**Fundamentals of Protein Chemistry**
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**Module - 05**
**Protein ligand interactions**
**Lecture - 25**
**Discussion Class**

In our last class on protein ligand interactions in this module 5, we will be discussing the several methods involved in protein ligand binding and work out some problems related to the discussions that we had considered in the previous lectures of this module.

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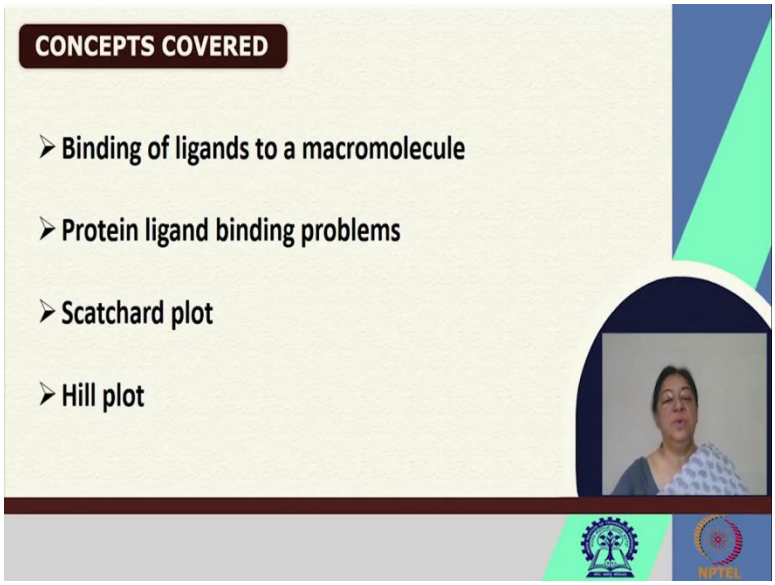


In these discussions we will be looking at the bindings of ligands to a macromolecule, which is what our protein ligand binding is all about and look at some specific plots, the scatchard plot and the hill plot with specific examples of how they may be calculated and how they may be understood further.

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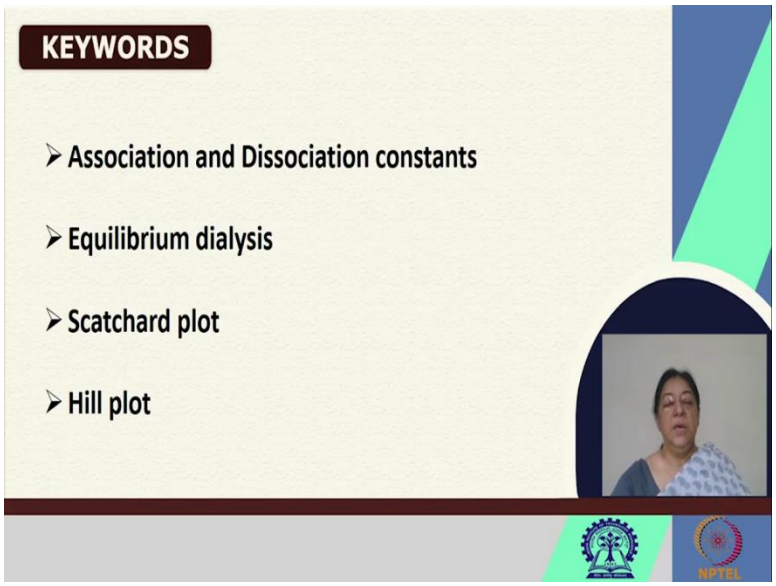


The association and dissociation constants are something that we looked at in the previous classes. Also other methods to identify protein ligand binding where we realize that we need a methodology to find out the protein ligand binding complex concentration or the free protein or the free ligand left after binding, to be able to get some meaningful information about the protein ligand complex and how the interaction proceeded.

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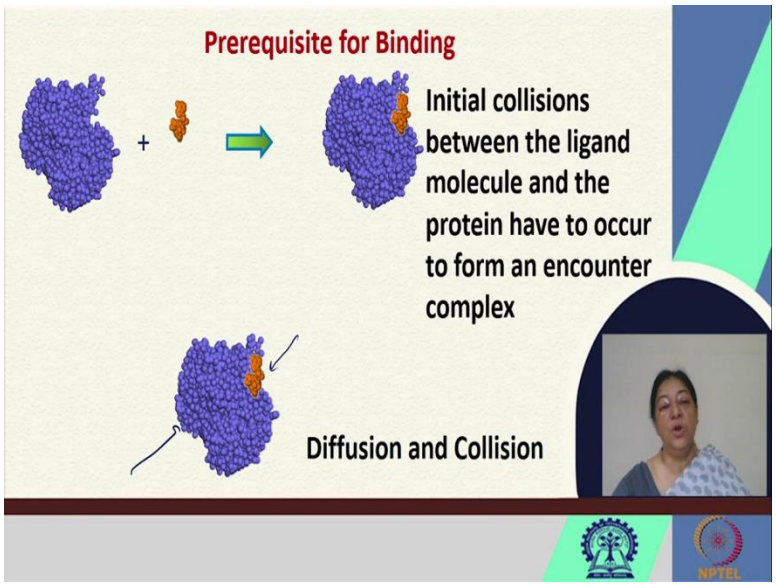


We understood that the prerequisite for binding, similar to an enzyme substrate complex formation, (which we will see later) requires diffusion and collision. In this specific case of a protein ligand bound together, we realize that with the proper orientation and the proper

proximity we would have this binding and the initial collisions will have to occur to form this [refer to slide] specific complex.

When we are working with enzyme and substrate, we realize that this is the enzyme and this is the substrate that has to bind to the specific pocket for a reaction to take place.

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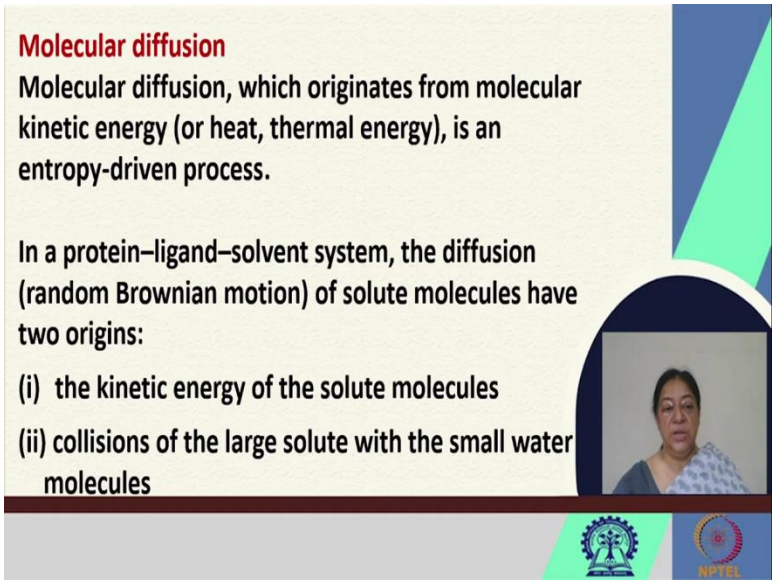


In our molecular diffusion and understanding, when we look at a protein ligand solvent system, we have the diffusion of the molecules, the diffusion of the solvent molecules.

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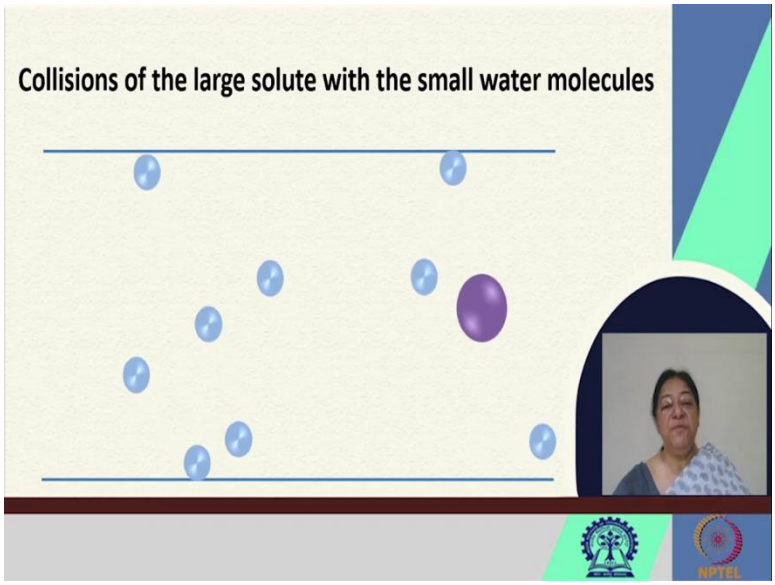

And we want a fruitful collision for a specific interaction to occur and in an enzyme substrate complex, for a specific catalytic reaction to occur.

