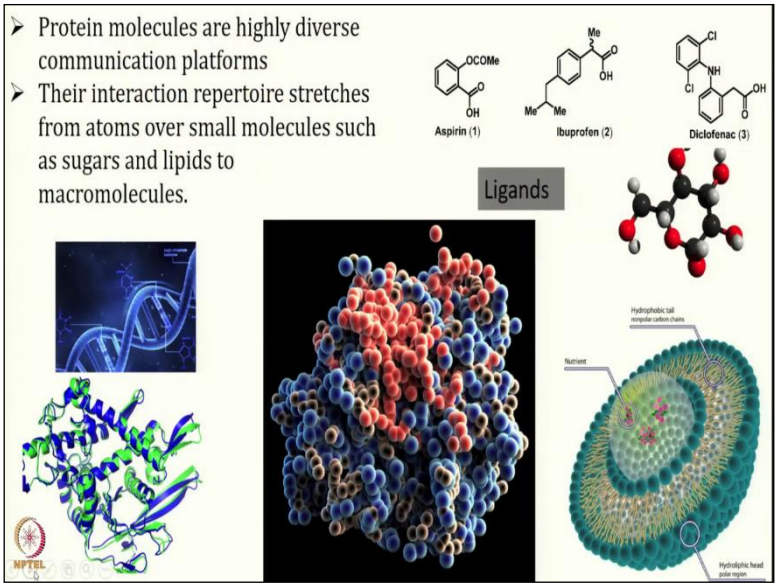
NMR spectroscopy for Structural Biology
Prof. Ashutosh Kumar and Prof. Ramkrishna Hosur
Department of Chemistry
Indian Institute of Technology - Bombay

Lecture: 41
Protein – Ligand and Protein – Protein Interaction

Welcome to today's class. So, in the previous lectures, we have seen how protein structure can be determined. Now here onwards, we are going to look at some of the application of those structures that we determined how we can understand the protein communication, the interaction, the drug interaction, drug-design and all those. So, here onwards I will be taking you through all these important concepts of protein-ligand, protein-protein interactions, and followed by how we can use these to design a drug molecule.

(Refer Slide Time: 00:55)



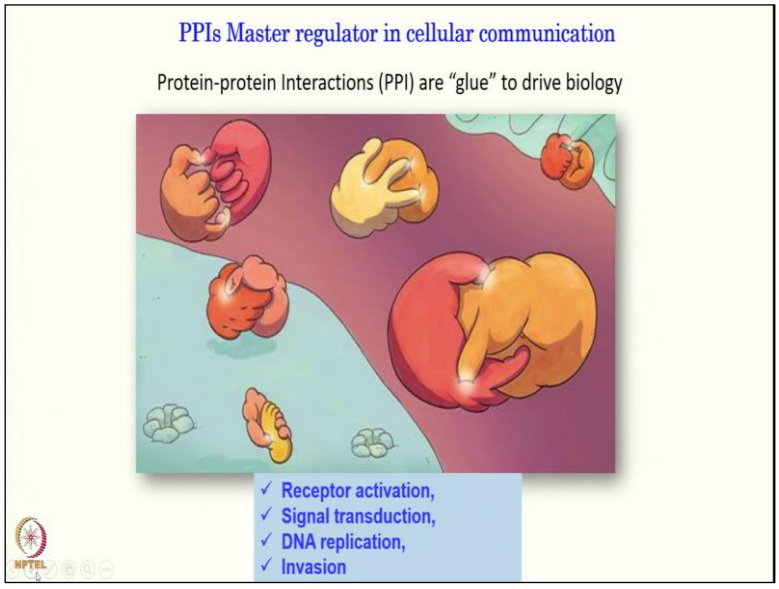
So, protein essentially are highly diverse molecule and they offer a communication pattern; actually they communicate with various molecules. It can be a small molecule, it can be big molecule, it can be even electron, protons, phonons, all sorts of light. It can interact with physically understandable molecules, like small molecules, like drugs. Protein interacts with a drug, many of the drugs you commonly use like aspirin, ibuprofen; some or other protein interacts with these molecules.

It can even interacts with lipid, all sorts of membrane protein are in lipidic environment and interacts with a lipid and regulates many of the important functions. It interacts with DNA; like

one of the DNA packaging machinery, nucleosome, is a nucleic acid-protein interactions or it can interact with other molecules. So, their interaction actually repertoire stretch from an atom to over small molecules such as, it can interact with the sugar, lipids, or macromolecule.

So, this dynamic personality of proteins through which it interacts, actually offers a very elegant tool to understand many of the biological functions where protein is involved.

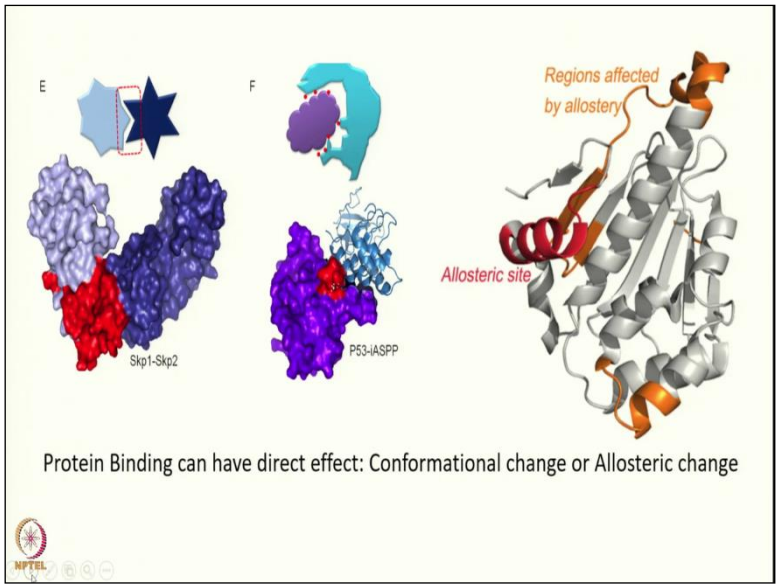
(Refer Slide Time: 02:29)



