Experimental Biochemistry
Prof. Soumya De
School of Bioscience
Indian Institute of Technology, Kharagpur

Lecture – 48
Protein Ligand Interaction (Contd.)

Hello welcome back. So, this is the second part of the lecture on Protein Ligand Interaction. In the last lecture, I talked about the thermodynamics of protein ligand interaction, and I talked about a few experimental techniques by which we can determine the dissociation constant. In today's lecture, I will focus on kinetics of this protein ligand interaction, and I will again go through the basics basic setup of this type of experiment. And then I will discuss two techniques by which we can measure the kinetics of a protein ligand interaction.

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So, I will talk about the basic experimental setup just like I did in the last lecture. So, this setup will talk about we will discuss how you setup this experiment, and what how do you measure. And even though this is the basic setup, we will see that according to the system we will have to modify our experiment and also choose the experiment. So, as I said I will talk about two kinetics experiment and I will also discuss the practical aspects of measuring kinetics.

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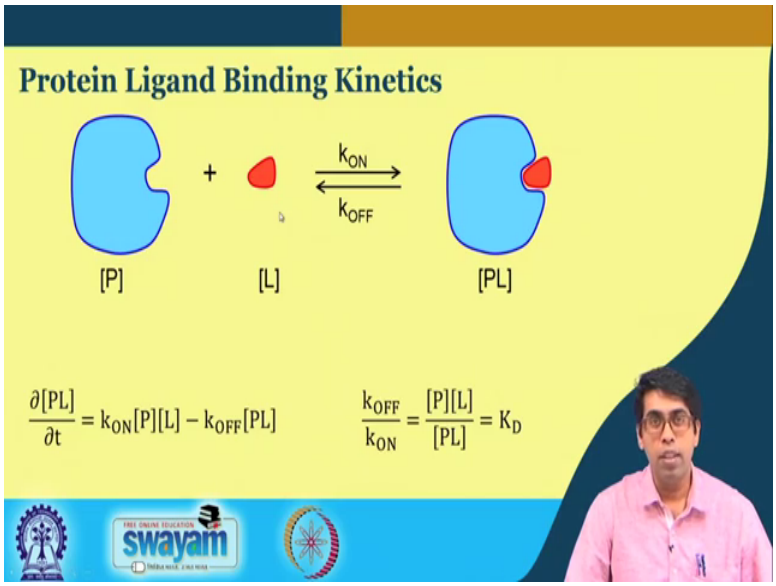

So, here is an overview of protein ligand binding kinetics. So, again this is a cartoon of our protein and this is the ligand. Now, here the ligand is shown as the small molecule, but it can be a large molecule another protein or it can be a macromolecule like DNA or RNA. So, in these two bind, you get the protein ligand complex. So, this is the simplest type of protein ligand interaction that you can see.

The rate of association is given by k ON, and then the rate of dissociation the rate at which this complex dissociates into the free protein and the free ligand is given by k OFF. So, the rate of formation of the protein ligand complex, so this is PL the rate of formation of this PL is given by this equation, where the formation rate is given by k ON multiplied by the concentration of the free protein and the concentration of the free ligand. And you have to subtract the reverse reaction because the moment some of this complex is formed, it will also dissociate into the two components. So, minus k OFF multiplied by the concentration of the protein ligand complex.

We already saw that in the last lecture, so under equilibrium what happens is this rate of change of protein ligand concentration is 0. So, there is no change in concentration. It means that the rate at which it is formed is the same at which it is dissociated. So, the left hand side becomes 0, and what we get is the ratio of the k OFF to k ON equals to the thermodynamic constant K D, which is the dissociation constant.

