**Fundamentals of Protein Chemistry**
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**Module - 05**
**Protein ligand interactions**
**Lecture - 23**
**Experimental Methods in Protein Ligand Interactions**

We continue our discussion on protein ligand interactions. In this specific lecture, we will be looking at experimental methods in protein ligand interactions.

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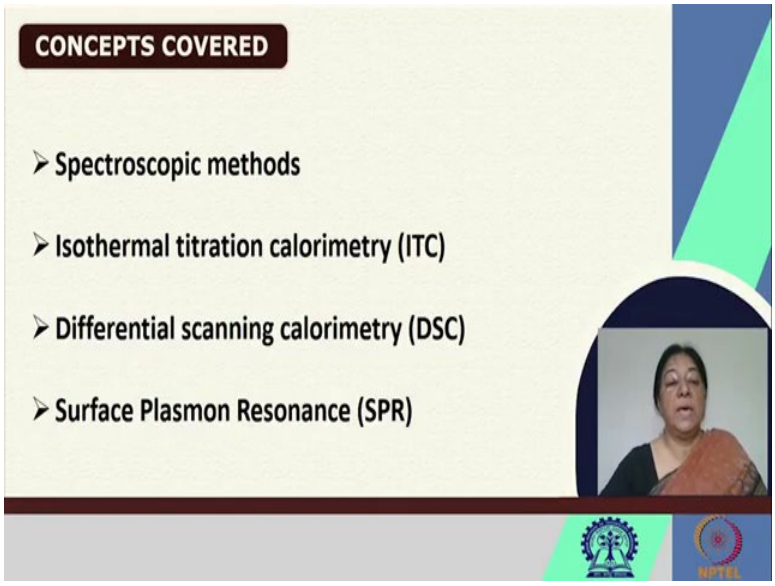


In the specific methods that we will be looking at we will visit spectroscopic methodologies, ITC that is isothermal titration calorimetry, differential scanning calorimetry, surface plasmon resonance. We have to remember that these are some of the techniques that are used, there are many techniques that are used to determine the protein ligand interactions that we have discussed in the previous lectures.

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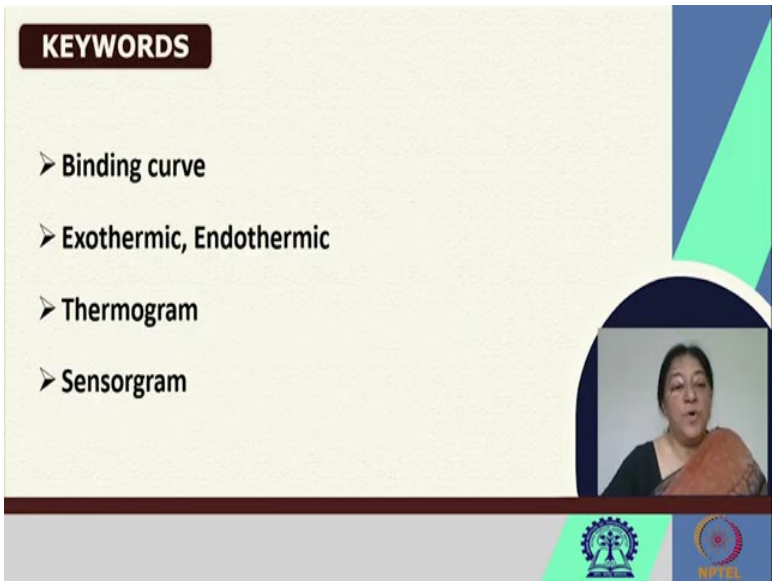


For example, the construction of the binding curve; how do we know whether we have an exothermic or an endothermic reaction; can we discuss the thermodynamic parameters; how are we supposed to determine the thermodynamic parameters from the data that we have.

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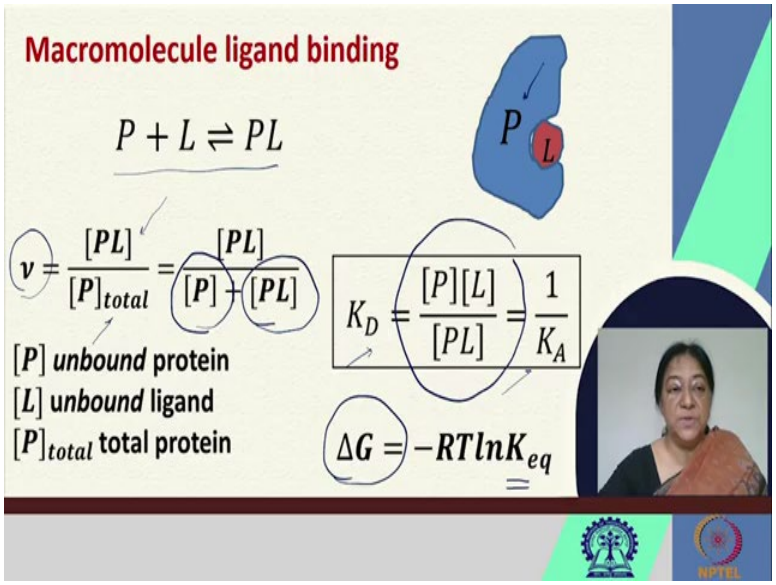


When we look at macromolecule ligand binding we see protein and we see ligand. Now based on the equilibrium associated with this, we have:

$$\nu = \frac{[PL]}{[P]_{total}}$$

We remember that the total amount of protein has to be either in the free form or in the bound form.

From this: $K_D = \frac{[P][L]}{[PL]} = \frac{1}{K_A}$

we can get the dissociation constant or the association constant depending upon how we write our equilibrium condition. From the dissociation constant, from the equilibrium constant we can get the free energy associated with this at the specific temperature of the experiment.

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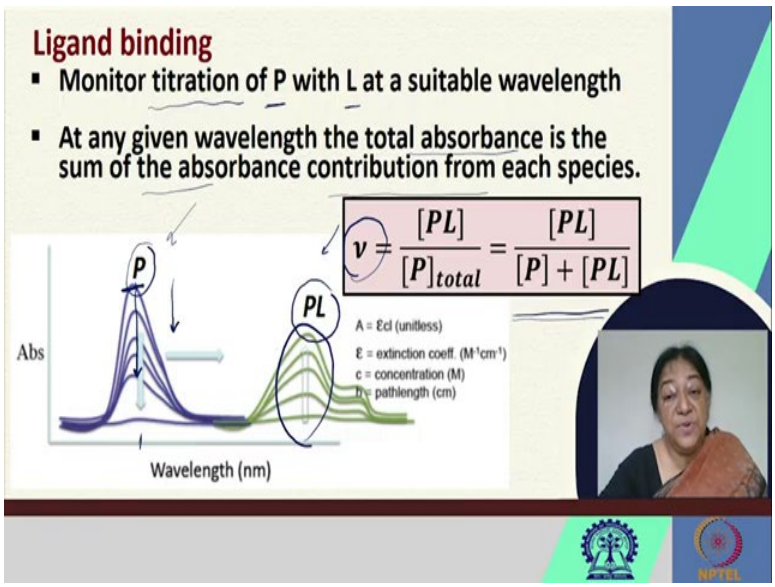


We looked at this [refer to slide] diagram initially where we have our protein and we are adding a particular ligand to the protein, forming the protein-ligand complex. As we add the ligand, we can monitor the titration of the protein with the ligand at a specific wavelength, where we see the peak. Now, at any given wavelength the total absorbance is the sum of the absorbance contribution from each of the species that we have here.

Given that we are looking at this particular wavelength where we have the concentration of the PL increase as the ligand is being added to the solution. This is the peak where we do not have the ligand, the total protein and as we go along this curve we see the amount of ligand added and from that we can find out our value of ν and the protein-ligand concentration as well as the free protein that is available to us.