So, what all essentially it does. So, actually protein-protein interactions is a master regulator in cellular communication. All the cellular communication happens through protein signalling or protein-protein interactions. So, they act as a glue to drive important phenomena in biology. For an example, in receptor activation there is some protein-protein interaction signal transduction, like a DNA replication some protein comes and interacts with a nucleic acid to start the DNA replication.

Even invasion, it is a viral invasion, bacterial invasion, some pathogenesis is driven by protein-protein interaction. Therefore it becomes paramount of importance if you want to understand how cellular communication happens; we need to understand the dynamics, thermodynamics, structural aspects of protein-protein and protein-ligand interaction and that is what we are going to do mostly this week.

(Refer Slide Time: 03:35)

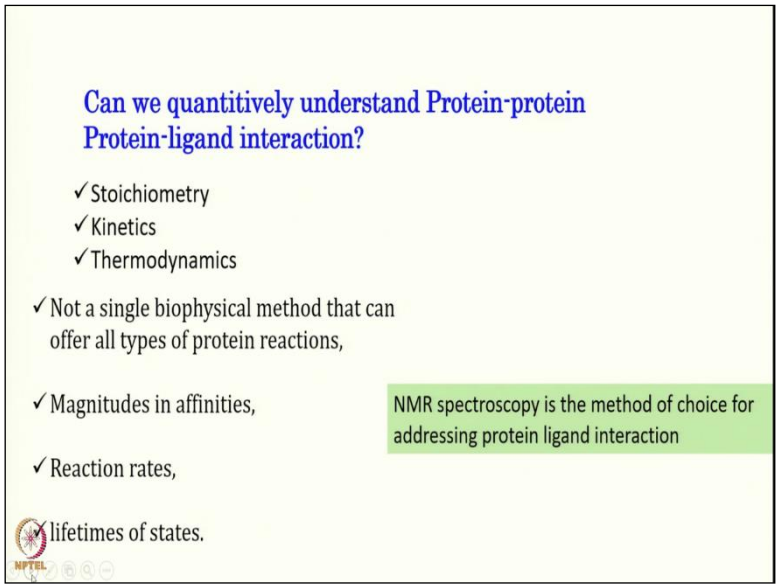


So, protein-protein interactions come in various shapes and size the two proteins can find a shape complementarity where they can interact or even the disorder protein can bind to a protein and takes order. So, binding effect binding can have a direct effect. When two protein interact, they can directly interact and they can bind with each other and do its function like a signal transduction or it can have allosteric effect.

So, direct effect – how they do? Basically, upon binding some type of conformational change happens and that conformational change lead to signal transduction, so that is a direct effect. It can have an allosteric effect; allosteric effect in a simple term it binds to allosteric site and the effect of this binding is seen somewhere else. So, the region which is regulated by allostery, binding happening somewhere else the effect is transduced somewhere else. So, that is the allosteric effect.

So, protein-protein interaction or protein-ligand interaction in general can have a direct effect, binds somewhere, changes the conformation and this conformational change actually initiates the cascading effect and that is how the signal gets transferred or it can have a allosteric effect and that is how the signal goes from one place to other place.

(Refer Slide Time: 05:10)



So, if this is so important protein-protein interaction, protein-ligand interaction, because it tells about the cellular communication, cellular regulation; can we understand this quantitatively? Quantitatively I means, like if two proteins or one protein one ligand is interacting, what is the stoichiometry? In which ratio they are interacting. So, what is the stoichiometry, what is the kinetics of their interactions, what is the forces involved, energetics or thermodynamics involved in their interactions.

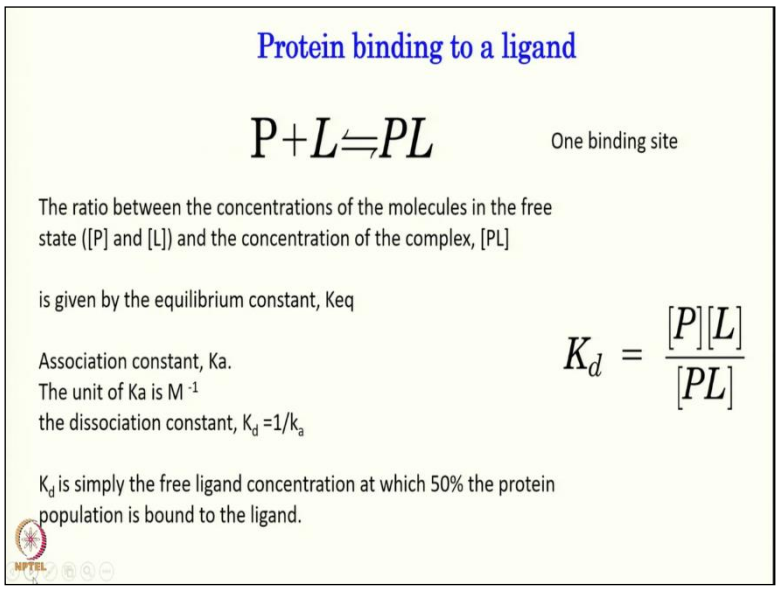
So, these are the some of the critical parameter that we need to understand the stoichiometry, that means ratio of their interaction, the kinetics the rate with which they are interacting k_{on} rate, the rate with which they bind, k_{off} rate, the rate with which they goes. So, k_{on}-k_{off} rate we can understand. What is the thermodynamic parameter like what is the enthalpy involved, what is the free energy,