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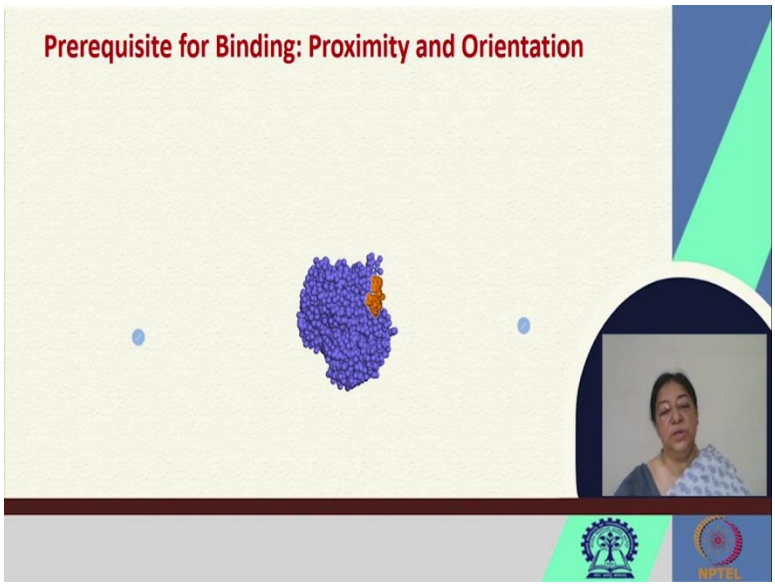


The binding that we look at in the collisions of the large molecules with the small water molecules, can affect the entropy and the enthalpy of the ligand binding and we realized that we needed a spontaneous change in the free energy to result in a fruitful protein ligand interaction. So we have our solvent molecules, we have our ligand molecules and our protein molecules and they have to come together to give us a specific complex formation.

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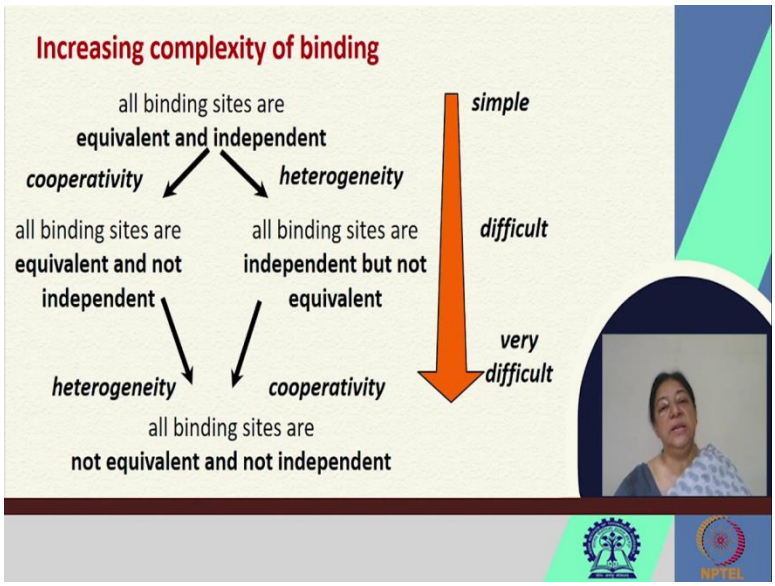

We see there are different levels [refer to slide] of binding. The different levels are such that we could have the binding sites on the protein, that are all equivalent and independent of each other.

This could require cooperativity of heterogeneity. We will look at cooperativity in this lecture and then later on when we have a special lecture on hemoglobin and myoglobin binding to oxygen; probably the most important protein ligand interaction known.

In cooperativity we have all the binding sites that are equivalent, but they are not independent of each other and in heterogeneity we have all binding sites that are independent, but not equivalent. As we look at heterogeneity and cooperativity, we can have all binding sites that are not equivalent and not independent of each other either. So this gets from a simple situation to a gradually difficult and a very complex situation, in understanding protein ligand binding all together.

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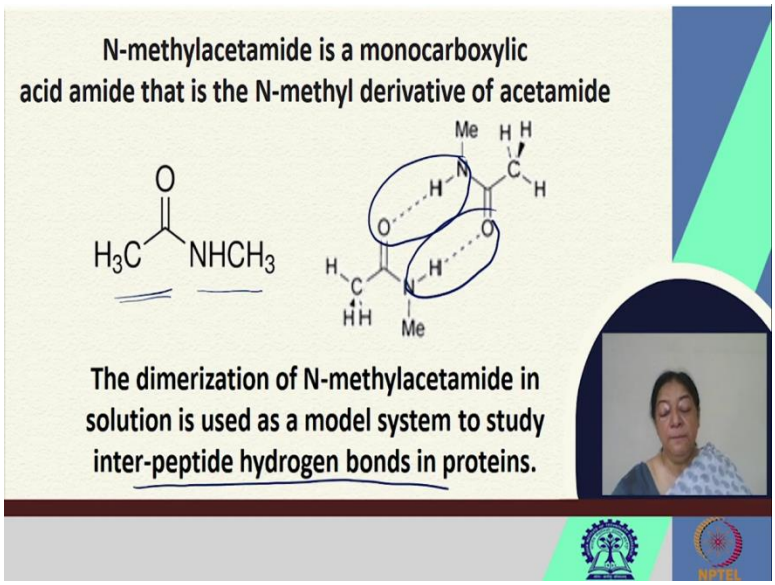


The N-methylacetamide which is a monocarboxylic acid amide that is an N-methyl derivative of acetamide, is chosen for a lot of studies as the dimerization of this compound is a model system to study the dipeptide, as you can see the -CONH- that gives us inter peptide hydrogen bonds in proteins that is the important measure to understand their interactions. So this is sometimes used as a model in many studies.

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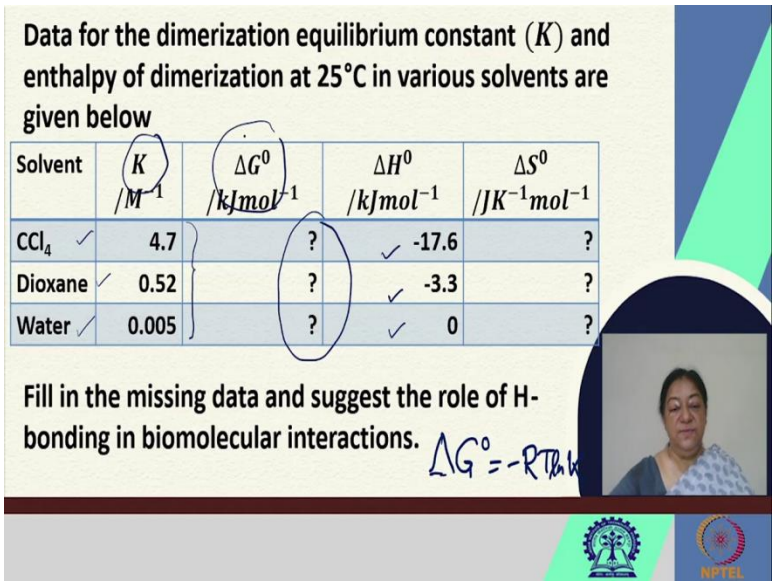


If we look at a specific problem related to this and we have data for the dimerization equilibrium constant K, the enthalpy of dimerization in various solvents and we would like to see the hydrogen bonding capability in these solvents. So we look at the solvent; we can see we have carbon tetrachloride, dioxane and water and these are the equilibrium constants in the different media.

From the equilibrium constants we can determine the change in free energy and from a knowledge of the values of our enthalpy changes, we can find out the corresponding entropy changes.

We can fill in the missing data and suggest the role of the hydrogen bonding in biomolecular interactions, by considering the spontaneity of the reaction in terms of when we calculate using our $\Delta G^0$ = - RTln K. What we have here is our K value we have our $\Delta G^0$ value.