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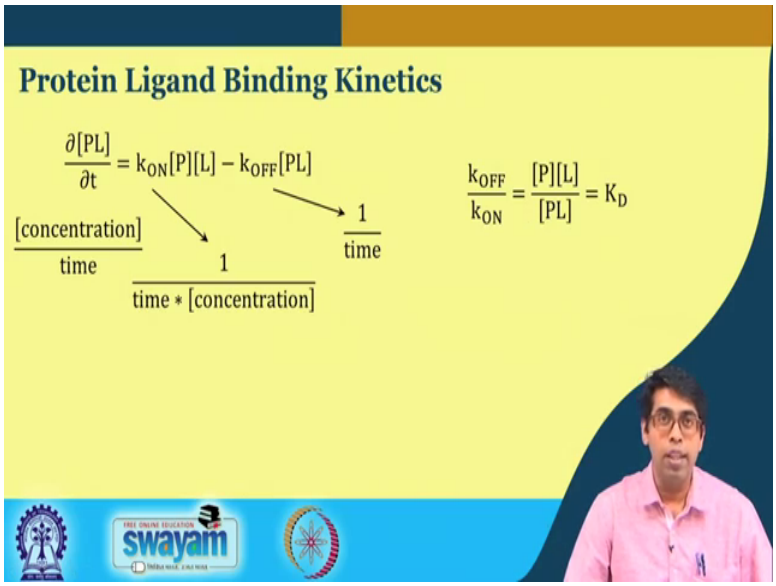

So, before we get into the actual experiments, it is important to just discuss slightly the units of each of these parameters that we are going to look at. The on the left hand side, we have the rate of change of concentration of the protein ligand complex. So, on top it is concentration. So, this will be in the units of concentration, it will be micro molar or milli molar or molar, and the bottom is time. So, it can be in seconds or it can be in minutes. So, the unit for this is concentration over time. For k ON, if you see so each of these should have exactly same as this dimensions. So, these are the two concentrations. So, on the left hand side, you have concentration over time, and then there are two concentrations getting multiplied. So, if you divide concentration over time by concentration square, you get the unit for k ON which is 1 by time multiplied by concentration.

So, the unit for k ON will be per molar per second or per micro molar per second or per micro molar per minute something like that. If we see this term this is k OFF multiplied by just concentration. So, here it is concentration, concentration, they cancel out. So, k OFF is will be only 1 by time. So, the unit of k OFF will be in per second or per minute or per milli second, but some unit of time.

So, you can do this yourself if you know since you know the unit of k ON and k OFF, and you also know the unit of K D which should be concentration. You can check whether if you when you take the ratio of these two, when you take the ratio of these two

units, you get back concentration which is the unit of K D. So, using dimensional analysis, you can actually confirm that the equation that you have written is correct. And this thing will actually help you in setuping the experiment, we will see that.

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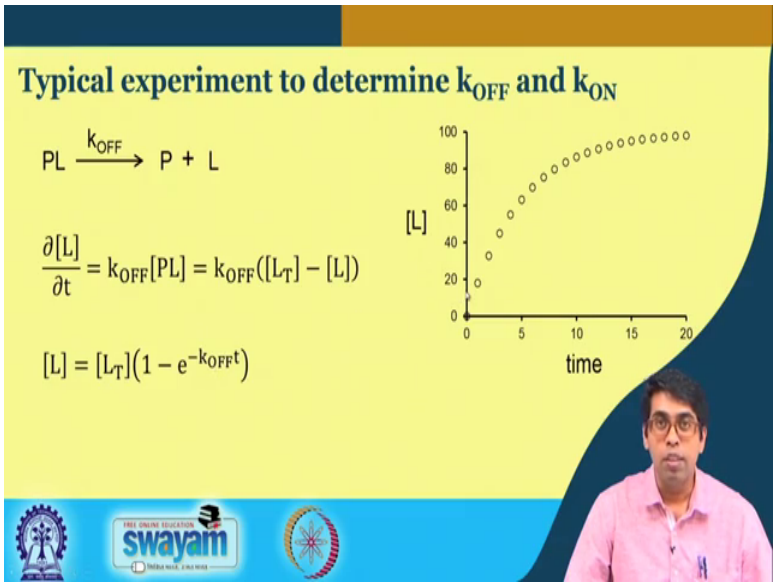

So, here is a typical experiment to determine this kinetics. We have already seen how you can determine K D using different experiments. So, today we are going to focus on experiments that we can use to determine the kinetics. So, one of the tricks that you will see we use to determine the kinetics is somehow convert this equilibrium reaction into a non-equilibrium reaction. So, for example, the way I have written here is that P L, so you already have the protein ligand complex, and somehow using some experimental trick we have converted into a unidirectional reaction, so it is an irreversible reaction. So, this is getting dissociated to the free protein and the free ligand and the reverse reaction is not happening.

