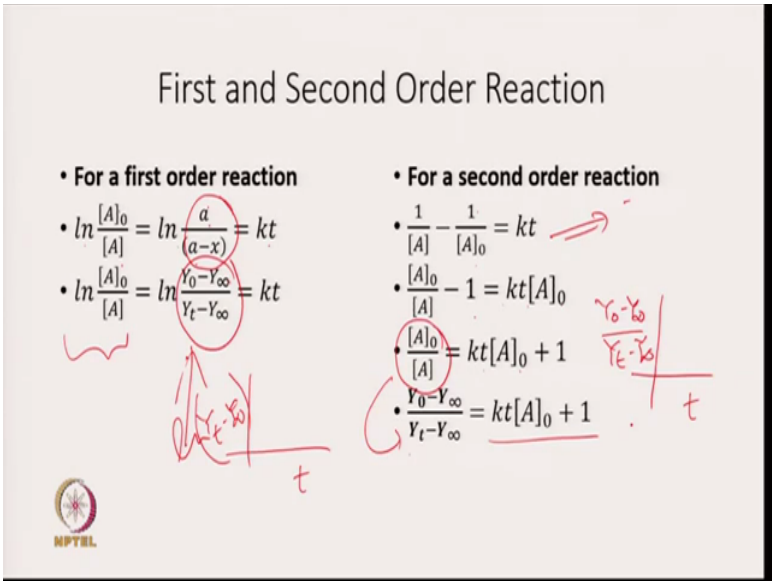

So now we can write first order equations, first equation is what? Log A naught by A is equal to log a by a minus x is equal to kt. And we have already calculated this ratio, we I wrote a minus a by a but you can write also a by a minus x. And that is equal to Y 0 minus Y infinity

by Y t minus Y infinity and so now you see I do not need this just by having the, your signal spectroscopic signal in this case, fluorescence at different time I can know this ratio. And now I can plot simply log Y t minus Y infinity versus t to get the value of k to get the value of k rate constant.

For a second-order equation we know this is integrated rate law, what we need to do is? We need to express this equation as a ratio of A by A naught or A naught by A. The way we can do is just multiplied this equation by A naught, so we will get A naught by A minus A naught by A naught which is one is equal to kt into A naught. A naught we generally know we our problem is to measure A at different time during the reaction.

So we can write A naught by A is equal to kt A naught plus 1 and this already we have calculated in the earlier slide. And that is equal to Y 0 minus Y infinity by Y t minus Y infinity and that is kt A naught plus 1. So now we can just plot Y t minus Y infinity or Y 0 minus Y infinity by Y t minus Y infinity versus t to get the value of k. So spectroscopic signal not only gives us a tool to measure Delta G, k, Delta H or Delta S it also gives us a tool to measure the kinetic parameters kinetic parameters. So I will stop here and thank you very much next time I will discuss the time resolved spectroscopy.

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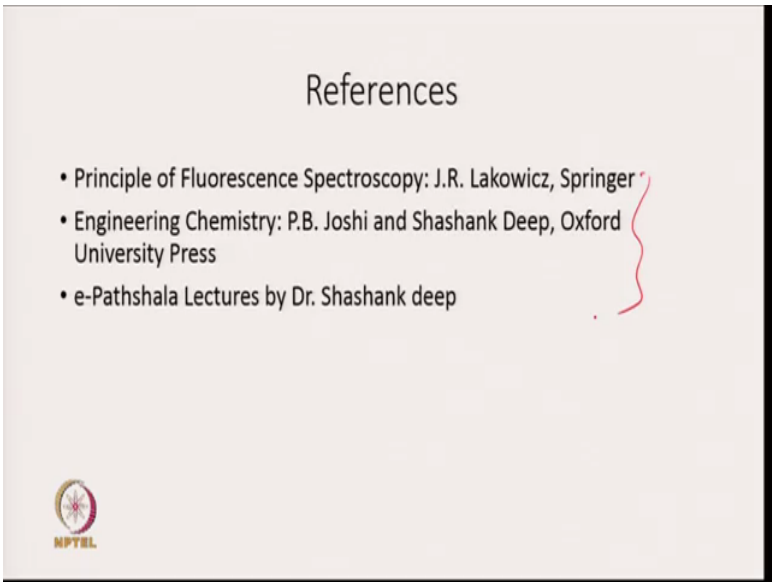

These are the some references so thank you.