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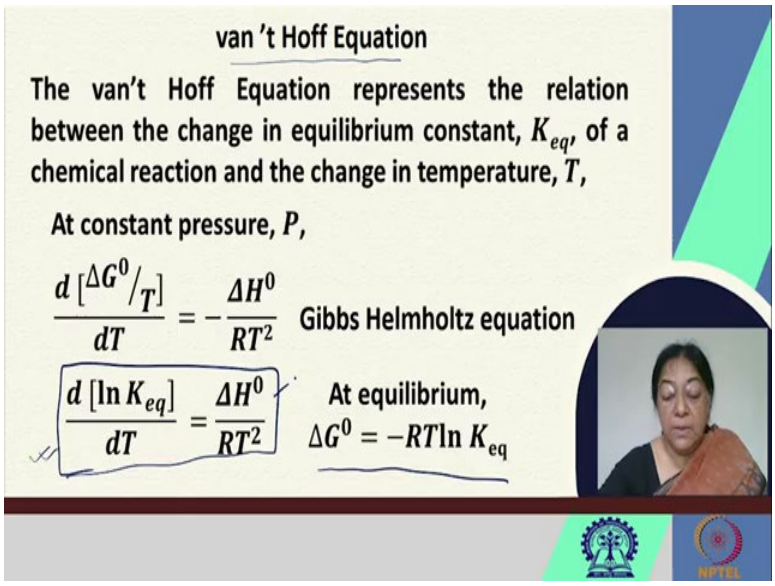


Another aspect of determining thermodynamic parameters comes from the van't Hoff equation. In the van't Hoff equation this represents the relation between the change in the equilibrium constant that is K equilibrium, of a specific chemical reaction and the change in the temperature T. Now at constant pressure we have the Gibbs Helmholtz equation. We know at equilibrium that $\Delta G^0 = -RT\ln K_{eq}$ and from this we can work it out in a manner that we get this:

$$\frac{d\left[\ln K_{eq}\right]}{dT} = \frac{\Delta H^0}{RT^2}$$

in terms of the enthalpic contributions.

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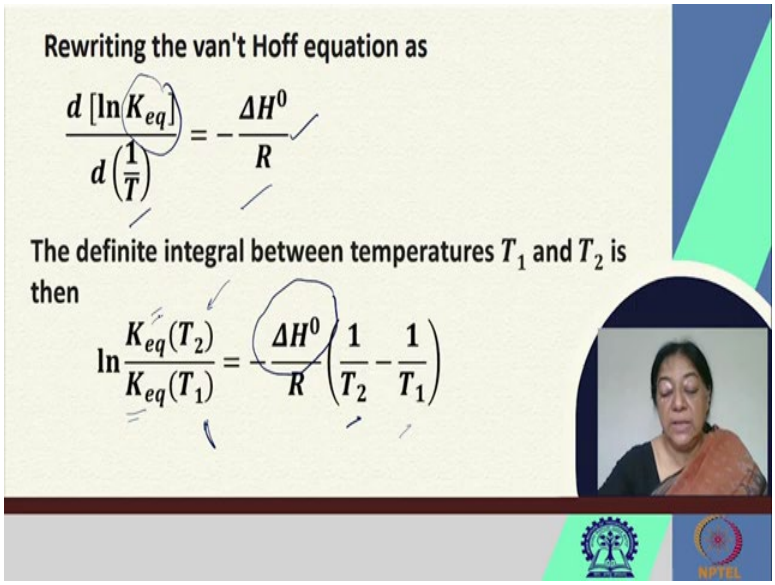


Looking at this specific relationship we can rewrite it as this expression;

$$\frac{d[\ln K_{eq}]}{d(1/T)} = -\frac{\Delta H^0}{R}$$

We know how to find the $K_{eq}$ and from this we have to see what information we can plot to get the specific thermodynamic parameters. So, we can have the equilibrium constant determine at two different temperatures and from the slope of the curve or by just plugging in the values of the $T_2$ and the $T_1$, from the $K_{eq}$ obtained at these two temperatures we can get the value of $\Delta H$.

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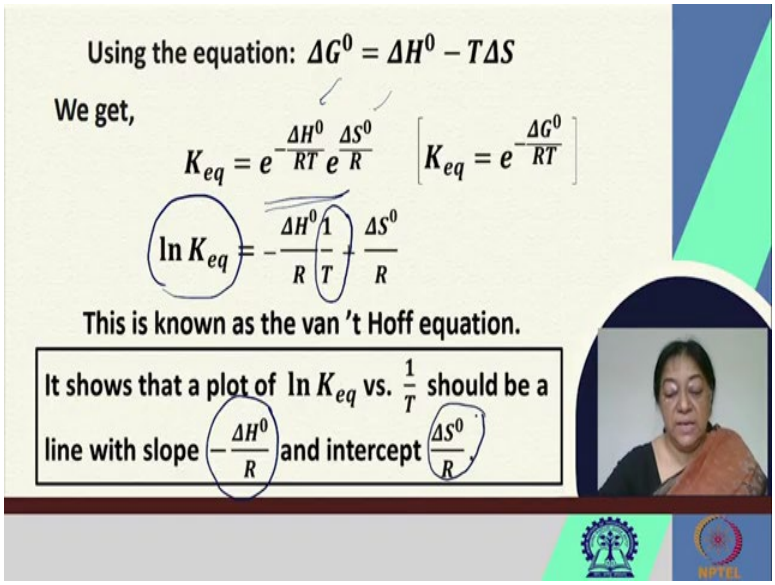


In addition to using this particular equation $\Delta G^0 = \Delta H^0 - T\Delta S^0$, we get a specific expression in terms of looking at the $\Delta H^0$ contribution and the $\Delta S^0$ contribution, given our $K_{eq}$. This expression is known as the van't Hoff equation and it indicates that if we plot it, it is of the form of $y = mx + c$. If we plot $\ln K_{eq}$ versus $1/T$, we can see that the slope is $-\Delta H^0/R$ and the intercept is $+\Delta S^0/R$.

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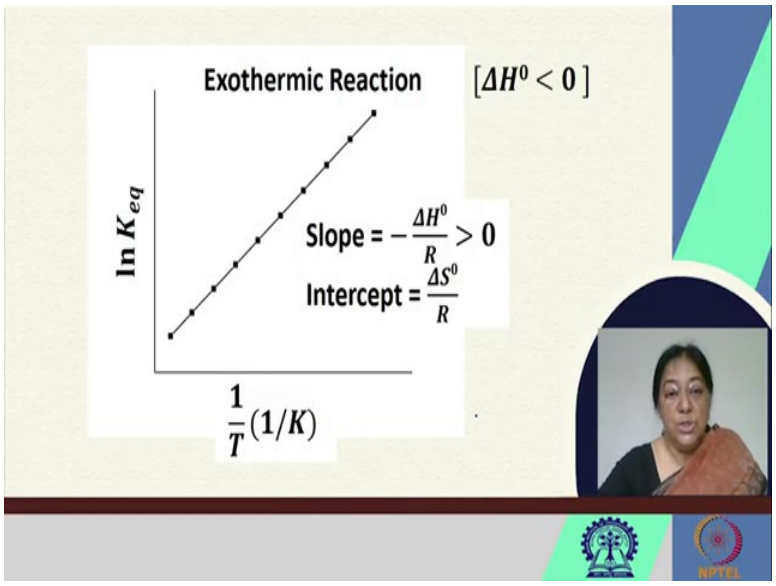


We can have an exothermic reaction where we will get $\Delta H^0 < 0$.

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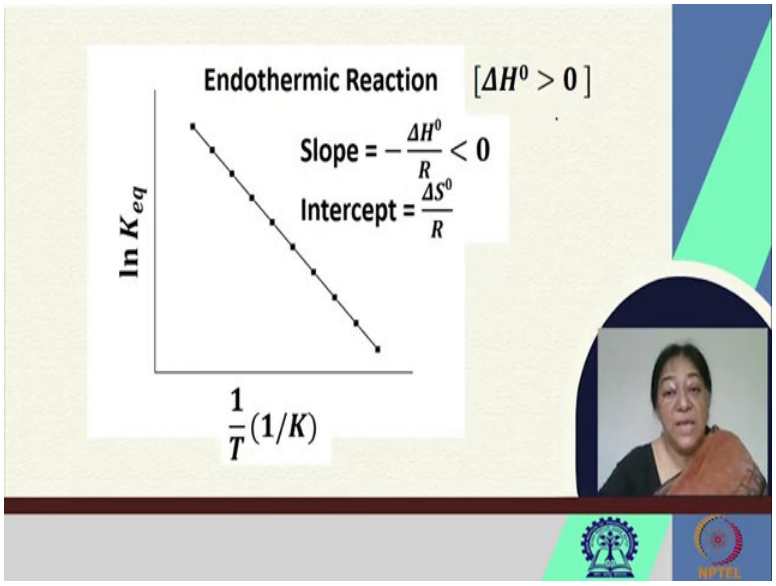


We can have an endothermic reaction where our $\Delta H^0 > 0$ and we see the slope of the plots in each of the cases because in this case we have - $\Delta H^0/ R > 0$ in a positive slope and in the endothermic case we have the - $\Delta H^0/ R < 0$. So this gives us an indication of how we can determine thermodynamic parameters.