what is the entropy? Can we understand all those thermodynamic parameter quantitatively – stoichiometry, kinetics and thermodynamics? So, for doing that there are various biophysical methods are there, which actually offers some of these, where we can understand the protein-protein interaction, protein reaction quantitatively. We can understand the magnitude in affinity like with the strength with which they are interacting.

They can even monitor the kinetics of interaction the rate with which they are interacting or like what is the lifetime of the complex formed, like how long they stay in this complex form that also they offers to understand. However, we look at some of these biophysical method that

are used; but NMR spectroscopy is a method of choice that basically address the protein-ligand interaction in a very elegant way that also we are going to look at.

(Refer Slide Time: 07:27)



So, let us start with some of the basic concepts of protein-ligand interaction. Suppose a protein has one binding site where ligand is binding. So, we can write a simple reaction like protein binding to ligand and forming a complex and this PL is in equilibrium with the free protein free ligand right. So, assumption is protein has only one binding site. So, we can write this equation.

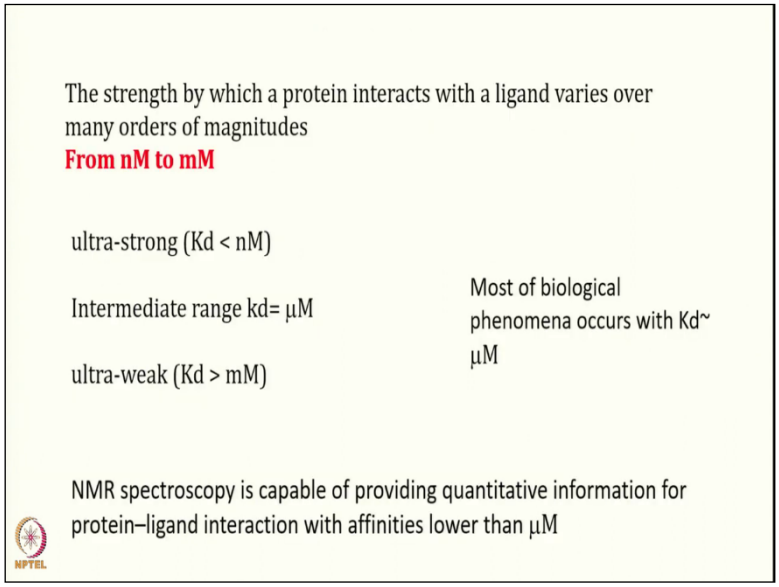
$$P + L \rightleftharpoons PL$$

So, the ratio between the concentration of the molecule in the free form, free state of protein and free of a state of ligand and the concentration of the complex PL, if we get this concentration we can get the equilibrium constant. So, we can write it K_{eq}, how much protein is in the free form, how much protein is in the complex form, and that is how we can get the decay equilibrium right on this reaction, so basic chemistry.

So, then we can get a rate, the rate with which they are associating, k-association and rate is more inverse, then we can have k-dissociation, the rate with which they are essentially dissociating, so like they are going back to the protein-ligand form. So, we can know the rate of k_{on} or k_{off}. k_{on} means k-association, k-dissociation and we can determine the dissociation constant of a protein which can be seen like this. So, K_d is a simply the free ligand concentration at which 50% of protein population is bound to the ligand.

So, that is a K_d. So, like you have heard about various K_d will be explaining those K_d little more in detail, but actually it is a term that determines the ligand concentration at which 50% protein is bound in the ligand form. So, looking at this simple reaction, we can understand something about rate of association, rate of dissociation, and the dissociation constant.

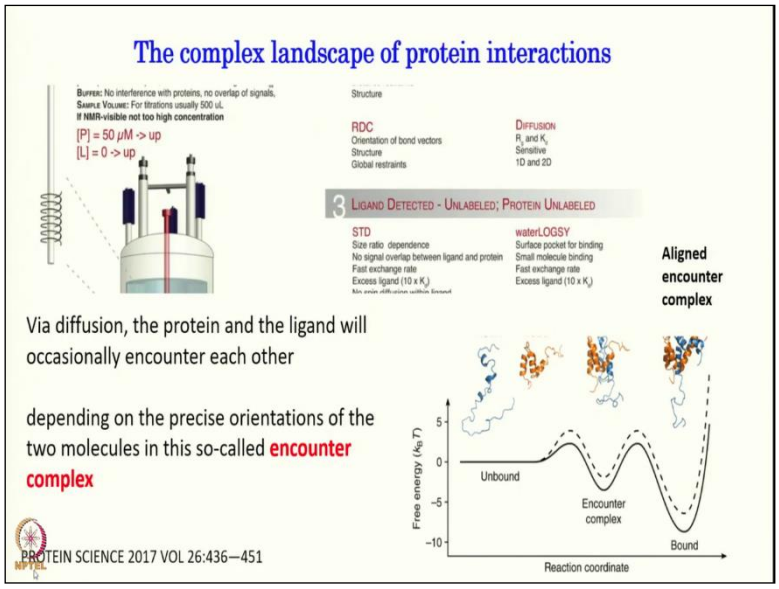
(Refer Slide Time: 09:44)



Now these strength of the binding can vary depending upon what sorts of interaction is happening and they can vary like order of magnitudes. It can be nanomolar to millimolar, nanomolar K_d means very strong binding, ultra-strong binding is nanomolar. We can have a ultra-weak binding which is like a more than millimolar, and we can have an intermediate which comes somewhere in the micromolar range.