In practical, what we what happens is the reverse reaction is very small, so that practically it becomes a irreversible reaction. So, somehow we have to do that. And once we do that we can then measure the concentration of either the free ligand or the free protein as a function of time. And from that we can get a measure of our OFF rate. So, this is again the equation. If we look at let us say our ligand is labeled, so that we can monitor the ligand as a function of time the free ligand as a function of time. And if we have converted this reaction into a irreversible reaction, then the concentration of the

ligand will keep on increasing with time, so that will be the rate of formation of the ligand will be given by the OFF rate multiplied by the concentration of the protein ligand complex which is written here.

Now, we know that this protein ligand complex is nothing but total ligand minus free ligand. We have already seen this equation in the last lecture, so that is what we use here we replace this P L by total ligand minus free ligand. So, this is our variable which keeps on increasing with time. And this is constant because we have used a fixed amount of ligand concentration, and we are watching the experiment as a function of time. So, we are not changing the total ligand concentration. So, this is constant.

Now, if you integrate this equation d L d t equals to k OFF multiplied by total ligand concentration minus ligand concentration. Remember, this is the constant; this is a constant. So, if you integrate that, you will get this exponential function, where the ligand concentration depends on the total ligand concentration, and it increases exponentially. So, if I plot the concentration of the free ligand as a function of time, I will get something like this. So, this is time and this is the concentration of the free ligand. So, you will see that it keeps on increasing with time.

And we can, so this will be your experimental data where you will get points like this. And we already know this equation. So, we can fit this experimental data to this equation and calculate the L total and k OFF, these are the two variables that we will fit. L total is something that is not very useful to us, but we are mostly interested in k OFF. So, from this we can actually get the OFF rate.

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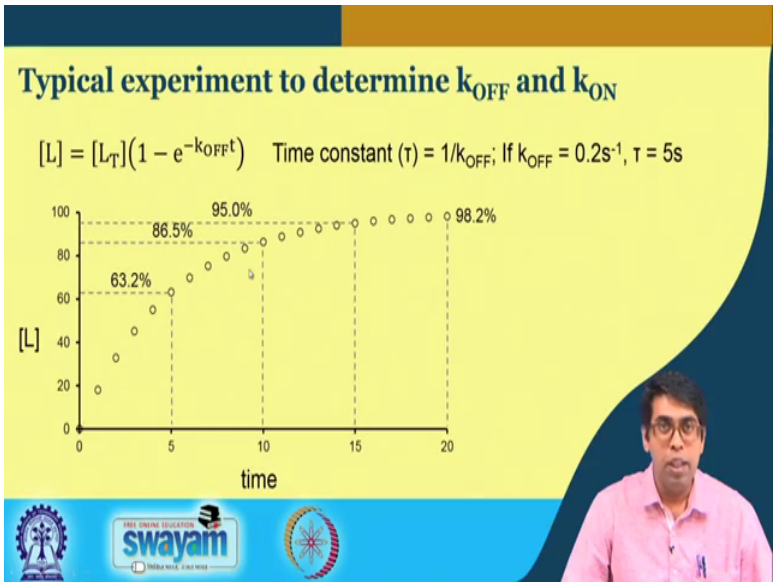

So, there are few things that we have to consider when we design a kinetics experiment. It is true that we do not know the exact values of the rate constants. So, k OFF or k ON, but we have normally just like the K D experiments, we have some idea whether this OFF rate will be in seconds or whether it will be slow, and it will be in minutes or whether it will be very fast, and it will be in milli seconds. So, we have some idea about that. And depending on that we have to choose the experiment that can measure data points in the time scale that fast, so that we can measure fast reactions or we can measure slow reactions. And also we have to consider how long should we collect our data.