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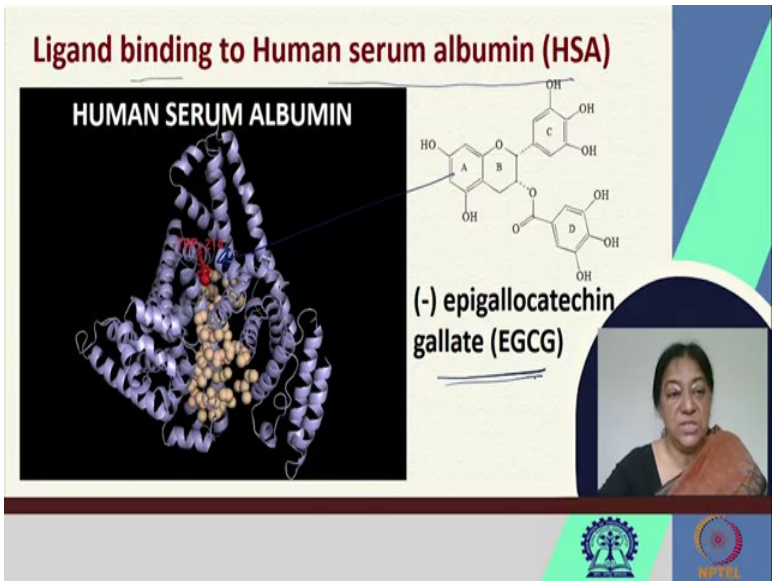


Let us look at a specific example of the ligand binding to human serum albumin, the abundant protein in our plasma. This [refer to slide] is the structure of human serum albumin.

If we want to look at a specific ligand binding to HAS, we can opt for this tryptophan residue and see how the fluorescence of the tryptophan residue is affected by the binding of the ligand. In this particular case, we can choose this ligand epigallocatechin gallate EGCG and see what difference in the spectra we observe on binding of EGCG.

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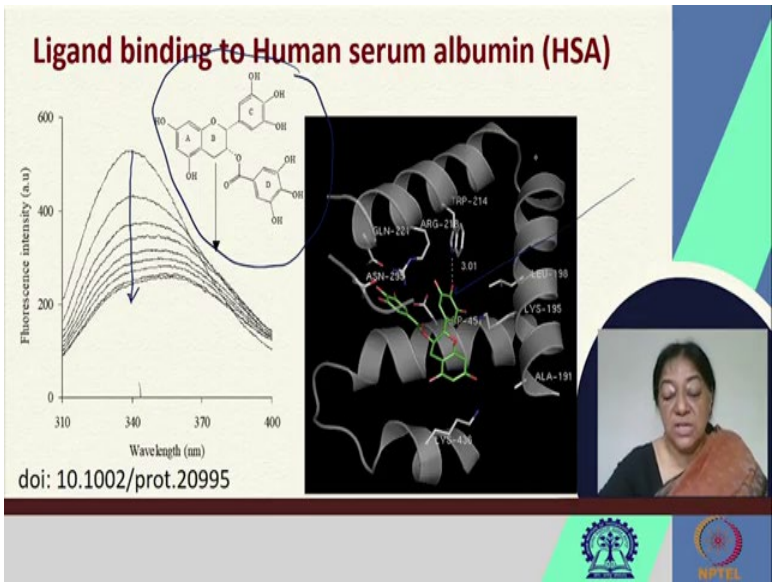


This is our structure of EGCG and this is what happens to the fluorescence intensity at the specific wavelength that corresponds to the tryptophan residue and the reduction in the fluorescence intensity, at that point of the emission of the tryptophan indicates that this particular ligand is going to the site where the tryptophan is present.

Fortunately HSA has only one tryptophan. It is an indication that this EGCG that is marked here, is going to the site where the tryptophan is present. We will see how we can look at this data or how we can construct such diagrams in the next lecture.

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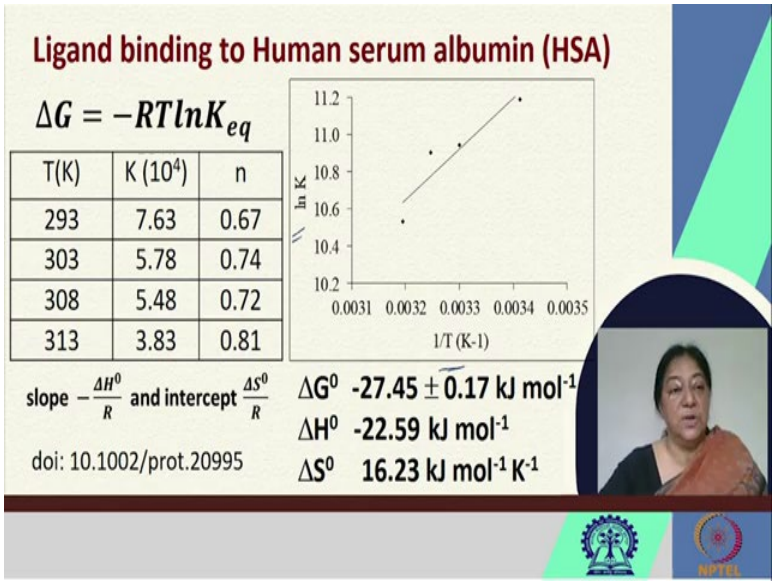


When we look at this ligand binding now to human serum albumin, we can conduct this specific experiment at different temperatures and from a Scatchard plot we can get the value of n that is the number of the stoichiometry of the equation and from this information we can plot a van't Hoff plot, where we have the ln K versus the 1/T, we have a positive slope. So from this we can get our slope and our intercept, getting our thermodynamic parameters.

This is a way in which we can determine the thermodynamic parameters for ligands binding to proteins. In this case we looked at a specific fluorescent spectroscopic technique. If our protein-ligand is such that there is a change in the UV spectra, we can also monitor that. Our idea is to monitor the reduction in protein concentration; the increase in protein-ligand concentration; to monitor the effect of the $K_{eq}$ that is observed.

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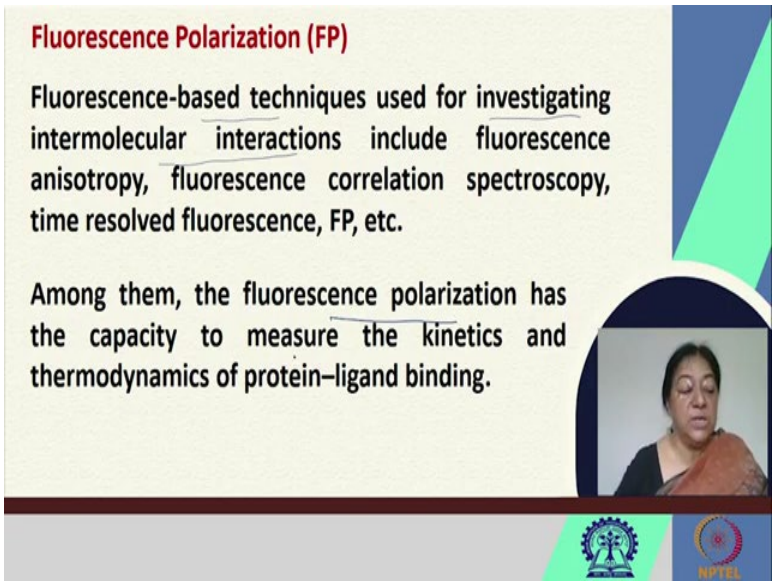


Another technique that is used is fluorescence polarization. These fluorescence based techniques are used for investigating the intermolecular interactions, including anisotropy, correlation spectroscopy, time resolved fluorescence as well as fluorescence polarization. This specific method has the capacity to measure the kinetics and the thermodynamics of protein-ligand binding.

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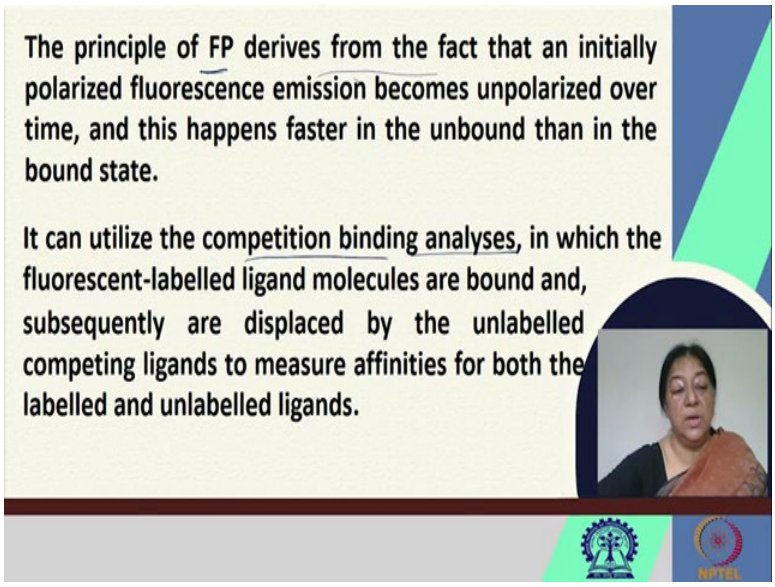


The principle of fluorescence polarization derives from the fact that an initially polarized fluorescence emission becomes unpolarized over time and this would happen when the ligand is in the unbound state. As such it can be utilized for competition binding analysis, in which the ligand molecules are bound and then they can be used for competing ligands to measure the affinity for both labelled and unlabelled ligands also. Let us see how this works.