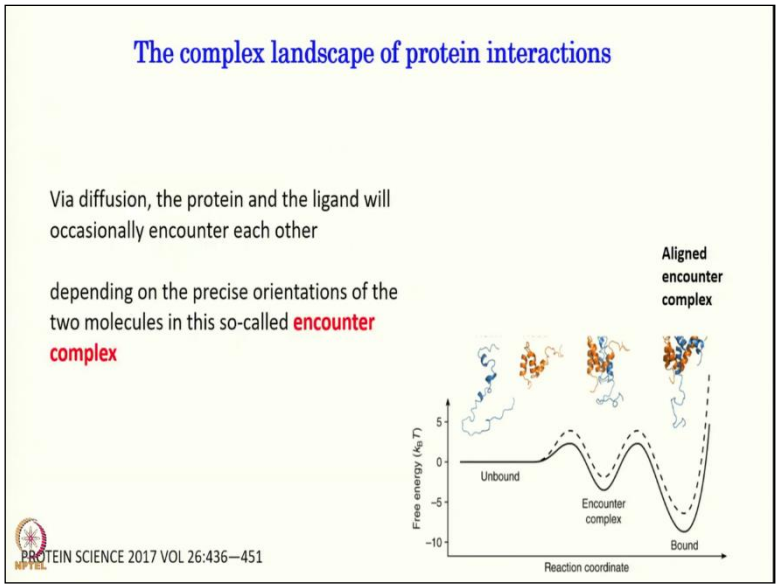
So, we can have all sorts of K_d, most of the biological phenomena occurs where the K_d is in micromolar range, like they are transiently binding with a micromolar strength and they goes off. So, these essentially form lots of cellular communication. So, basically NMR spectroscopy is capable of providing quantitative information of protein-ligand interactions, affinity which is even lower than the micromolar range. Actually NMR can report about the K_d ranging from nanomolar to millimolar range

(Refer Slide Time: 10:58)



But before we go to the details of that, let us go little bit more detail that what actually the complex formation landscape for protein-protein interaction is needed. So, to start with, we can determine the protein structure using various NOE-based experiment or RDC-based experiment; we can even get the parameters like the diffusion of the molecules, like hydrodynamic radii, and the K_d and these are sensitive one 1D and 2D.

(Refer Slide Time: 11:35)



So, let us look at complex landscape of protein interactions. So, when two molecules are in solution, they diffuse all the time, like they are tumbling, they are diffusing, they are doing translational diffusion, even rotational diffusion, they come and encounter with each other. So, say protein, a big protein molecule we have here and a small ligands they are diffusing and then they are coming and colliding right.

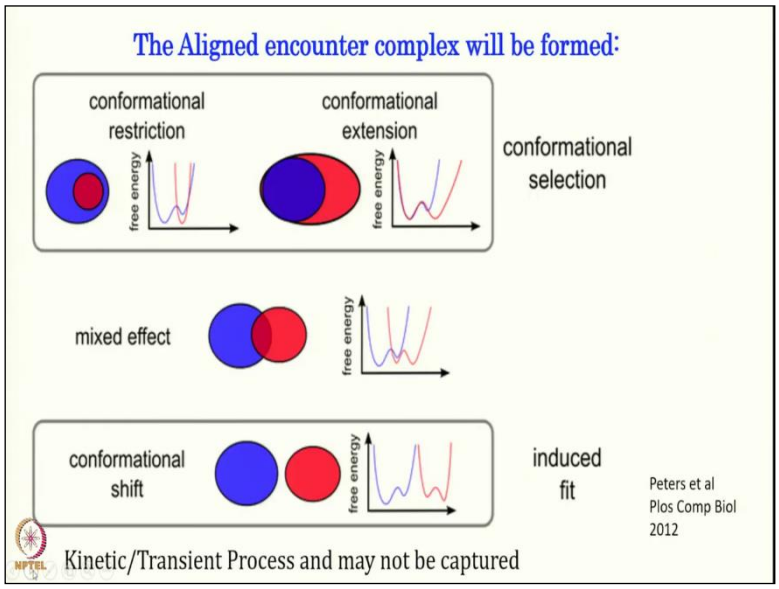
So, they occasionally encounter each other and depending upon how precise the orientation is, so here is suppose binding site and here is ligand. So, through searching the appropriate binding site, sometimes it happens that they find the precise orientation and then two molecules from something called encounter complex. So, here is say binding site and here is my ligand.

So, it searches all the possible sites and finally when it finds the right site, it comes and binds, that is a correct binding happening. So, when they form a precise orientation, that will be called encounter complex, and encounter complex is needed for the proper interactions. So, the energy landscape we can say, the two proteins, here quite disorder protein and here little order protein, they are in unbound form, they have one energy landscape which is like a zero.