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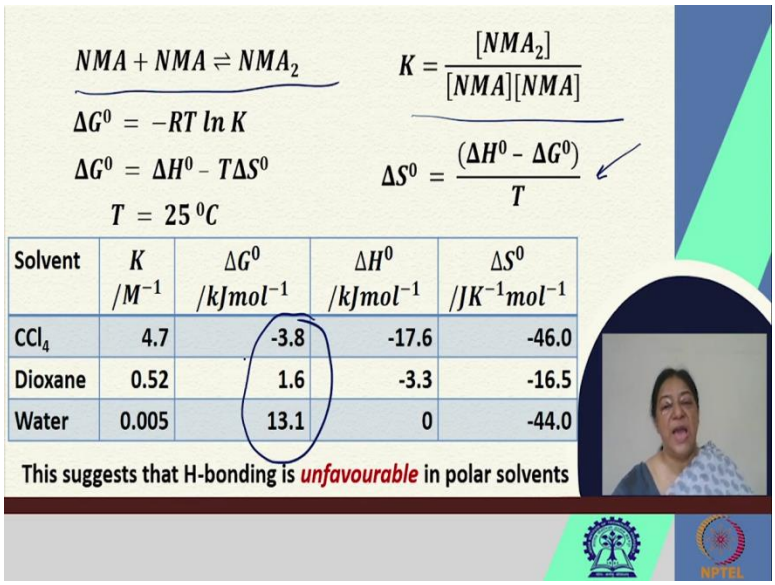


We can actually look at the dimerization procedure, which gives us our $\Delta G^0$ = - RTln K and we know that this K is a measure of the law of mass action applied to this particular set. So when we look at a $\Delta G^0$ we know it is $\Delta H$ - $T\Delta S$. So, the $\Delta S$ can be given by a small rearrangement, giving us this value and we know that T is 25$^o$ C.

We can calculate our values and we can fill up the table. And based on the table, we can look at the $\Delta G^0$ values and determine what hydrogen bonding aspect would be considered for this particular dimerization reaction. And so this suggests that when we have polar solvents, the hydrogen bonding is unfavorable.

This is an indication that we can look at the way we would study reactions such as these, in the determination of free energy changes, enthalpy changes and entropy changes.

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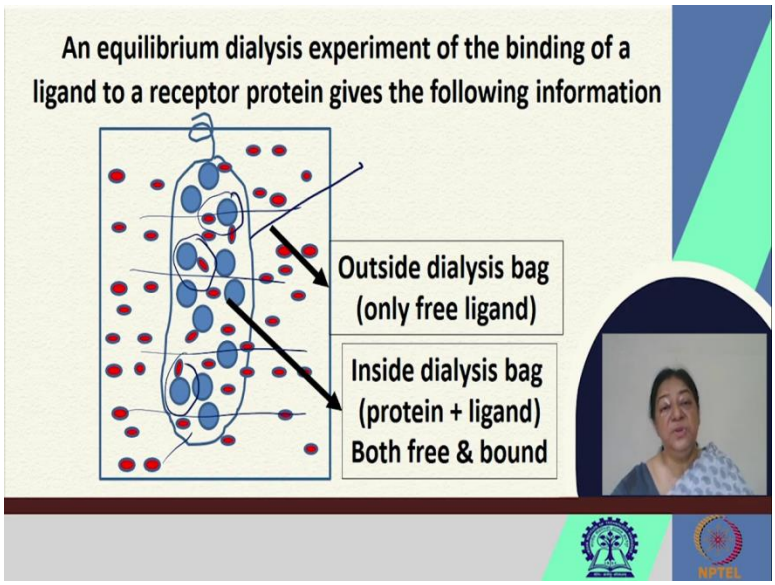


In the equilibrium dialysis experiment, we will look at a problem related to this. We learnt that we have a specific dialysis bag that is permeable to specific molecular weight. We have this bag that has a specific molecular weight cut off, allowing any molecule that has a molecular weight less than the cutoff of the dialysis bag, to pass freely through the membrane.

In the dialysis bag we only have free ligand and the free ligand concentration outside the dialysis bag is equivalent to the free ligand inside the bag because the ligand does not see the bag at all and considers that we have an equal concentration of the free ligand throughout.

However, inside we have a protein ligand complex form and this protein ligand complex will not be allowed to leave the bag considering that the molecular weight is high enough to be retained in the bag.

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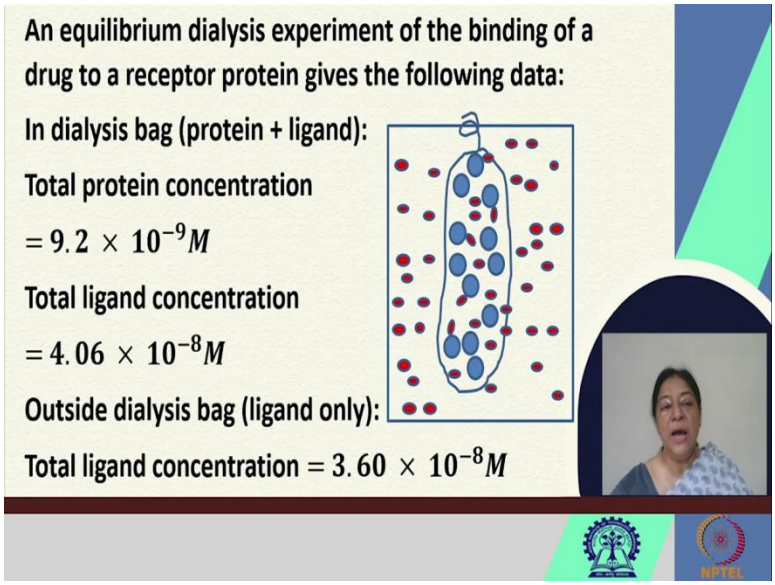

Based on an experiment like this if we get the data, where we have in the dialysis bag the protein plus the ligand, we have a total protein concentration, we have a total ligand concentration and from that we have outside the dialysis bag (that is only the ligand), we have a ligand concentration of 3.60 X $10^{-8}$ M.

So understanding now that we can determine the amount of protein that is free, the amount of ligand that is free and probably amount of protein ligand complex that has been formed; given that we know the concentrations and we are able to determine the concentrations.

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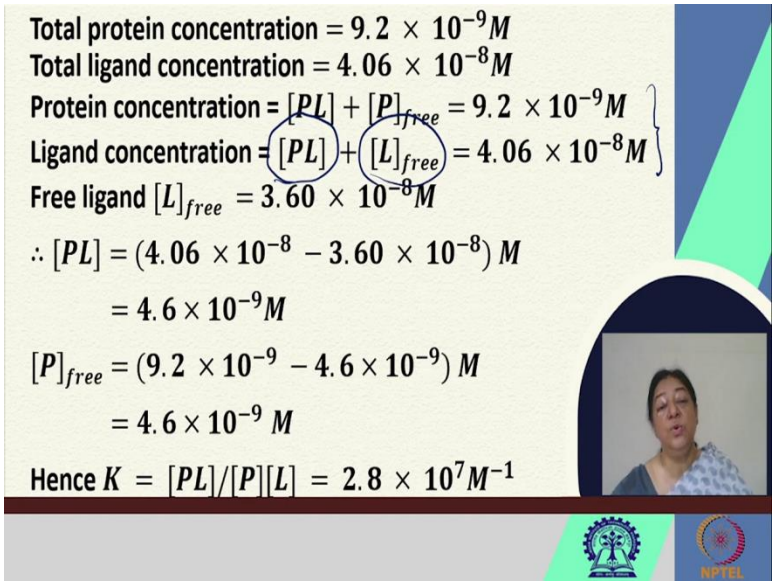


So we have the protein concentration, the ligand concentration and we know that the total protein concentration has to be whatever is bound to the ligand and whatever is free. The ligand concentration is what is bound to the protein and what is free.

The free ligand given by the concentration outside the dialysis bag, tells us the amount of ligand bound to the protein. From that we can determine the free protein and based on that we can determine our K or association constant.

From methodologies that are going to be used to determine the free concentrations of the ligand or the protein or the protein ligand complex, we will be able to determine the association constants which we are interested in.