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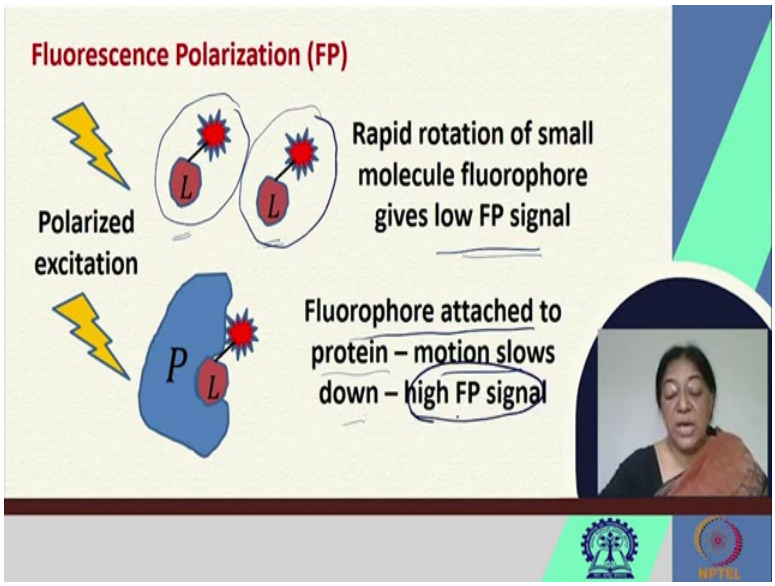


If we have a polarized excitation and we have a ligand molecule that has this [refer to slide] tag along with it because it is small in size, there is rapid rotation of the small molecule fluorophore, resulting in a low signal. However if this were attached to the protein, because of the larger molecule the macromolecule, that is this fluorophore, the motion of the molecule is slowed down. The rapid rotation is no longer possible, giving us a high FP signal which can be monitored.

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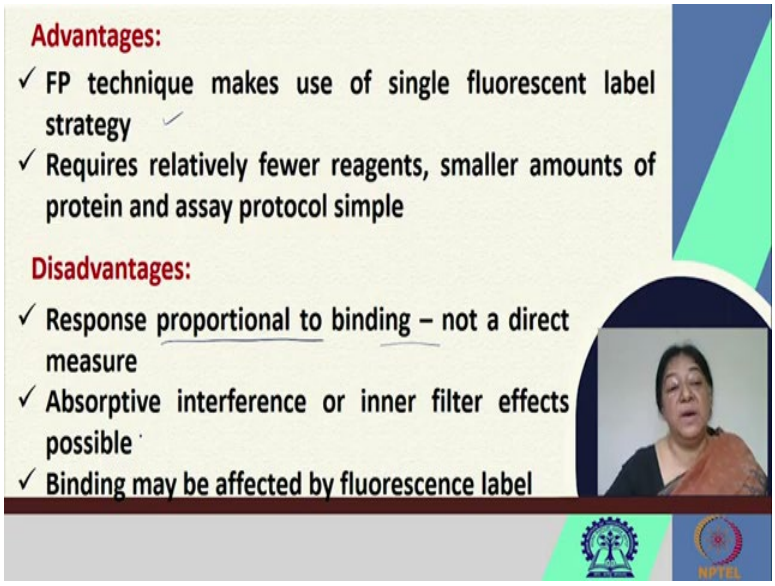


The advantages of this technique makes use of a single fluorescent label, but it also could be proportional to the binding and is not a direct measure and there could be absorptive interference

or inner filter effects and the binding to the protein may also be affected by the fluorescence label.

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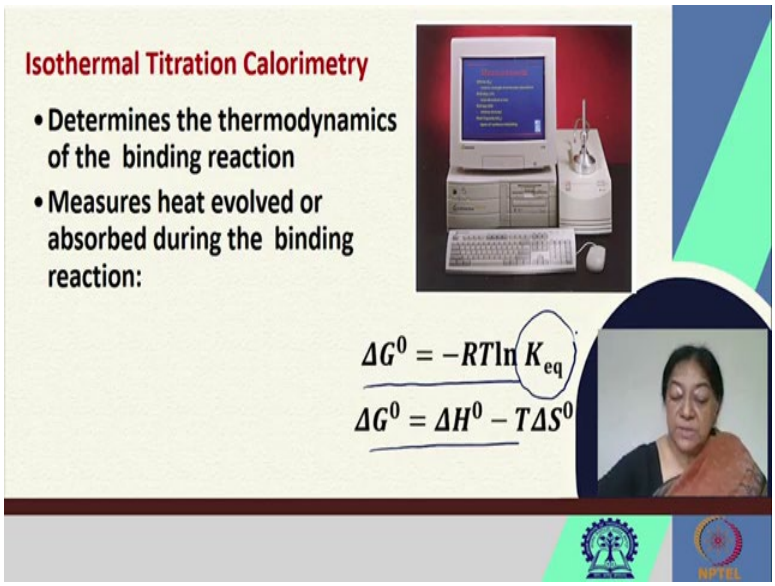


Another method that is commonly used to determine the thermodynamics of the binding reaction, measures the heat evolved or absorbed during the binding reaction. This is the isothermal titration calorimetry, where we look at the specific expressions that we know for

$\Delta G^0 = -RT\ln K_{eq}$ and $\Delta G^0 = \Delta H^0 - T\Delta S^0$.

Now, when we know how we can monitor the $K_{eq}$, given that we know a methodology to determine the free protein and the bound protein. From that we can get a Scatchard plot, from the Scatchard plot we can get the stoichiometry, we can also get the dissociation constant which is what we are interested in a protein-ligand binding experiment.

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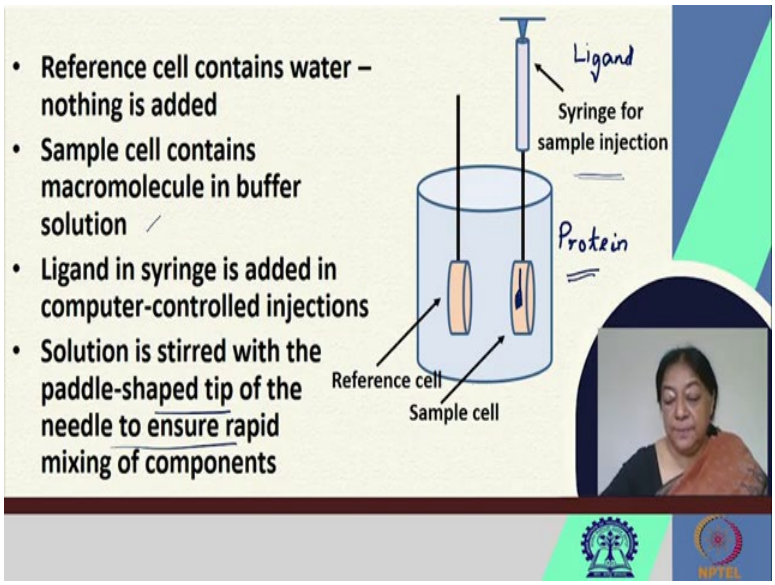


In this [refer to slide] particular diagram, we see that there is a reference cell and there is a sample cell and this is the syringe that is used for sample injection. The sample cell contains the macromolecule in the buffer solution and the ligand is present in the syringe.

This is where we have the ligand and in the sample cell here, we have our protein of interest or the macromolecule of interest. This ligand is then injected into the macromolecule, in this case the protein, by computer controlled injections. There is in here a small paddle shaped tip of the needle that ensures rapid mixing of the components as these micro injections are made.

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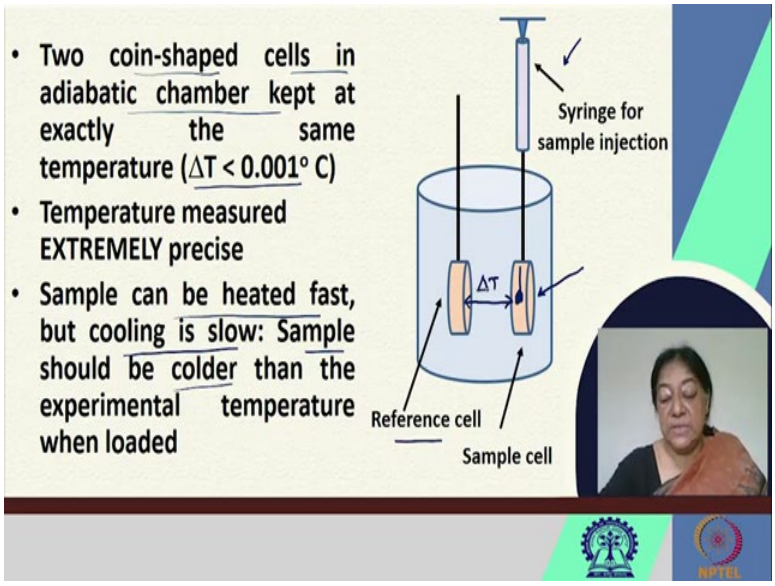


Following the micro injections, these coin-shaped cells that are in the adiabatic chamber are kept at the same temperature and the $\Delta T$ is very small between these two and the temperature measured here is extremely precise. We have to be careful because the sample can get heated fast

and cooling is slow. So the sample should be colder than the experimental temperature when the loading is done.