And when they form an encounter complex, when they are coming closer, but not so in precise orientation, they form an encounter complex, you see the energy is dropping down. So, that means they are becoming more stable and then they finally adjust to bind to the appropriate binding site that will be called aligned encounter complex. So, you can see energy further drops down and now that is forms the stable protein-ligand or protein-protein complex.

So, first thing we learn they have to come closer by diffusion, then they have to find a proper orientation, and finally when they fit each other, that is called aligned encounter complex, the energy is down in the bound form, this is the stable state that we have formed.

(Refer Slide Time: 14:00)



Now when this encounter complex forms, it can have various way of searching its right conformation. So, two or three already we have learned in the previous cases, it is called

induced fit or conformational selection; these are the two known concepts in the in the protein-ligand or protein-protein interaction case. So, what happens that confirmation selection says, that there are various multiple conformation at the protein energy landscape available, ligand starts searching the right kind of conformation.

And so, it searches between all equally probable energy states and one of the energy state which fits better it binds and lowers down the energy, that is called conformational selection. In induced fit what happens, that the confirmation of either ligand or protein changes and basically it finds the right way to energy stabilize. So, it can change its conformation from here to here and that is how energy state is stabilized.

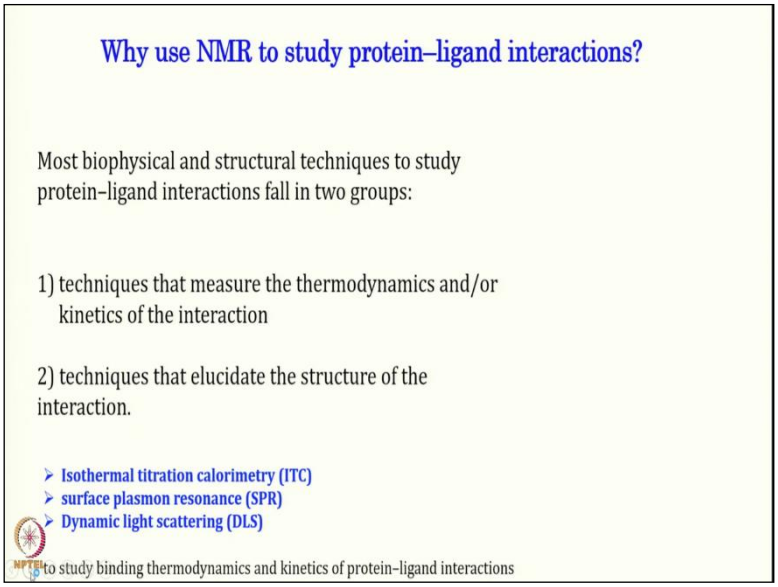
Now recently some of the more states have been has been discussed, which is called conformational restriction. So like in the previous slide we saw, that the two proteins in which there was one protein which was quite disorder, and now it binds and takes some more order; this kind of the conformational selection we can say conformational restriction is happening or it can even happen to accommodate a protein or a ligand, the other receptor proteins extended its conformation, that we will call is a complex conformational extension.

So, you can see here suppose red is a ligand and blue one is a protein. Now conformation getting restricted, here you can see confirmation of the red is getting extended. So, this is a conformational extension. Conformation means like a more equal probable energy states are coming and that is a conformational extension or it can have a mixed of some conformation restriction some conformation extension or it can even shift the conformation that is called induced fit. So, with one of these two methods basically protein interacts with each other.

So, if you look at carefully what is happening here; two things are happening shift in the energetics of protein-protein or protein-ligand interaction. So, that means the thermodynamics is involved here. Second thing what is happening here, it is a structural change happening because protein is changing its conformation either restricting or extending or like a induced fit also again conformation shift is happening.

So, structure plus thermodynamics both things are changing. So, if we want to understand protein-protein protein-ligand interaction, essentially we need to get hold of all those changes that is happening.

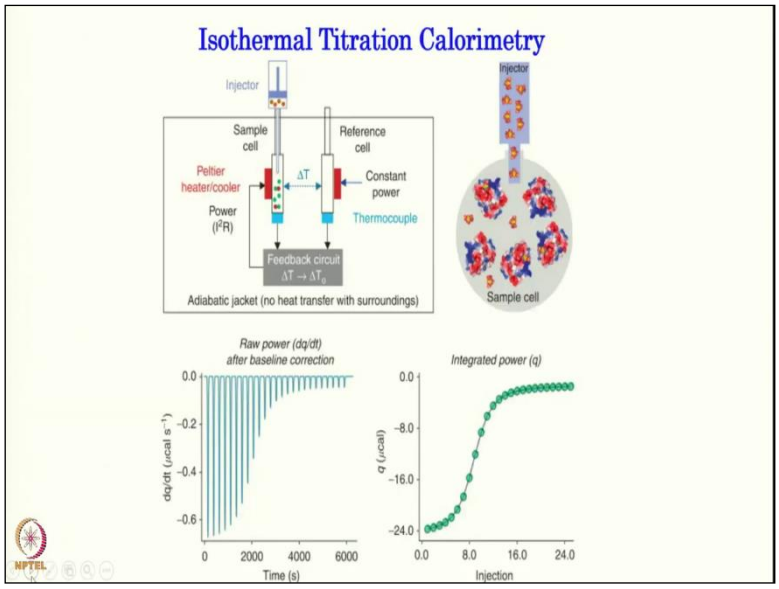
(Refer Slide Time: 17:16)



Now most of the biophysical or structural technique that are there to understand protein-ligand interaction, we can classify them that they fall in two groups: either they measure the thermodynamic or kinetics of interactions. Some of them we can classify as isothermal titration calorimetry which measures the essentially thermodynamics.