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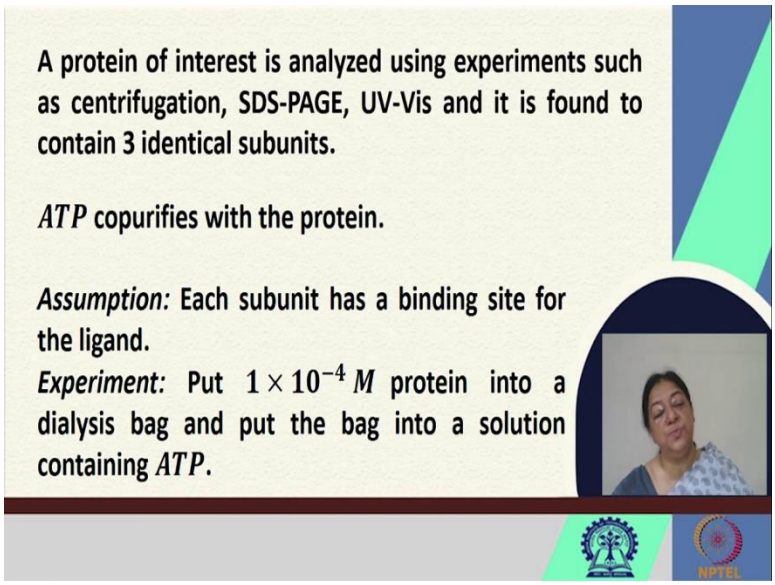


Similarly, if we have an example where we look at a protein of interest that is analyzed using several experiments and it is found to have now three identical subunits and we find that ATP copurifies with the protein.

We have a specific assumption that each subunit has a binding site for the ligand and an experiment is conducted, where a specific concentration of the protein is put into the dialysis bag and the dialysis bag is put into a solution containing ATP. Now we have to identify how much of the ATP is going to be bound to the protein.

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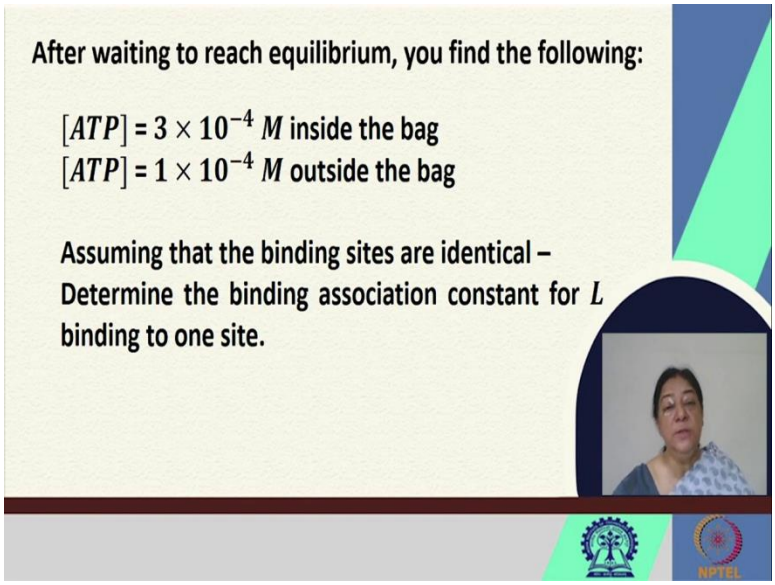


Based on this we wait for reaching equilibrium, this is extremely important because we are finding out the association constants in the assumption that the reaction has reached equilibrium. We find out that the concentration of ATP is 3 X $10^{-4}$ M inside the bag and 1 X $10^{-4}$ M outside

the bag. Assuming that the binding sites are identical we can find out the association constant for the ligand binding to a single site.

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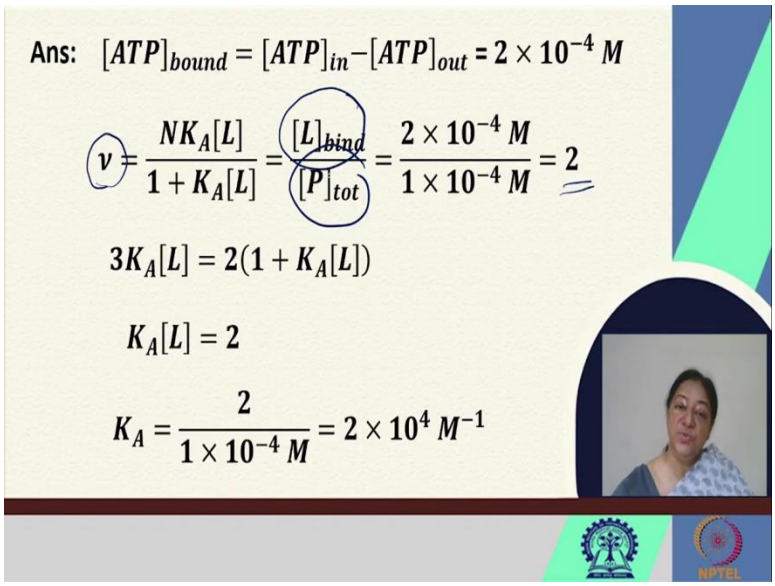


We look at the $[ATP]_{bound} = [ATP]_{in} - [ATP]_{out}$; given by the ATP concentrations that can be determined and from that we can look at our scatchard plot, find out the number of ligands bound to the total molecule or the macromolecule concentration present. So this [refer to slide] is the concentration of the ligand bound to the total protein present and from that again, with a bit of algebra, we can find out our value for the association constant.

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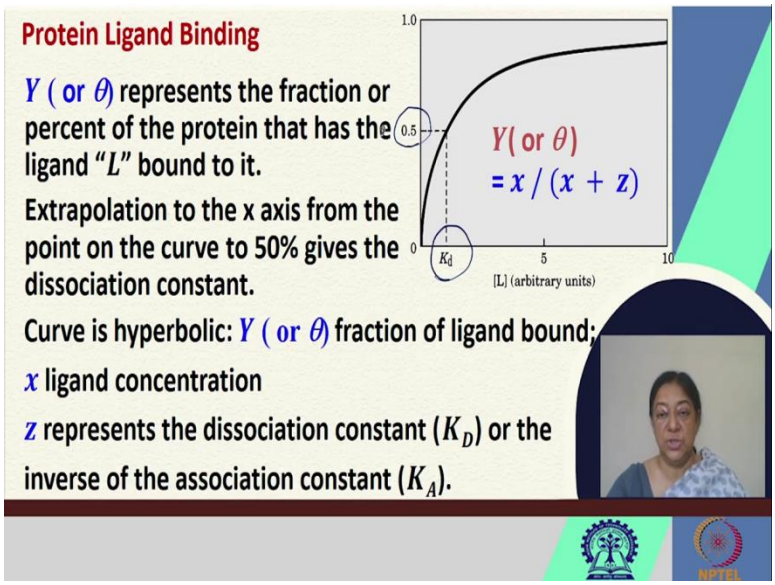

Let us look at protein ligand binding from a different perspective, in understanding the fraction or the percent of the protein that has a ligand bound to it. So if we look [refer to slide] at the specific way in which we have the ligand bound in the specific binding curve, we understand that if we have a specific fraction or a ligand bound to it, we know that when this reaches 0.5, we have the value of our dissociation constant.

The curve is hyperbolic in nature indicating the fraction of the ligand bound, x being the ligand concentration and z that represents the dissociation constant or the inverse of the association constant.

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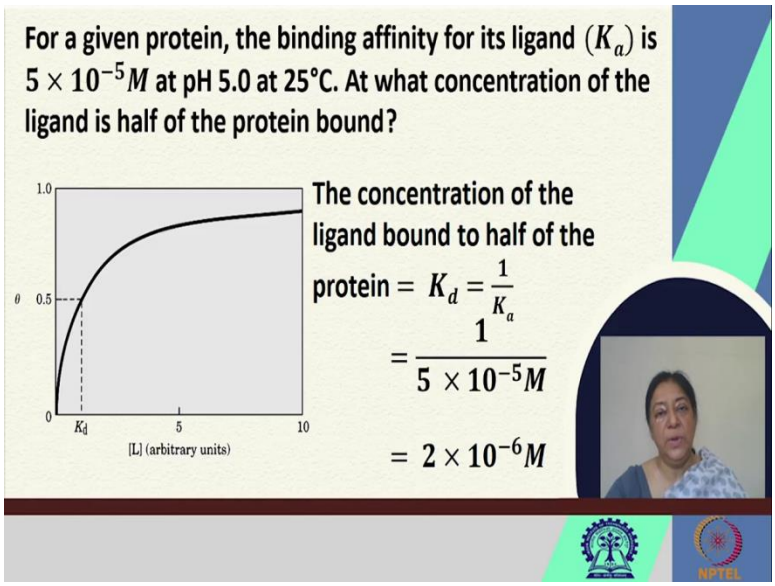


Along these lines if we consider an experiment where we have for a given protein, the binding affinity for its ligand that is given as 5 X $10^{-5}$ M at pH 5 at 25° C. If we want to know the concentration of the ligand when half of the protein is bound, it indicates the dissociation constant. So, the concentration of the ligand bound to half of the protein is equal to $K_d$, is equal to $1/K_a$ that gives us 2 X $10^{-6}$ M, that is 2 μM.