Given our experiment we have our protein in this [refer to slide] cell here, our reference cell here and the ligand being injected into the protein of interest. During the injections there is the occurrence of a reaction, an interaction that results in either the evolution of heat or the absorption of heat. What is going to happen in that case is there is going to be a temperature difference between the reference cell and the sample cell.

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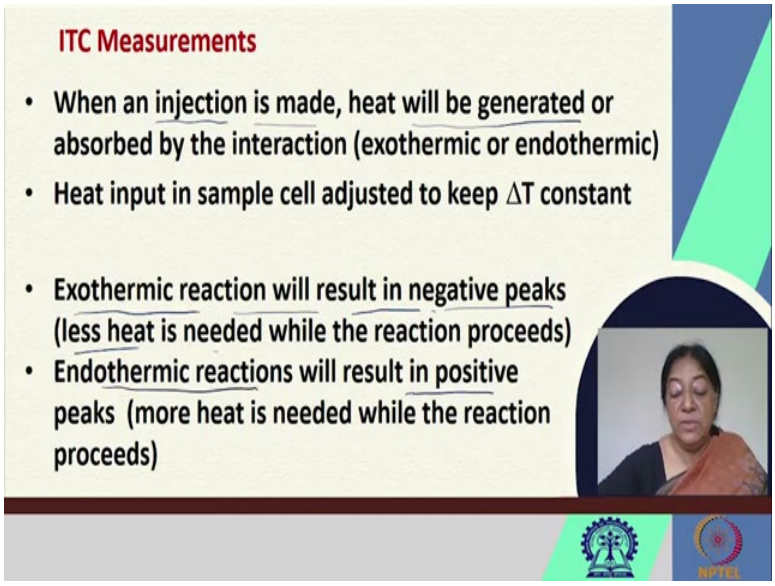


How is this treated? So, when this injection is made, heat will be either generated or absorbed by the interaction giving us either an exothermic or an endothermic reaction.

The heat input in the sample cell is adjusted to keep the $\Delta T$ constant. And, what we observe is we in the exothermic reaction, we see negative peaks because less heat is needed when the reaction proceeds. Conversely, when we look at an endothermic reaction, this result in a positive peaks because more heat will be needed when the reaction proceeds.

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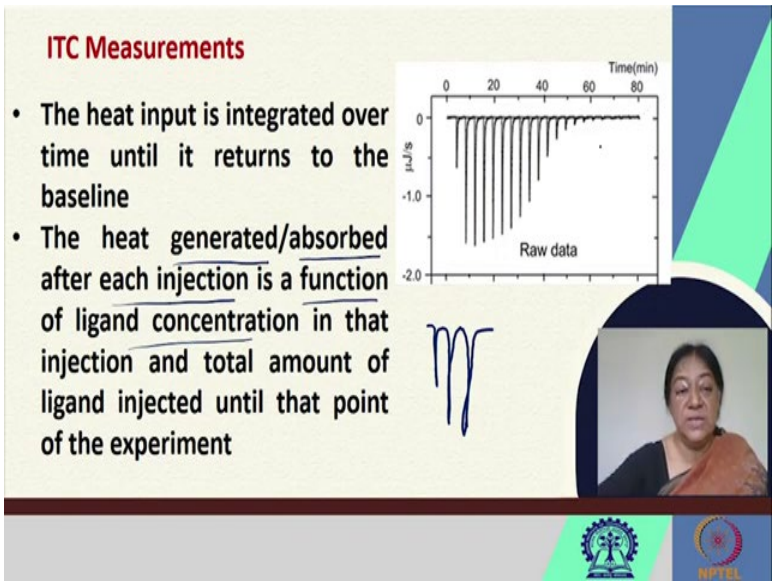


This heat put is then integrated over time until it reaches the baseline. The heat generated or absorbed after each injection is a function of the ligand concentration in that injection and the total amount of the ligand added till that point.

When we have the first injection we have heat variations. Heat changes, comes back to the baseline, leading to the next injection and so on and so forth. Depending upon the injections and depending up on it coming back to the baseline every time before the next injection occurs, we have to monitor the data.

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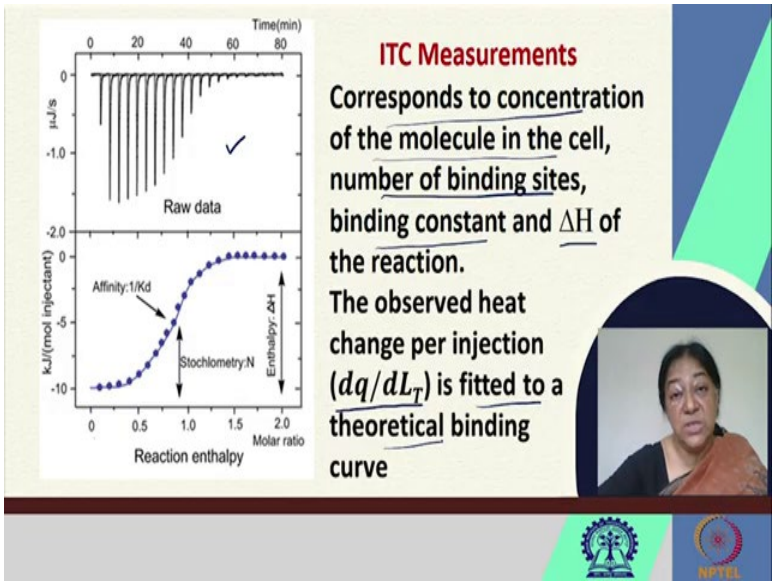


The ITC measurements have this [refer to slide] as the raw data and this corresponds to the concentration of the molecule in the cell, the number of binding sites present on the protein, the binding constant and the enthalpy of the reaction. The observed heat change per injection given

by $dq/dL_T$ is fitted now into a theoretical binding curve, from which we can get the stoichiometry, we can get the enthalpy and we can get the affinity. This is useful for determining the thermodynamic parameters of the particular protein-ligand interaction.

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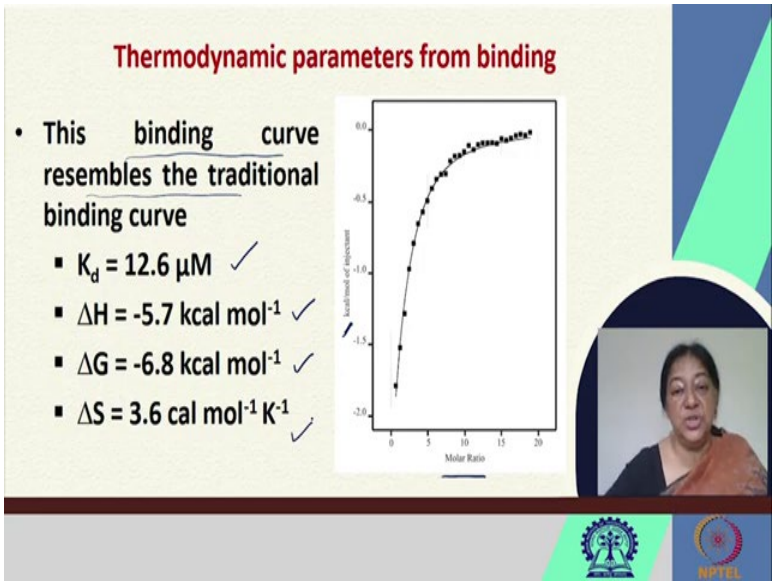


So how do we get the thermodynamic parameters? This is a typical binding curve which gives us the kcal per mole of injection of the injectant and the molar ratio that is observed. Following this, this binding curve as we recognize, resembles the traditional binding curves that we have seen. From this we can get the $K_d$ value the dissociation constant, we can get the $\Delta H$ value, the $\Delta G$ value and the $\Delta S$ value, following the thermodynamic experiment from an ITC instrument, the isothermal titration calorimetric.