The surface plasma resonance again that measures the k_{on} and k_{off} rate, some measures thermodynamics, dynamic light scattering essentially it measures the how shape and size of the molecule changes, briefly I am going to discuss all these. And then there are techniques which elucidate structure that may happen upon interactions. So, those again we are briefly going to study, the thermodynamic and structural techniques and then we will come why NMR can be used to study protein-protein interaction.

(Refer Slide Time: 18:24)



So, essentially let us focus on the thermodynamic parameters that are there. One of the prominent one is isothermal titration calorimetry. What happens here it is isothermal means same therm temperature, titration we are titrating it, and calorimetry because it measures the heat. So, same like we are maintaining the same T like therm, isotherm we are titrating two things and measuring the heat that is why it is called isothermal titration calorimetry.

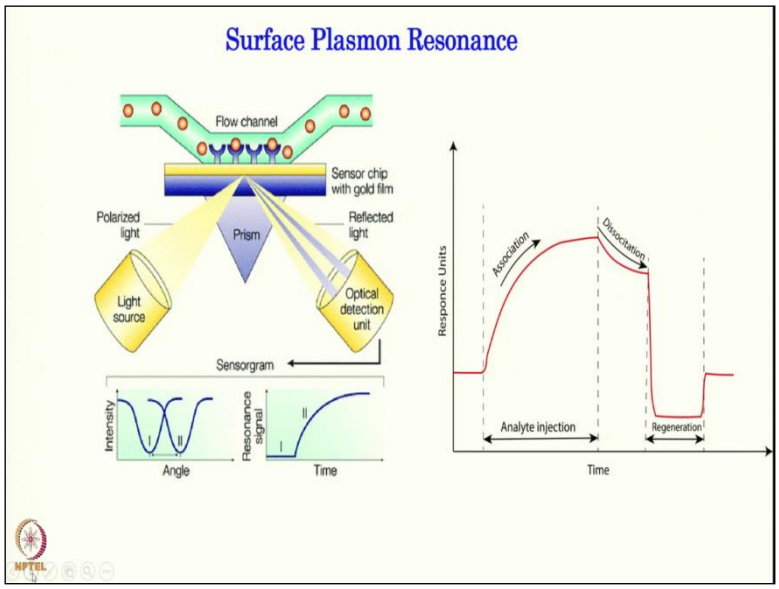
So, what essentially we are doing we have two cell, one is called sample cell another is called reference, they are maintained at isothorn which has some thermocouple and they are in adiabatic jet jacket. So, that no heat transfer happens with surrounding. Now here is a reference cell that has a feedback loop to maintain the temperature and here is my sample and here is my ligand, sample-ligand we are titrating.

So, we are injecting each time here. So, you can see injector and your sample cell. Now this is my protein shown in red and blue and orange and yellow is my ligand. So, we are each time we are adding some ligand; upon addition of ligand heat change happens, they then again it brought back because we have a reference cell and sample cell. So, brought back to the isotherm, again we add and then heat change happens.

So, because of this heat change happening, we are measuring here *dq/dt*, change in the heat with time. So, that is what we are measuring, change in heat with time, microcal-per-second, each addition of ligand there is a heat change. Now what happens, that after sometime, it gets saturated and you see there is no further heat change. Now what we will do, will fit this to equation which is here.

And from fitting of this equation, essentially we get various thermodynamic parameter ΔH, the change in enthalpy, change in entropy, ΔG and change in stoichiometry or n. So, how many molecules binds to one protein molecule, that is Δn, what is the free energy change? What is changing entropy and what is changing enthalpy? So, this isothermal titration calorimetry, is a wonderful technique to understand the thermodynamics of the protein-ligand protein-protein interaction.

(Refer Slide Time: 21:00)



The other one, which is used commonly, is called surface plasmon resonance it also measures the thermodynamics. So, it is like, here you immobilized your receptor on the sensor-chip and here is a prism, you have a light source, optical detection unit, and whatever. Upon binding, it forms plasmon, then reflective index is changed, and that essentially gives you the rate of association.

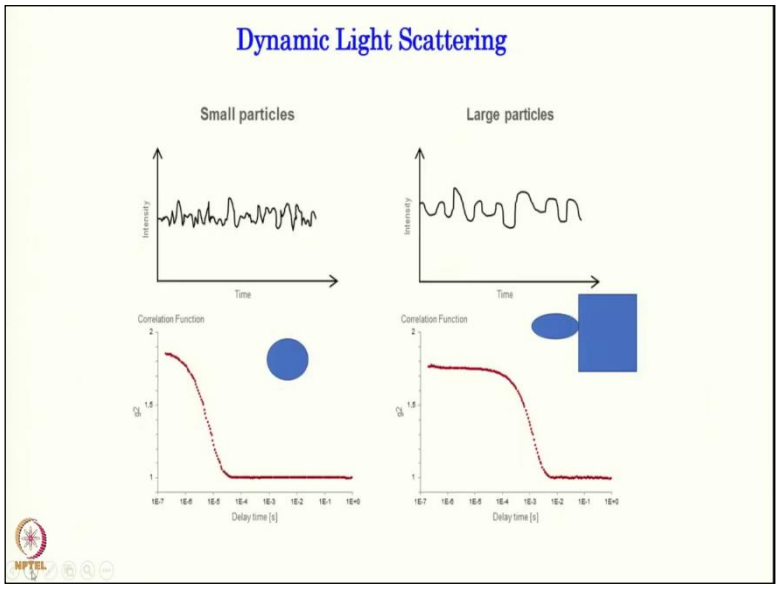
So, that refractive index change is measured in terms of response unit and like here you can see the angle changes upon binding when you flow some ligand. So, here is a receptor, here is my ligand coming, and upon binding some response unit change that you plot it. So, what happens, when it starts binding, it shows a curve which is called association curve.