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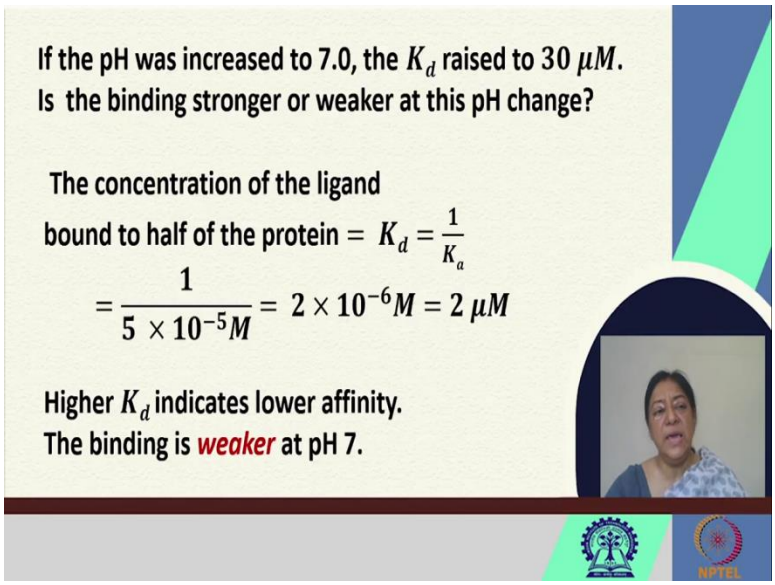


If we now have some additional information about our protein, say that if the pH was increased to 7 the $K_d$ raised to 30 μM will the protein be stronger or weaker at this pH change. So the concentration of the ligand that we found in the previous case, bound to half of the protein corresponding to the dissociation constant, now we have this say at 2 μM. So if the pH is raised to 7, the $K_d$ is 30 μM.

We know that a higher $K_d$ indicates a lower affinity. So, this means that the binding is weaker at pH 7. Given instances like this, we are able to understand the binding capabilities of our proteins, we are able to understand their binding at different pH values and have an assessment of how we would like to design ligands that could bind to proteins effectively.

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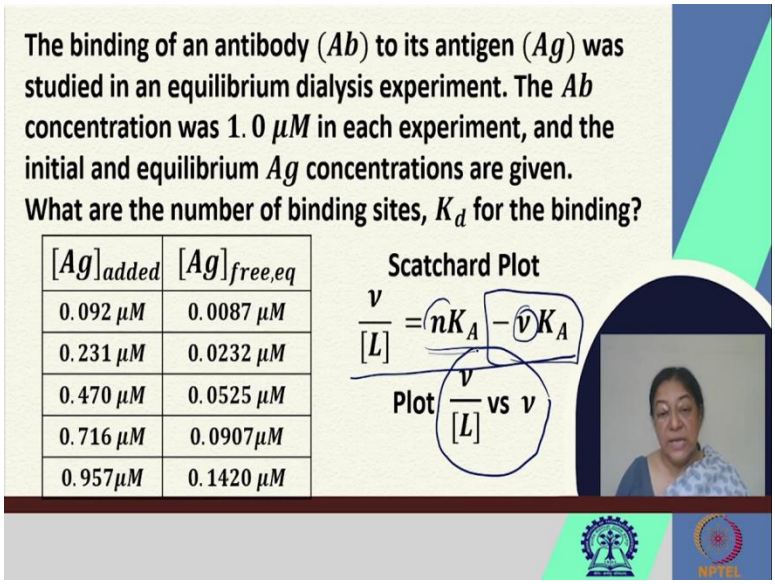

In another example let us look at antigen antibody binding. The binding of an antibody to its antigen we know is a very strong binding and this was studied in another equilibrium dialysis experiment where the antibody concentration was 1 μM.

The initial and equilibrium antigen concentrations have been given in the table here [refer to slide], we want to know how many binding sites there are present and the $K_d$, that is the dissociation constant for binding. Now to do this we need to plot a scatchard plot, as we have learnt in one of the previous lectures.

What we have is, we have this linear equation $\frac{\nu}{[L]} = nK_A - \nu K_A$

and we have to plot ν/[L] versus ν, that from the slope of which we will get the value of - $K_A$. and we get the intercept.

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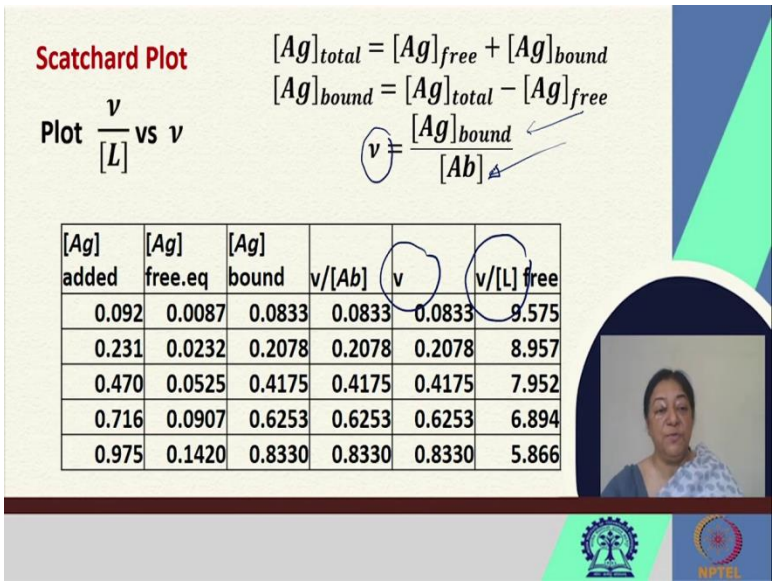


| [Ag] added | [Ag] free.eq | [Ag] bound | ν/[Ab] | ν | ν/[L] free |
|---|---|---|---|---|---|
| 0.092 | 0.0087 | 0.0833 | 0.0833 | 0.0833 | 9.575 |
| 0.231 | 0.0232 | 0.2078 | 0.2078 | 0.2078 | 8.957 |
| 0.470 | 0.0525 | 0.4175 | 0.4175 | 0.4175 | 7.952 |
| 0.716 | 0.0907 | 0.6253 | 0.6253 | 0.6253 | 6.894 |
| 0.975 | 0.1420 | 0.8330 | 0.8330 | 0.8330 | 5.866 |

If we look at this [refer to slide] scatchard plot, we have to do a bit of calculation in order to find the ν/[L] and the ν value which we now have to plot. So, with the knowledge of the $[Ag]_{total} = [Ag]_{free} + [Ag]_{bound}$, knowing that the total antigen concentration is what is free and what is bound together, we can find out the amount bound and the definition of the ν is the amount of ligand bound to the total amount of macromolecule, which in this case is the antibody.