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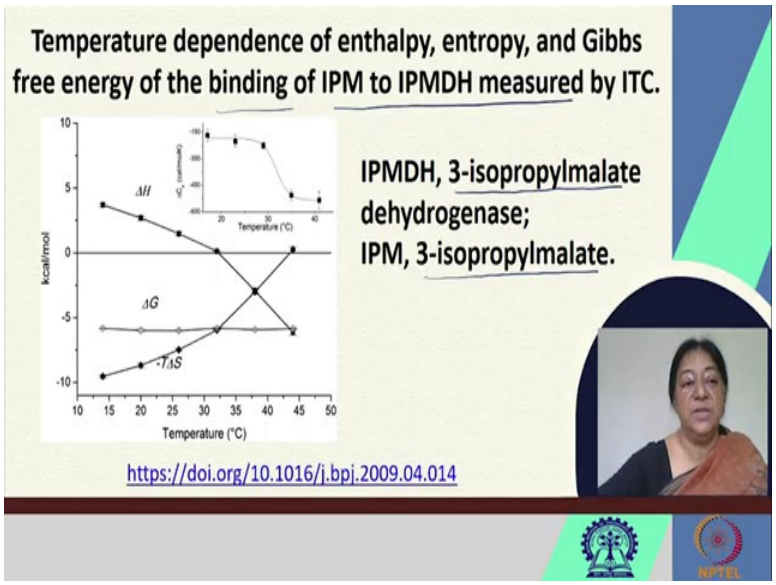


The temperature dependence of enthalpy, entropy and the Gibbs free energy of the binding of IPM to this particular dehydrogenase is now shown here [refer to slide]. So, we have a ligand that is 3-isopropylmalate and the protein 3-isopropylmalate dehydrogenase. This is the typical curve that we get from the change and from the temperature changes associated with this and this gives us an idea of the typical values.

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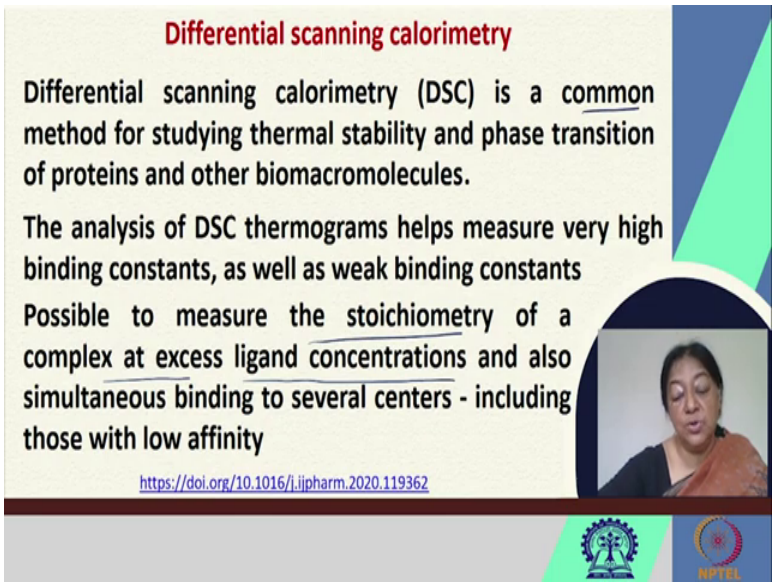


Differential scanning calorimetry method is also a commonly used method for studying thermal stability and phase transition of proteins as well as other biomacromolecules. In this case the thermogram as it is called helps us measure very high binding constants and in addition it can also measure weak binding constant.

It is also possible to measure the stoichiometry of the complex, at excess ligand concentrations and also account for simultaneous binding to several centers that could also have centers with low affinity. So, they could be sites that have low affinity for the ligand that also can be monitored through DSC.

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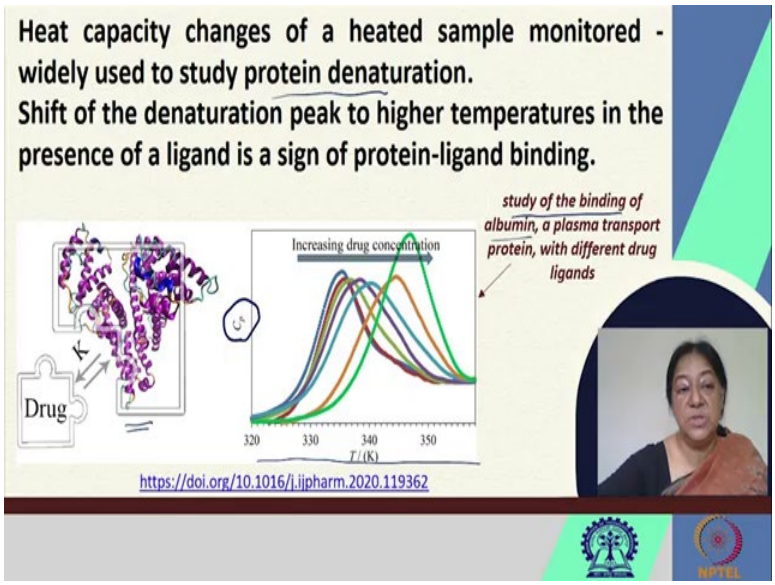


In this case also we are looking at heat capacity changes of a heated sample and this is widely used in the protein denaturation. In this case what happens, there is a shift of the denaturation peak to higher temperatures, in the presence of a ligand and this indicates that there is ligand binding.

If we have this is a structure of human serum albumin. If we have a drug with a particular dissociation attached to this, we have a typical increased drug concentration occurring here that gives us a variation in the heat capacity of our sample and from that we can determine the specific values. So, we are looking at the study of the binding of albumin with this particular ligand.

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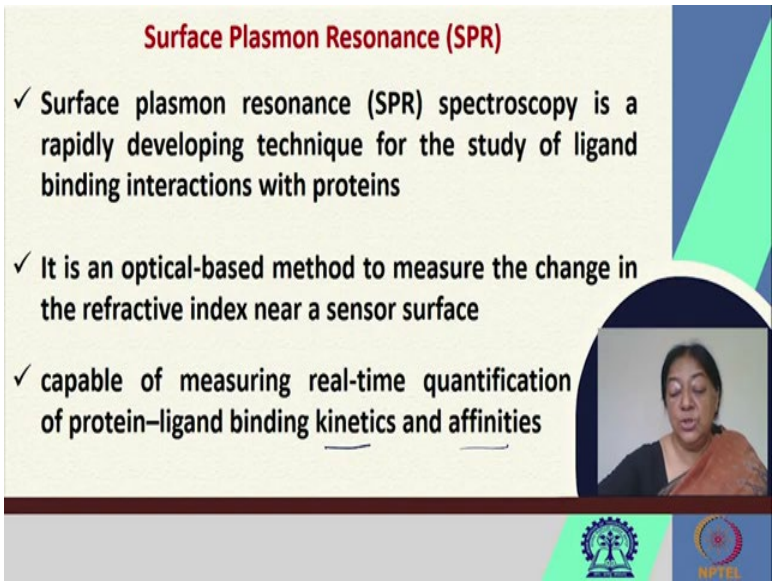


Another technique that is commonly used nowadays is surface plasmon resonance. Surface plasmon resonance spectroscopy, is a rapidly developing technique and is used for the study of ligand binding interactions with proteins which is the subject of our study.

This is an optical based method and it measures the change in the refractive index near a sensor surface. It is capable of measuring real time quantification of protein-ligand binding kinetics and affinities. So it has the advantage of giving us a time component in the understanding of the protein-ligand interactions.

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This allows a real time label free detection of biomolecular interactions. This occurs when we have the polarized light that strikes an electrically conducting surface at an interface between the two media.

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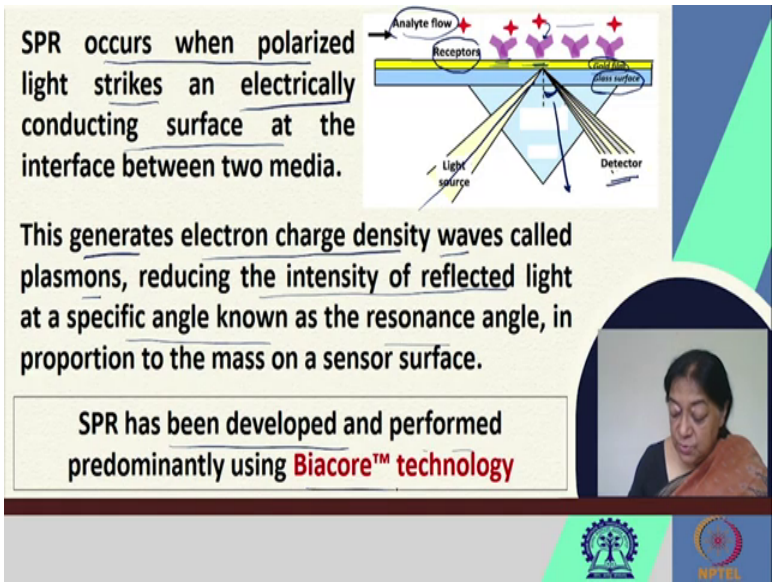


So, when we have the SPR occurring when the light strikes the media, this [refer to slide] is what is happening. We have our receptors that are attached to the gold film, that is on a glass surface of a glass slide. As our ligand or our analyte is flowed over the surface, there is an interaction between the receptor on the surface and the analyte that is flowed on the surface; if there happens to be an interaction.