So, for example, this is the equation that we have up here ok. So, k OFF and k OFF has the unit of per second and that is something normally that is not very intuitive. We cannot use a per second to determine the experiment. So, what we normally do is we take the reciprocal of this direct constant. So, 1 by k OFF is given by another constant, and it is called the time constant. So, for example, if the OFF rate is 0.2 per second, then this time constant tau will be 1 over 0.2, so it will become 5 second. So, let us say we have to design an experiment which has a time constant of 5 per second. So, the k OFF is a 0.2 per seconds, so that tau is 5, 5 second.

So, there are two questions how fast do you have to collect data, so that you can get a good estimate of the OFF rate, and how long do you have to collect your data, so that you can get a good estimate of the OFF rate. So, let us address the how long question

first. If we collect our data up to one tau value, so tau is 5 second. So, 1 tau value is 5 second. What you get is only 63 percent of your total saturation. So, with time the free ligand concentration will keep on increasing. And after sometime it will become more or less saturated. So, there will be not much change. So, you want to reach up to that point ok. If we wait for only 1 tau, we will be somewhere down here ok.

And what from experience or people have seen is that if you collect data only this much, then because this will have a lot of noise in them then the estimate of this k OFF that you will get will not be very good. If we go up to 2 tau, then you have reached only up to 87 percent of the end of reaction let us say. Up to 3 tau, it is 95 percent close to the end of the reaction. And when if you go up to 4 tau, you are almost towards 98 percent of the end of the reaction. So, it is advisable to wait up to 4 tau, so that the reaction is almost 98 percent complete.

And then the data points that you will have will give you if you feed this data set, you will get a very good estimate of your OFF rate. If I have if I collect data only up to this point, then the OFF rate that I will get will not be very good. There will be a lot of error in that OFF rate. But the OFF rate that you will get from this data set which goes up to 98 percent completion that OFF rate will have a very good, the errors will be very less, so that is one aspect how long you have to collect your data, and the other aspect is how fast you have to collect your data. So, if my tau is 5 seconds, you can see that we have collected data here at every second right, but it is also not necessary typically what you should do is you should collect data at a rate of 2 tau by 5.

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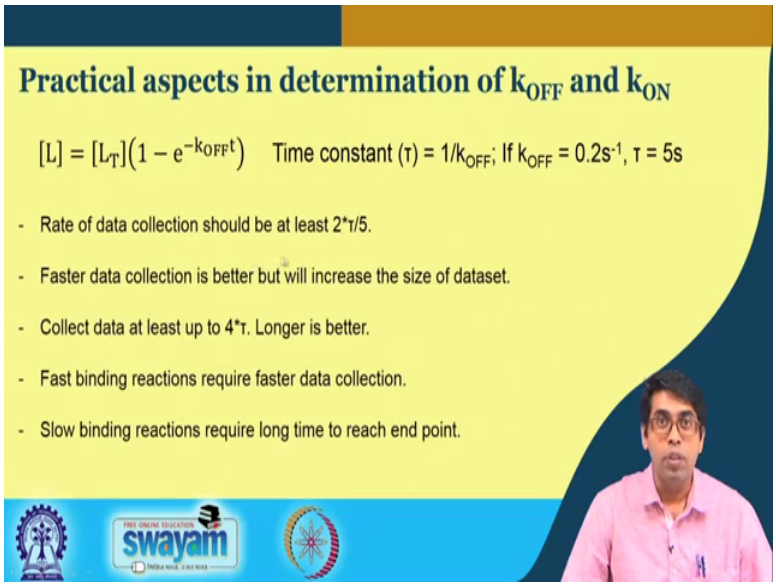

So, if my tau is 5 seconds, then 2 tau by 5 will be 2 seconds. So, you have to collect data at a rate of at least 2 seconds. Faster means you will get much more better data more data, but then the size of your data set will increase, and you will have to fit more points. So, it will also become computationally expensive. So, this, but this is the minimum that we try to achieve. As I mentioned before collect data at least up to 4 tau. Say if your tau is 5 seconds, then you should collect data up to at least 20, seconds and again longer is better.