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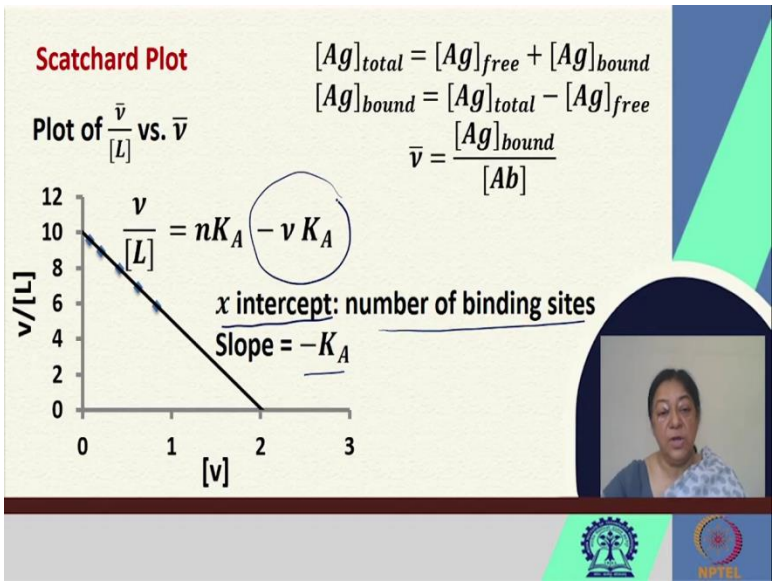


Given our plot of ν/[L] versus ν, we will get this [refer to slide] plot, given the values in the table. What we see from this value is that the equation that we get or the information that we get from the x intercepts, gives us the number of binding sites and the slope is equal to - $K_A$.

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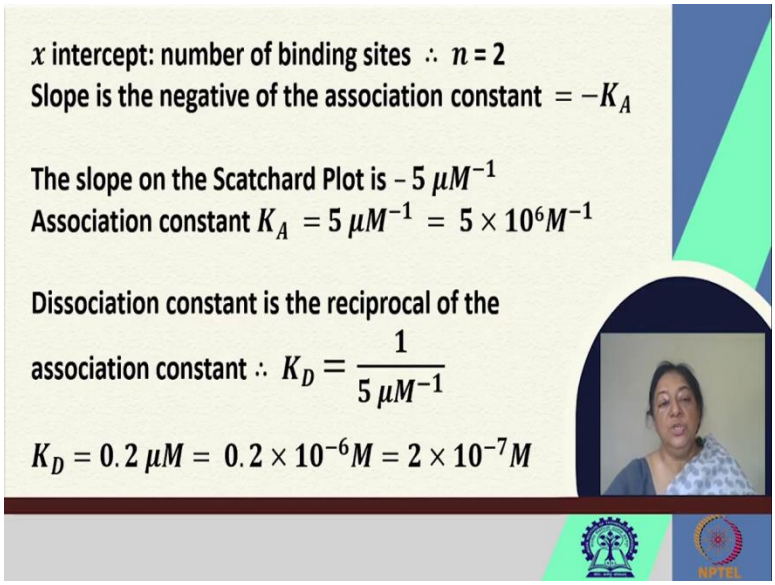


If we do a bit of calculation here we will see that we have found that the x intercept gives us the number of binding sites which is equal to 2. From the slope, we get the negative of the association constant - $K_A$. Now, when we look at the slope on the scatchard plot, we therefore, get - 5 $\mu M^{-1}$ which means that the association constant $K_A$ is given as 5 $\mu M^{-1}$ = 5 X $10^6 M^{-1}$.

So we know that the dissociation constant is the reciprocal of the association constant. From this we can get the value of $K_D$ that follows to be 0.2 X $10^{-6}$M that is 2 X $10^{-7}$M. So, this gives us an

indication of how we can use the scatchard plot to find out the number of binding sites and the association and dissociation constants of protein ligand binding.

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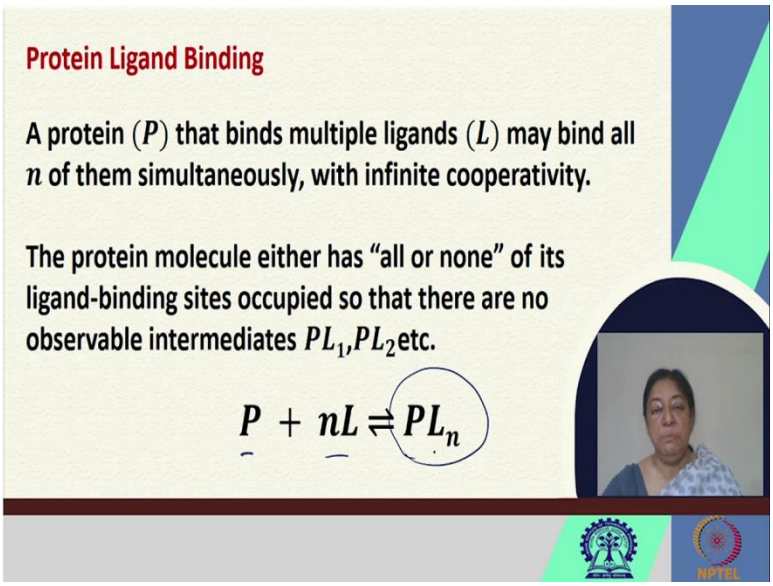


Now we look at another aspect of protein ligand binding in the sense, that we have the protein binding multiple ligands. If they bind all of them simultaneously, we call this infinite cooperativity. So we have P, we have nL and we have $PL_n$ formed in the complex formation of multiple ligands. The protein molecule in this case has an all or none situation, where either all its binding sites are empty or all are occupied which indicates that there are no intermediates PL 1, PL 2 et cetera.

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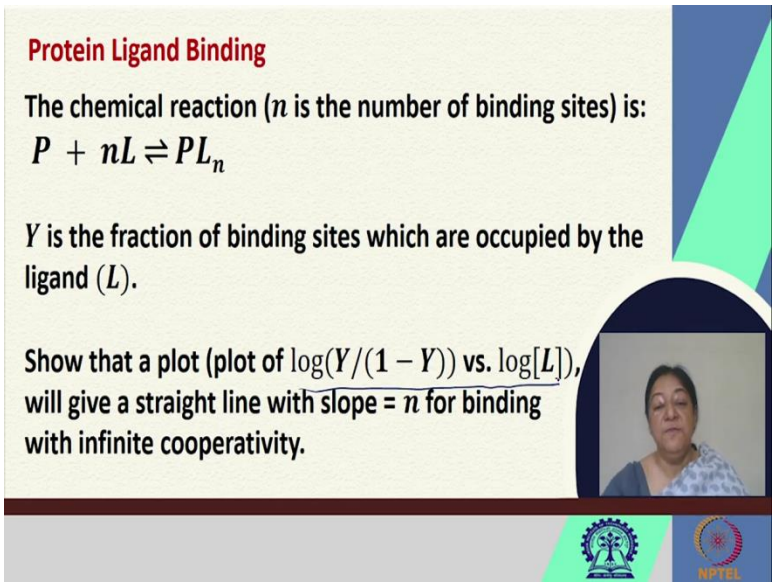

If we look at the association with this, the chemical reaction therefore is P + nL giving us $PL_n$ and Y as we considered in the previous, can be the fraction of binding sites which are occupied. Now, if we can actually show that a plot of this logy/(1-Y) versus log[L] will give a straight line with the slope = n for binding with infinite cooperativity, which says that all of our ligands bind together to the protein.

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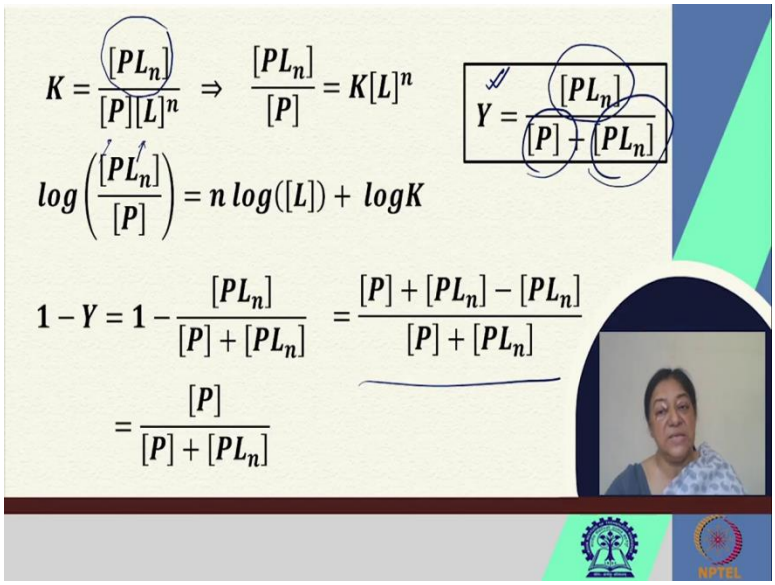


So let us see how this works. This [refer to slide] is our association constant, where by the law of mass action we have $[PL_n]$ and [P] and $[L]^n$ as the two reactants. Then we can look at a rearrangement of this separate equation and what we get is a fraction bound.