In that case, this generates electron charge density waves that are called plasmons, that reduce the intensity of the reflected light at a specific angle, that is the resonance angle. So, here we see the specific angle [refer to slide], that is our resonance angle. This is our light source and if there is any change in the mass, if this ligand is bound to the receptor, then this can be detected. This has been developed and performed predominantly using this technology, Biacore technology.

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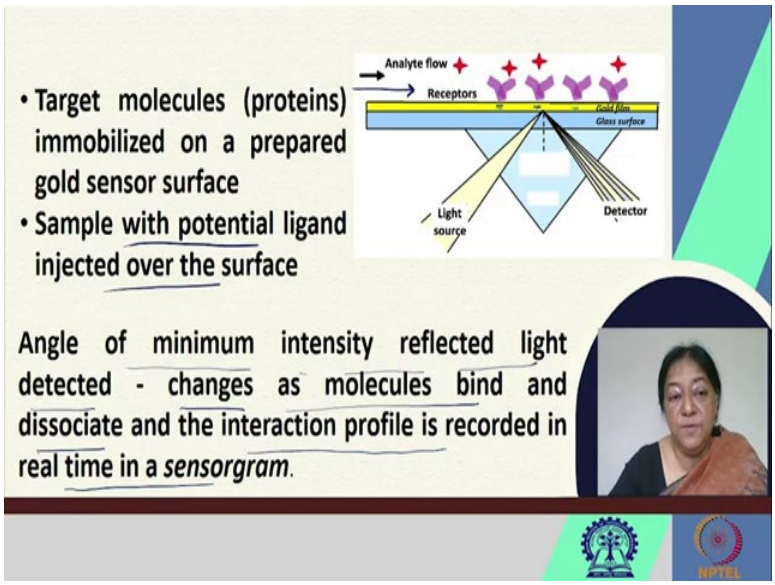


Let us see how it works. The target molecules (the proteins), are immobilized on the surface, that is the gold film on the glass slide. The sample with potential ligand is ejected over the surface, where we have the flow of the analyte. If there is an interaction, the angle of minimum intensity reflected light is detected.

This gives a change as the molecule binds and dissociates and the interaction profile is recorded in real time, in what is called a sensorgram. So, we see an association followed by a dissociation depending upon how strong the affinity of the ligand is for the receptor molecule.

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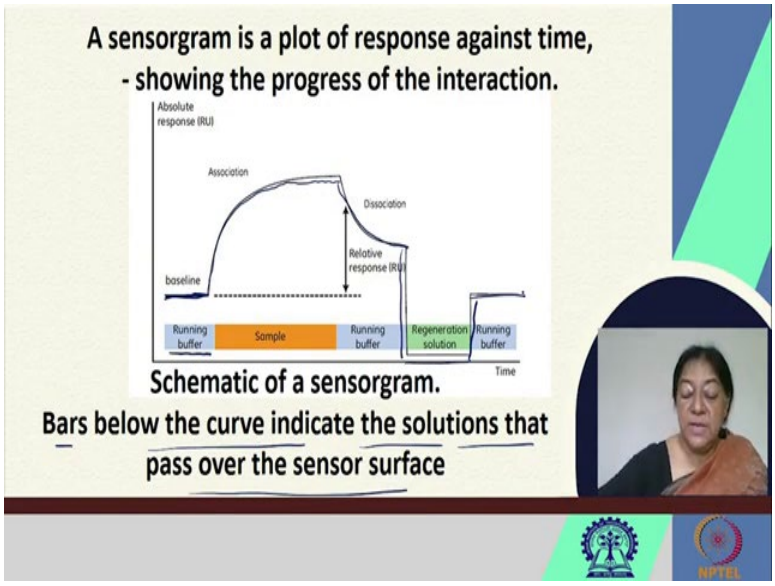


This [refer to slide] is a typical sensorgram which is a plot of the response against time, showing the progression of the interaction. So, we are looking initially at the baseline that corresponds to

the running buffer. This is a schematic of a sensorgram, the bars below the curve here indicate the solutions that pass over the sensor surface.

Initially we have a baseline because this is just the running buffer. As we have the sample injected, there is association that is given by this sensor, then there is dissociation, followed by regeneration and then goes back to the baseline, where a further injection can be done or further flow of the analyte can be done.

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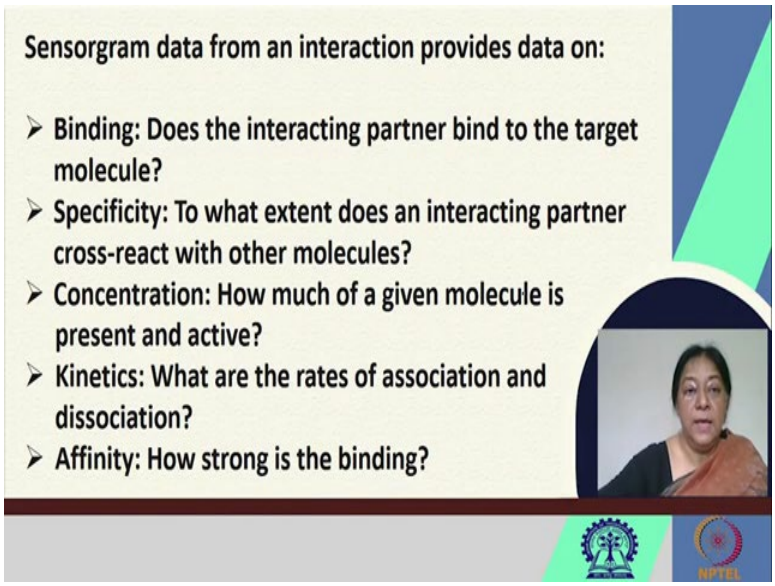


Now, when we look at the sensorgram data from an interaction, this provides data on binding, answers the question: Does the interacting partner bind to the target molecule? It tells us about the specificity – to what extent does the interacting partner cross-react with the other molecules.

It tells us about the concentration, how much of a given molecule is present and active and most importantly it tells us about the kinetics of the particular reaction going on, what are the rates of association and dissociation that can be measured from the specific sensorgram and how strong is the binding; whether we have a low affinity binding or a high affinity binding from the specific data.

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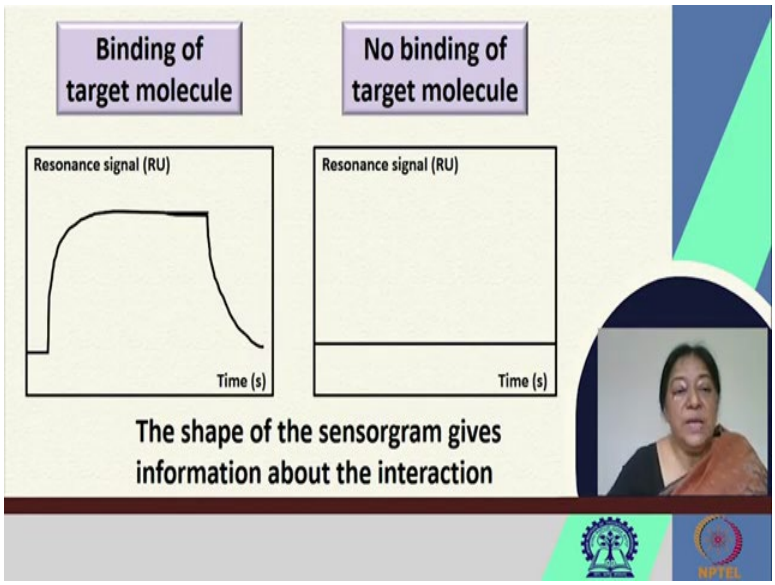


When we look at the shape of the sensorgram, this actually gives us information about the interaction. So, we are looking at the resonance signal we are looking at the binding of the target molecule. In this case we have the association, followed by the dissociation. If there is no binding to the target molecule, there is no change of the resonance signal with time as would be expected.