So, this means that if your binding reaction is fast, so if the tau is not in seconds, but say it is in milli seconds, then you will have to collect data much fasters. So, you will have to choose an experiment where you can collect data points in milli second time scale in every 1 milli second ok. But if the reaction is slow, then if the tau is in minutes, then you can collect data in 10 seconds or 20 seconds or 30 seconds. So, you can use a experiment where you do not have to collect that fast data, data at that fast rate. Also longer tau means that you will have to wait for long time to reach the end point. If the tau is short, then the end point is reached very quickly, so that you can actually repeat your experiments much faster. But if the reaction is slow then you will have to wait till you reach the end point, and then again you can repeat your experiment. So, these are some of the considerations which will determine what type of experiment you can use. And this will be more or less clear from the two examples that I am going to discuss next.

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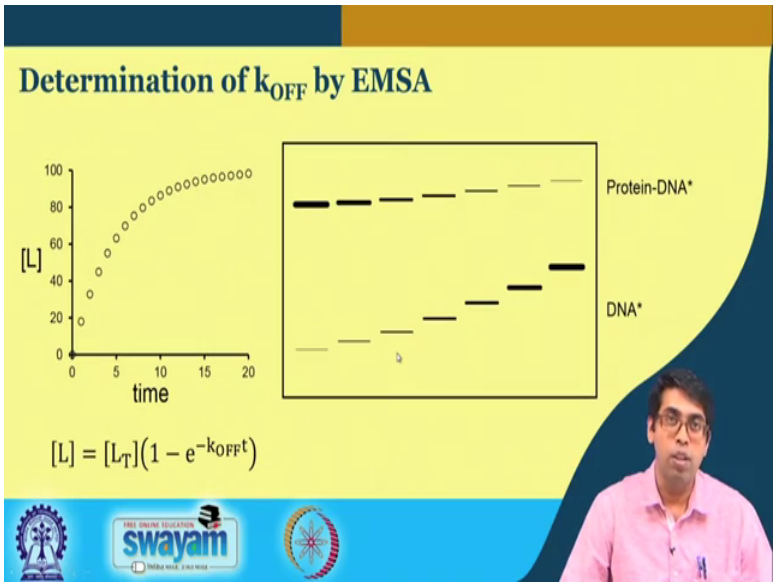

So, we have already seen EMSA. And we saw that we can use EMSA to determine the K D. And in this case the gel is non-denaturing. So, we are using a (Refer Time: 15:22) gel. So, we are going to do the same and which means that we are going to look at protein DNA interaction. So, in this case, we are not going to do any titration, we have only one reaction. This is our DNA which is labeled with the radioactive label or it can be florescence tag, and then we have added protein such that we have our protein DNA complex.

Now, we have to somehow. So, if you let this set, it will reach equilibrium. Now, we have to somehow trigger the dissociation and make it an irreversible reaction. The way you can do that is by adding the same DNA, but now the DNA is not tagged ok. So, there is no florescent level or there is no radio level on this DNA. And you add this DNA in much more excess compare to this DNA. So, if this DNA is added in some concentration, this DNA will be added in almost 100 fold excess to this concentration which means that the moment this protein DNA complex is dissociated, the chances of that protein binding this DNA is very less, it will most likely bind this unlabeled DNA, so that this DNA becomes free ok.

So, in that case, you will have you can just take out samples from this reaction mixture and run a gel and see as a function of time how much free DNA you have. So, to do that, you run this gel in a slightly different manner. So, you basically start running your gel.

And while the gel is running, you add some in let say the first lane, then you wait for a minute or so, then add again may be 10 micro liters to the second lane, wait for another few minutes add 10 microliters to the third lane so and so forth.

And what you will get is something like this. So, you have added your reaction mixture 10 microliters lets say at time 0. After 1 minute, you added here; after another minute, you added here; after another minute, you added here. So, you keep on doing that while you are running the gel. So, it means that you have to be very careful because large amount of current is passing through this gel.

So, what you will see is that the free DNA band will show up like this. Since these lanes are loaded later on the migration is still up here ok. So, this has migrated more, this has migrated less, because this has less time this has more time to migrate, but these are all coming from the free DNA. And these are coming from the bound DNA, where the label DNA is bound to the protein. As time progresses the concentration of the protein DNA complex where the DNA is labeled keeps on decreasing and the concentration of the free level DNA keeps on increasing. So, we can if we can quantify each of these bands, then we will again get a plot like this.