$$Y = \frac{[PL_n]}{[P]-[PL_n]}$$

We get the fraction bound with the ligand in this case which is defined by our Y. So, we can rearrange this equation to give us what 1 - Y is and from that we can do a bit of algebra to determine that 1 - Y corresponds to : $\frac{[P]}{[P]+[PL_n]}$

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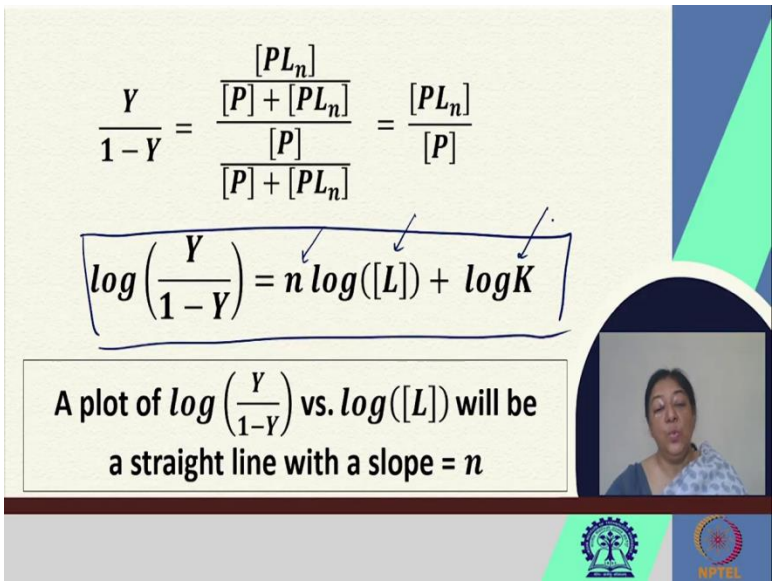


So, Y/(1-Y) will give us this [refer to slide] expression which will work out to a specific expression which we get which is log (Y/1-Y) = n log ([L]) + log K. So based on this if we plot log (Y/1-Y) versus log ([L]), we will get a straight line with the slope = n.

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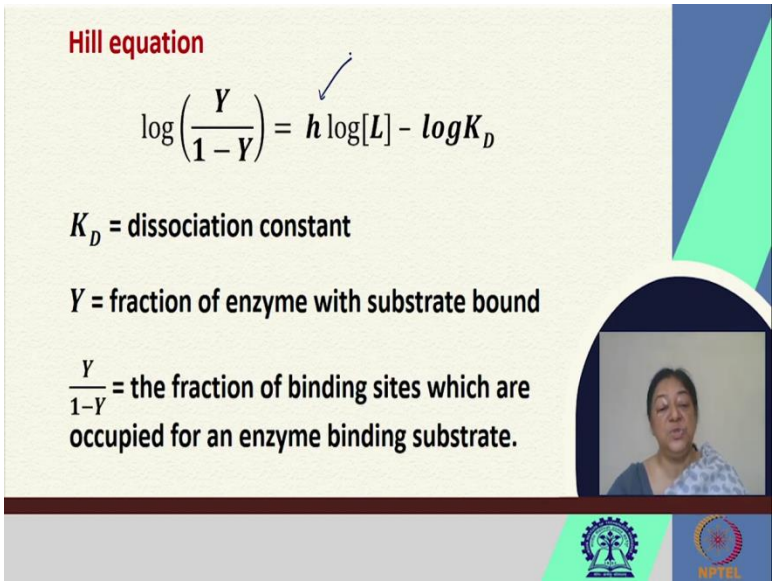


This is called the Hill equation. So we have log (Y/1-Y) = h log [L] – log $K_D$. We will see what this h means in a moment, $K_D$ is the dissociation constant, Y is the fraction of the enzyme with substrate bound and (Y/1-Y) is the fraction of the binding sites which are occupied for the enzyme binding substrate.

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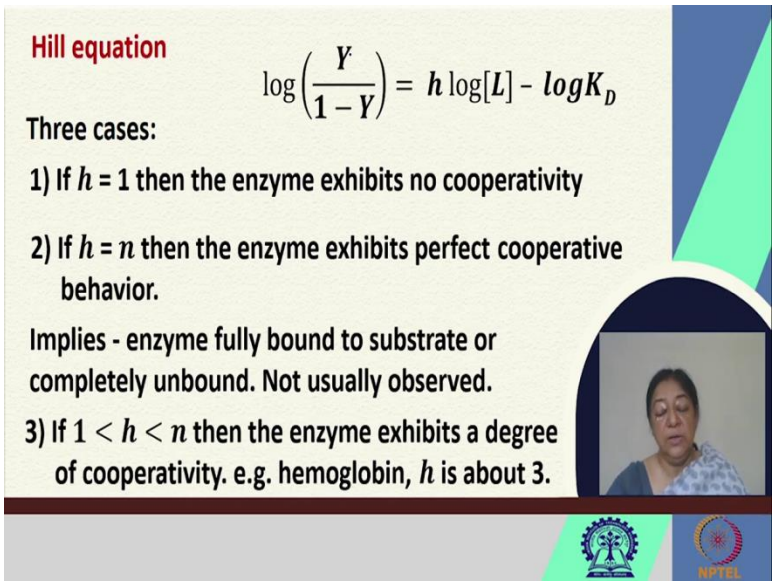


Now if we look at 3 cases, if h is equal to 1 then the enzyme exhibits no cooperativity as it is called. The cooperativity factor means that the binding of one ligand facilitates the binding of the other ligand to the binding site.

So if h is equal to n, then the enzyme exhibits perfect cooperativity saying that the number of binding sites are occupied immediately. So, the enzyme in this case will be fully bound to the substrate, if we are looking at an enzyme substrate situation or the protein fully bound with the ligand, but this is not usually observed in reality.

In reality we have a value that is between 1 and n, then we say that the enzyme or the protein exhibits a degree of cooperativity. For example, hemoglobin that has four binding sites which we will see in a subsequent lecture, shows us an h of about 3. If now we work on this hill equation looking at a hill plot and see how we can work on a specific example.

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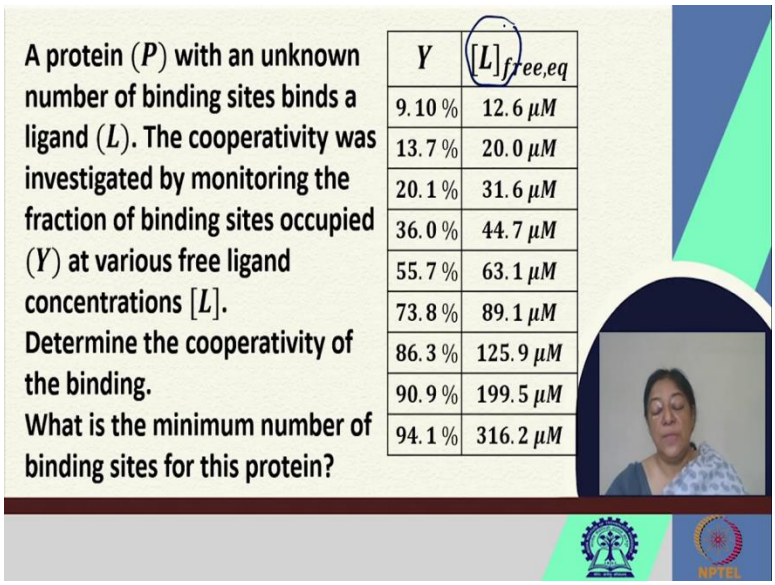


If we have a protein P with an unknown number of binding sites say with the L, the cooperativity has been investigated by monitoring the fraction of the sites occupied with free ligand concentration.

We want to know the cooperativity of binding and also the number of binding sites, the minimum number of binding sites of this protein. Given that we know the fraction of binding sites that are occupied at the specific free ligand concentrations that we have here, let us work to find out the table that needs to be constructed to get our plot.