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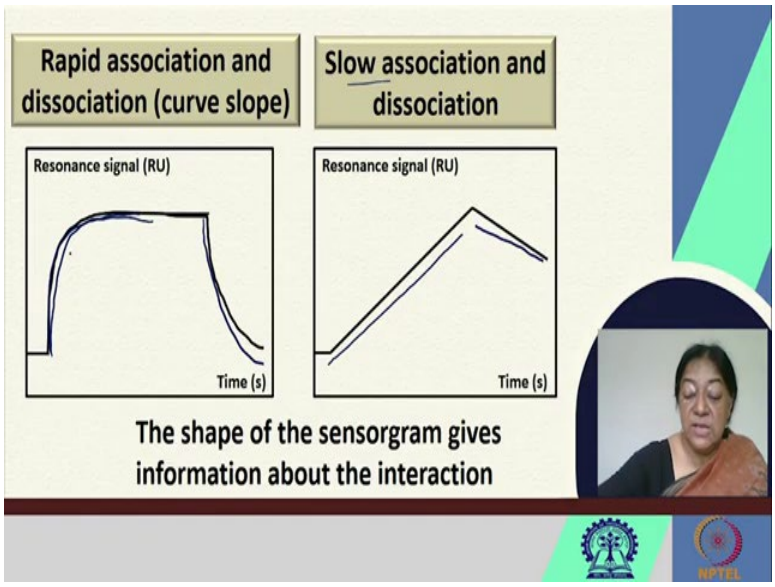


If we now look at a rapid association or a slow association and dissociation, a rapid association would mean a very fast binding, similarly a fast dissociation. We see this [refer to slide] indicative of a slow association and this indicative of a slow dissociation; meaning that we would have the affinity less in a case where there is slow association because we would expect for a rapid association, a sudden binding and a sudden increase in the resonance signal.

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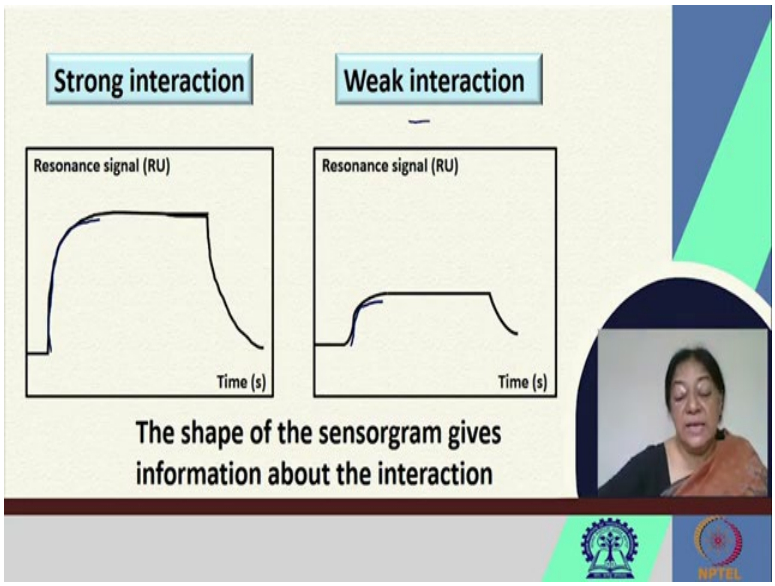


So this [refer to slide] is indicative of a strong interaction and resonance signal being lower is indicative of a weak reaction. We see that the shape of the sensorgram gives us information about the interaction, about the affinity, about the association and dissociation characteristics.

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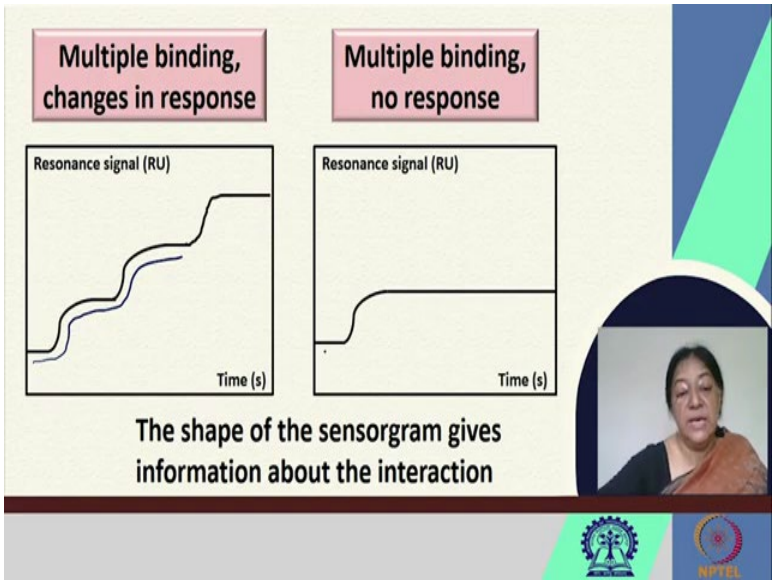


Additionally, it can also give us information about multiple binding, where we could have these changes in response and we could have multiple binding associated with different kinds of response in the sensorgram.

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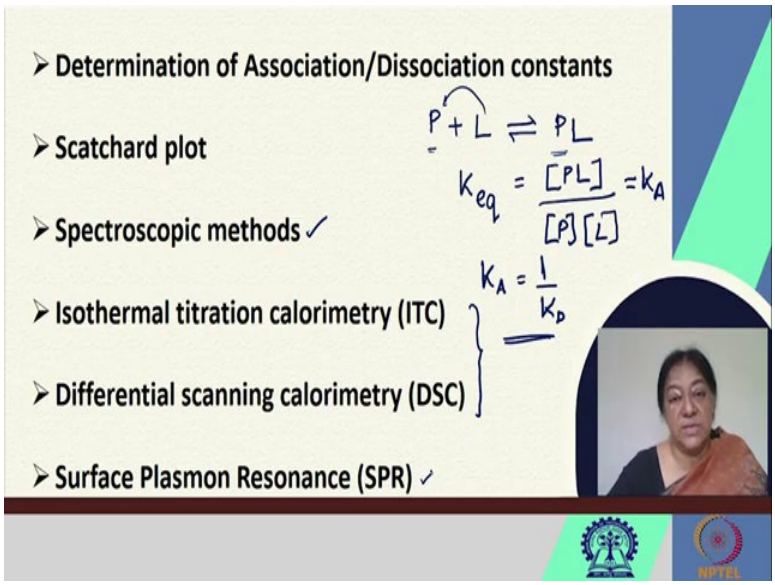


What we have looked at in the previous lecture is the determination of association and dissociation constants from a knowledge of our protein concentration, our ligand concentration and how they interact to give us our protein-ligand complex in an equilibrium situation.

We understood how we can plot the Scatchard plots, that give us an indication about the stoichiometry, give us an indication about the variations in our interactions in this case. We look at an association constant in the typical law of mass action, where we are looking at an equilibrium situation in the thermodynamics of the specific interaction.

The $K_A$ value we know is now the inverse of our $K_D$ value. We looked at specific spectroscopic methods particularly fluorescence and fluorescence depolarization, to see how the interaction of the ligand with the protein can change the characteristic spectrum of the protein, to get a knowledge of the protein-ligand concentration or the depletion of the protein concentration.

Other interactions that give us thermodynamic characteristics of our specific interaction between the protein and the ligand are given by isothermal titration calorimetry, differential scanning calorimetry. And in surface plasmon resonance, we can follow the kinetics of the association and the dissociation and we can measure the binding, the specificity of binding, the ligand affinity and we can follow this association with time.

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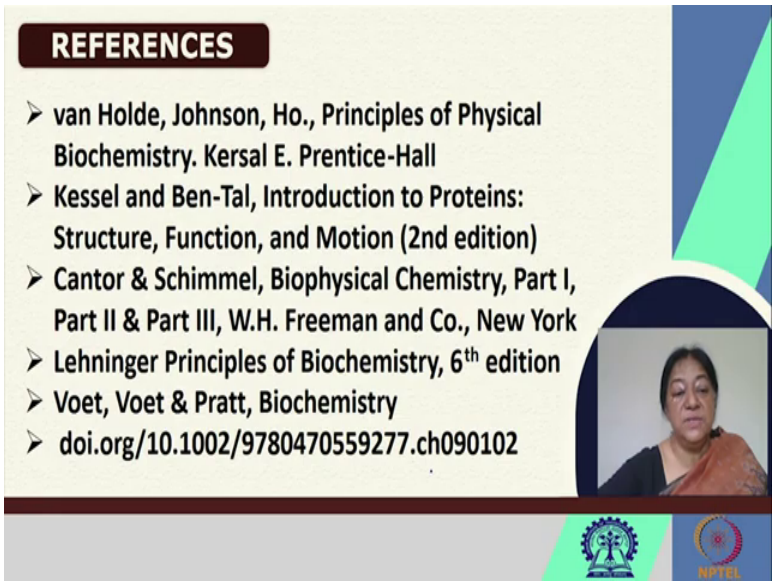


These [refer to slide] are the references that have been followed.

Thank you.