So, these free DNA concentrations are plotted here as a function of time and we can fit this experimental data to this equation and calculate the OFF rate. So, you can imagine that since we are taking out aliquots and adding it to a gel which takes sometime so this the time points that we are collecting are in minutes. So, you are adding this may be after 1 minute or 2 minute or 5 minutes right. So, it means that the OFF rate that can be measured using a technique like this has to be very slow. The tau has to be in 10s or 20 of has to be at least 10 minute or 20 minute or so on right, so that you can actually do an experiment like this. If the OFF rate is fast and if the tau is in seconds, then you cannot use an experiment like this.

So, it turns out that when a complexes when a complex is a very tight binding complex let us say it is in the range of nano molar or pico molar dissociation constant, the OFF rates are very slow, because there is a limit to the ON rate. And if you remember the equation k OFF divided by k ON equals to K D, so you can back calculate if the on rate is limited by the rate of diffusion which is 10 to the power 9 per molar per second. And if the K D is let us say 1 pico molar, then what will be your OFF rate. And you can see that

the OFF rate will be very slow. So, for very tight binding complexes which is case in many times for protein gain interactions, you can use EMSA to determine the OFF rate. If the binding constant is weak in let us say micro molar range, then you cannot use EMSA to determine the OFF rate.

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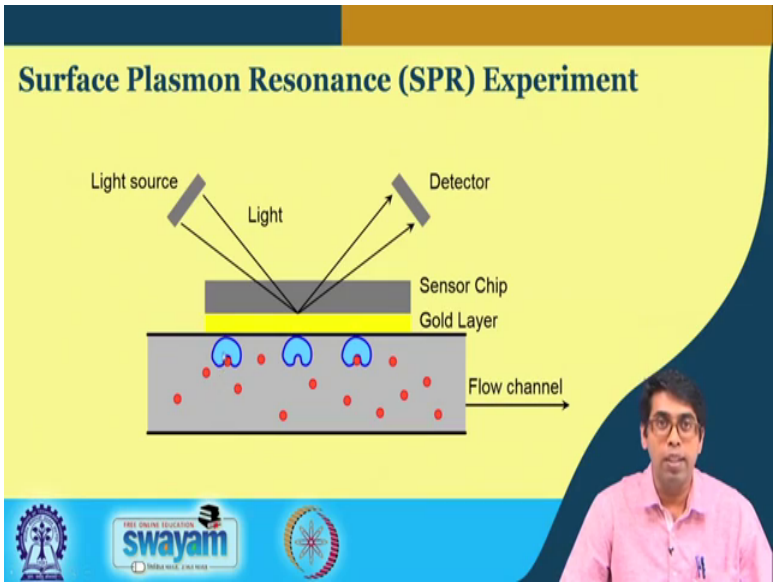

The other technique which is very commonly used to determine binding kinetics and also the binding constant is surface plasmon resonance experiment. This is the very versatile experiment which can be used for different types of molecules and in a wide range of binding constant and also kinetics. So, the basic setup of this experiment is as follows. This with these two lines represent flow channel. And this grey part is actually your buffer, which is flowing through this channel. This yellow bar is a gold layer. So, it is a metallic layer. On top of that there is a sensor chip.

So, this is sort of the setup that is used in surface plasmon resonance experiment. The protein that you want to see test binding that protein is immobilized on this chip. And it is inside this flow channel. And what you do is you flow your ligand through this channel. When the ligand is flowing some of it will bind, and the once which are not bound will flow out ok. So, when there is no ligand, so under this conditions what is done is a light is incident, and there is a total internal reflection, because of this metallic gold layer the refractive index is changed, and there is a response of binding.

So, the signal that you get is basically the angle of this total internal reflection. When ligand binds to the protein, the refractive index of this system is changed which means that the angle is also changed. To achieve the same total internal reflection you will have to change the angle of this light, so that is the signal. And it is a very sensitive method by which we can detect binding of ligands to proteins. So, again typically what you get is some signal as a function of binding of ligand to protein.