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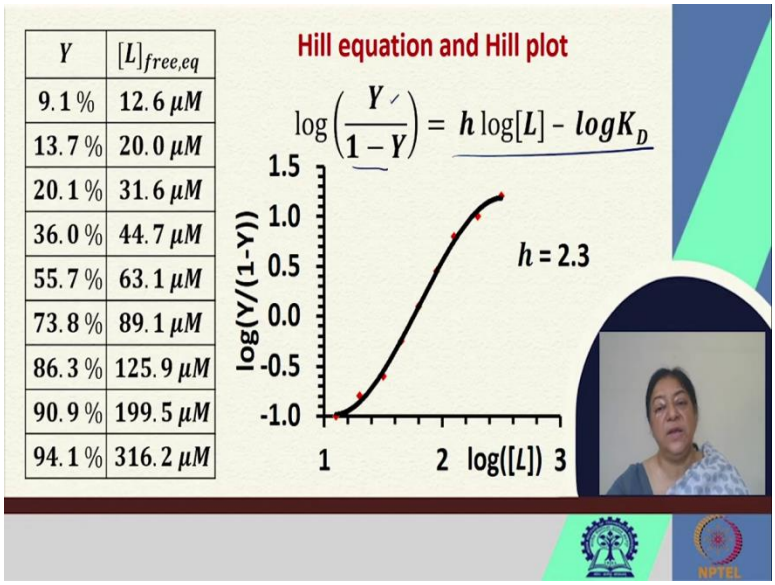


So, we have Y and [L] and we need to find log (Y/1-Y) and this h log [L] – log $K_D$ value here and what we will get is from the slope we will get the value of h. If we plot the graph, this [refer to slide] is the observed plot that we get for the data shown on the left. What this means now is

we it we need to find out what h is. h turns out to be from the linear part of the curve a value of 2.3.

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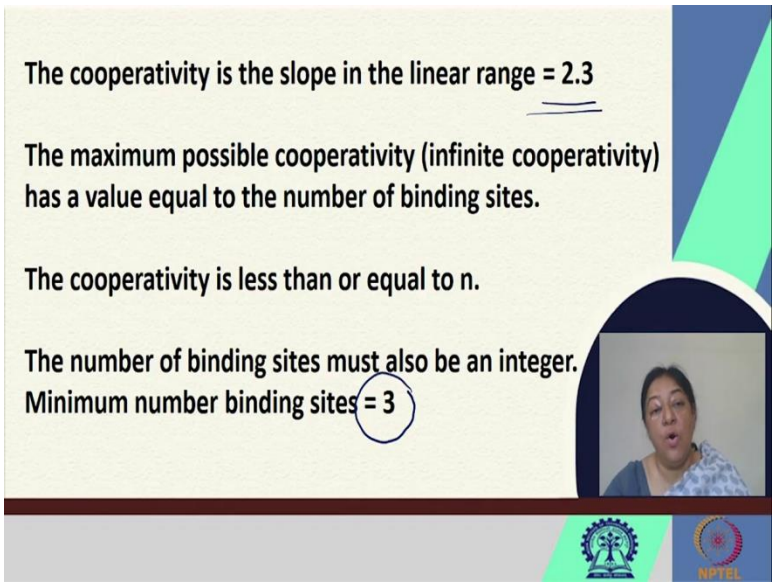


This means that the cooperativity is the slope in the linear range that comes out to be 2.3 and the maximum possible infinite cooperativity has a value equal to the number of binding sites. But in this case the cooperativity has to be less than or equal to n, n means there is infinite cooperativity.

The number of binding sites also must be an integer and what this indicates that the minimum number of binding sites is 3 and this being a cooperative nature of the order of the value of h being 2.3, indicates that there is some cooperativity in the binding of this ligand with the protein. That means that once one ligand molecule bounds, this will help the other ligand molecules bind to it.

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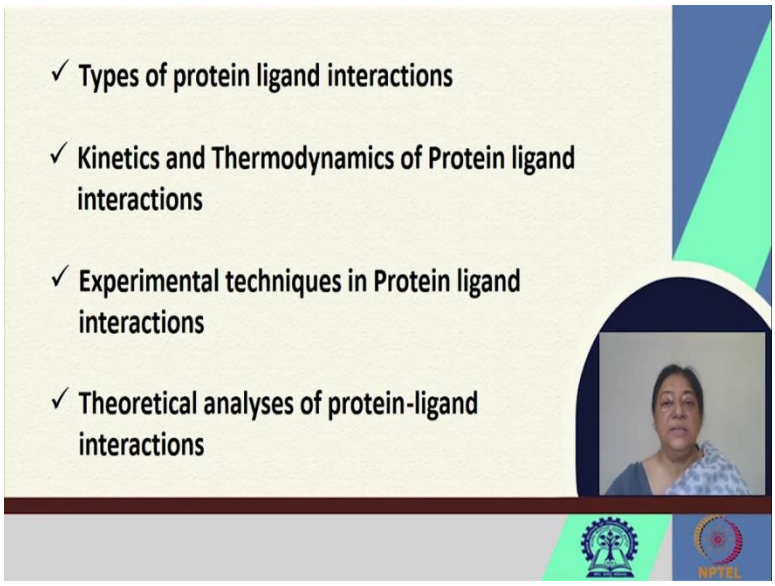


What we have looked at in the series in this module are, the types of protein ligand interactions, the kinetics and thermodynamics of protein ligand interactions, followed by experimental techniques in protein ligand interactions and in the previous lecture theoretical analysis of the protein ligand interactions in terms of docking methods and other methodologies.

In the final lecture of this module we have looked at specific types of plots, what we can do from an equilibrium dialysis experiment with actual data, how we plot a scatchard plot to determine the number of binding sites and a hill plot from a hill equation to determine the cooperativity of binding.

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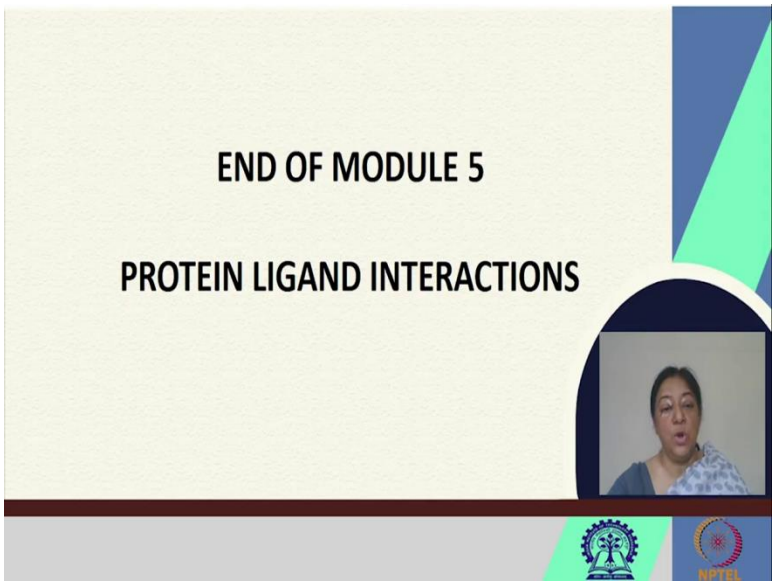


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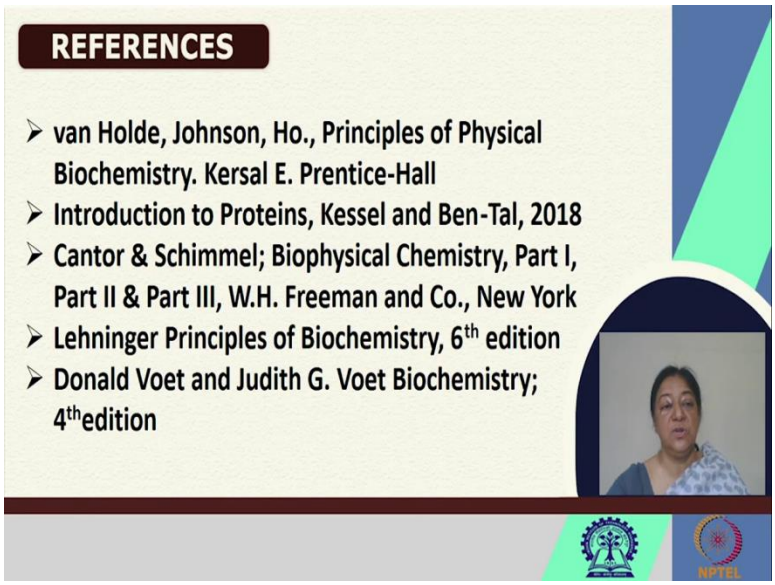


This ends this module 5, that dealt with protein ligand interactions. The references have been given in each of the lectures which are also listed here [refer to slide].

Thank you.