So, k_{on} – association curve and then finally you wash with buffer, then it dissociates and then you regenerate your chip so that the next set of experiment can be done. So, here is analyte injection you do, it associates then it saturates, you can see here saturation happening, and then

you dissociate it. So, with time we can measure the k_{on} rate, k_{off} rate and that again gives you the K_d, precisely surface plasma resonance give you k_{on} rate,

what is the rate with which ligand bind to a protein, what is the rate with its get dissociate, and then finally you can calculate the K_d. So, very important it measures the k_{on} rate and k_{off} rate.

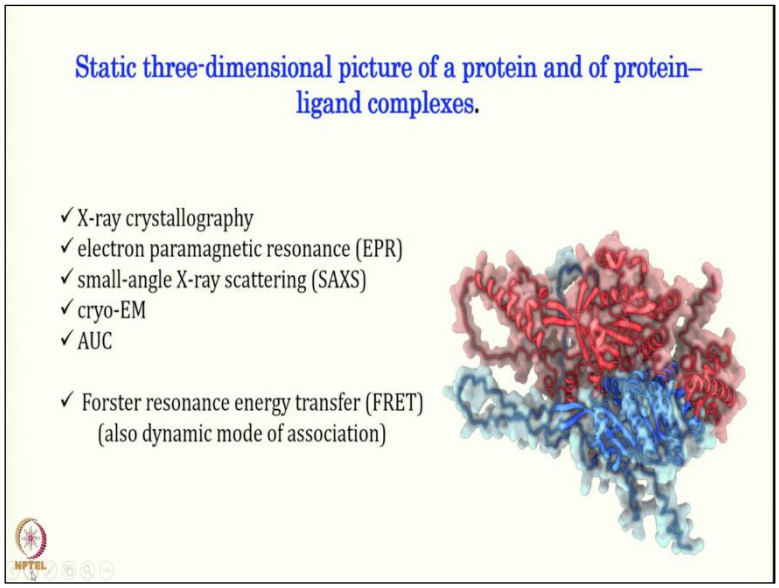
(Refer Slide Time: 22:45)



The third one that we talked is essentially dynamic light scattering, that also gives some idea of protein-ligand protein-protein interactions. So, essentially this measures how the molecules fluctuate in the solution. So, if you have a small particle that is fluctuating, you can plot a correlation function, and small particle tumbles very fast, so you can have a here correlation function, that shows the decay is happening fast.

The large molecule slowly tumbling, you can see the intensity of this large molecule is quite broader. So, here you can see when it form a complex, you have a different kind of correlation function, and you can plot it to get how the shape upon interactions have changed, how the molecule has become bigger. So, these are the three techniques; essentially this does not give the thermodynamics, it gives idea about the change that happening and this is again low resolution technique.

(Refer Slide Time: 23:54)



So, now coming back to some of the high-resolution techniques that gives you a complex structure, the static three-dimensional picture of protein-protein or protein-ligand complexes. One of them leading technique that is used is essentially X-ray crystallography. So, what happens that you take a protein and ligand or two proteins, you crystallize them, diffract it, and using the electron density map you can get the three dimensional structure of protein-protein interaction.

Then another one not so precise but now it is quite used because it is a sensitivity is called EPR. Again, you have you have to have a paramagnetic tag, and then one can get the protein complex interactions, how the paramagnetic tag interacts with the other partner, one can get the structure of a protein. The third one is small angle X-ray scattering. So, here essentially, it is kind of a x-ray scattering, but does not give high-resolution, atomic resolution structure, gives overall shape and size of a molecule.

So, here the complex which forms bigger will give a different kind of a scattering pattern. So, you know that now complex form has happened. The recent phenomena of cryo-EM, that is excellent tool for understanding the bigger complexes is coming more and more profoundly which understand the three-dimensional structure of a protein-ligand or protein complexes.

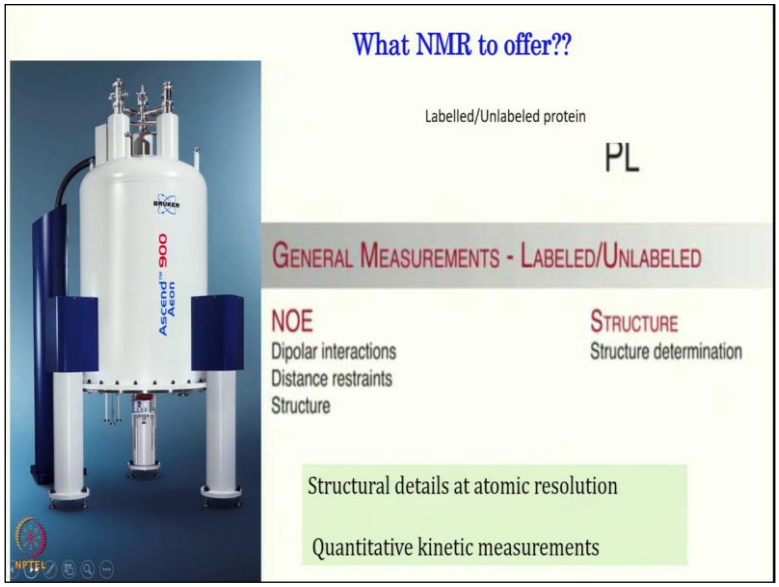
Another very important technique called analytical ultracentrifugation, essentially that also reports upon complex formation, how the molecule sediment. So, you can measure the sedimentation coefficient and that also says that the complex formation happens. So, essentially

the sedimentation coefficient is lower when it is in the free state and when it is in bound state it becomes higher.

So, essentially you get this shape and size of a molecule that can be complementary to dynamic light scattering. So, AUC, cryo-EM, SAXS, EPR and X-ray crystallography gives you a static three-dimensional structure picture. Another important techniques, where you can even get the dynamic mode of association is called Froster Resonance Energy Transfer a fluorescence based technique.

Where you have a two fluorophore attached on two molecules, can be ligand and protein and how they come closer and get associated you can measure the distance between these two fluorophore using this fluorescence technique called FRET and then measure the dynamic mode of association, even the mode of complex formation. So, with these all techniques thermodynamic and the structural mode of interactions can be studied.

(Refer Slide Time: 26:41)



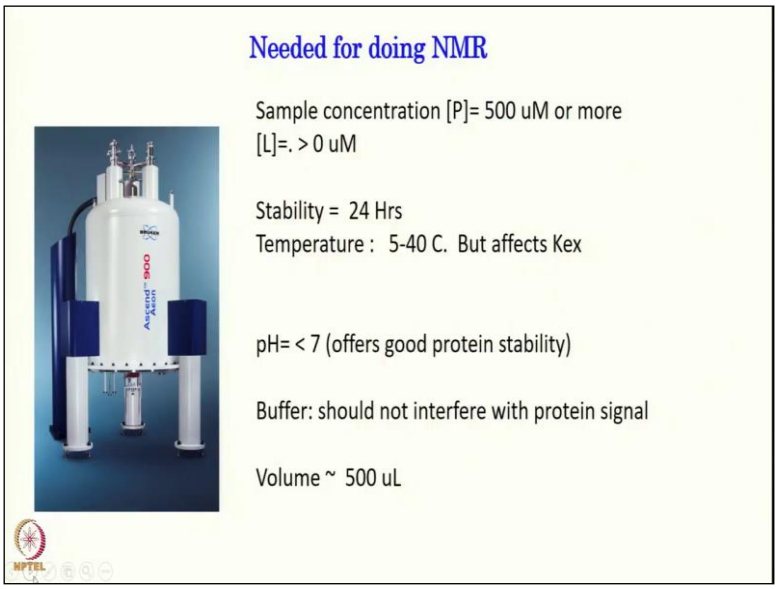
Now the question remains then what NMR can offer? Can NMR offer simultaneously both of these aspects structural as well as the thermodynamic and kinetic. So, we know that one can understand the structure of protein, already we have seen that can be determined using NOE based restraints, the dipolar interaction, the distance restraint helps us to get the structure of the molecule and we can determine the structures.

Once the structure is there, the static or complex structure, we can get these structural details for that probably you need a labelled protein and you can have an unlabeled ligand. So, using

these we can determine this structure, what you need a spectrometer and most of the time protein labelling is needed. We can determine the structural details at atomic resolution and we can measure the kinetics of binding thermodynamics of binding.

So, here onwards we are going to delve deeper into how we can measure the thermodynamics and kinetics simultaneously along with the structure of a protein-ligand or protein-protein interactions.

(Refer Slide Time: 28:07)



So, what you need for doing NMR? Essentially the protein concentration which should be like a 500 micromolar or more you need a ligand depending upon binding constant, you need to have a more than zero micromolar. The stability for this interaction, protein should be stable for 24 hours or so. Temperature is also important, many proteins are stable in this range.

So, temperature is also important, what temperature we choose. But one caution is there, the exchange rate depends upon the temperature. So, if you go at higher temperature, the on-off rate can be different. So, you need to choose an appropriate temperature, we want to understand the complex formation. The pH is very important, pH should be less than 7 and it should offer the good protein stability.

Preferably less than 7, because amide proton does not exchange, and you can detect all the amide protons. So, pH for doing NMR experiment which should be less than 7; which buffer, a buffer which does not interfere with the protein signal. Many of the buffer like a MES buffer and all those has lots of proton in itself that overcrowds the protein spectrum. So, we should

avoid those kind of buffers, phosphate buffer is the best buffer to choose, but actually it depends upon where your protein is happy and stable.

So, buffer has to be selectively chosen and the typical volume for doing this experiment should be 500 microlitter. So, if we have all these, we are ready to go for protein-ligand interaction and that is where we are going to start in the next class, how we are going to now use NMR spectroscopy to study the protein-ligand interaction and getting the thermodynamic, kinetic parameter from it.

Structural part we have already seen but wherever needed I will come back and explain that. Thank you very much and looking forward and to see you in the next class, thank you.