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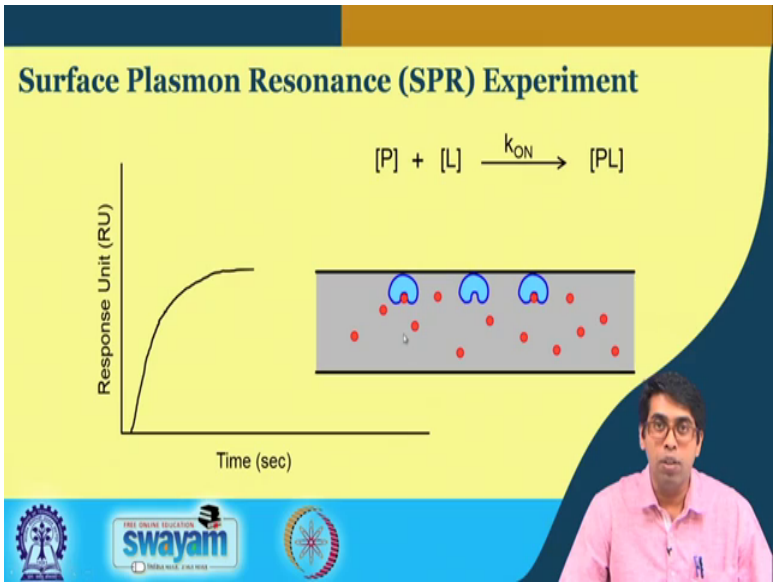

So, now how do you convert it to a irreversible reaction. So, as I said that this is a flow channel which means that ligands are continuously flowing through this. Ligands which get bound to the protein will stay there, and anything that is not bound will flow out of the channel. So, initially the proteins all the proteins are free, and as the ligands are flowing in they will start binding. So, at that point, what we get is a change in response unit like this.

So, again if I go back as the ligand starts flowing in, there will be binding of ligand to the protein. And what you will see is the response unit keeps on increasing. And after sometime depending on the concentration of the ligand, some of the proteins will be bound. So, you will reach an equilibrium where binding of the ligand and dissection of the ligand becomes constant. So, there will be no change in the response unit. At that point, what we do is we stop flowing the ligand and we start flowing only buffer which means that there are no ligands are coming in. And the moment some of this ligand is

dissociated out it will be carried out by the buffers. So, the chance of the same ligand going back and binding to this protein or some other protein is very less. So, in this second part of the experiment, the response unit keeps on decreasing, because the ligands are all flowing out and at the end no ligand is bound, so that it again goes back to the base line.

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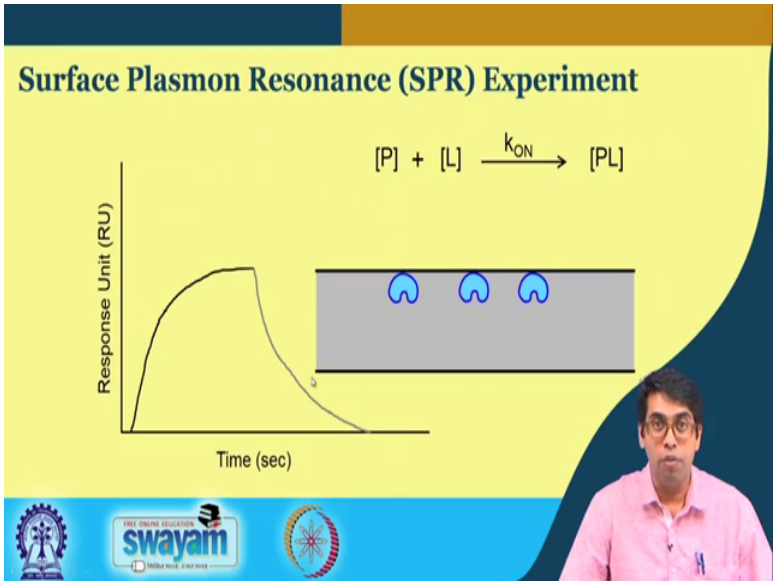

So, there are two parts of this experiment.

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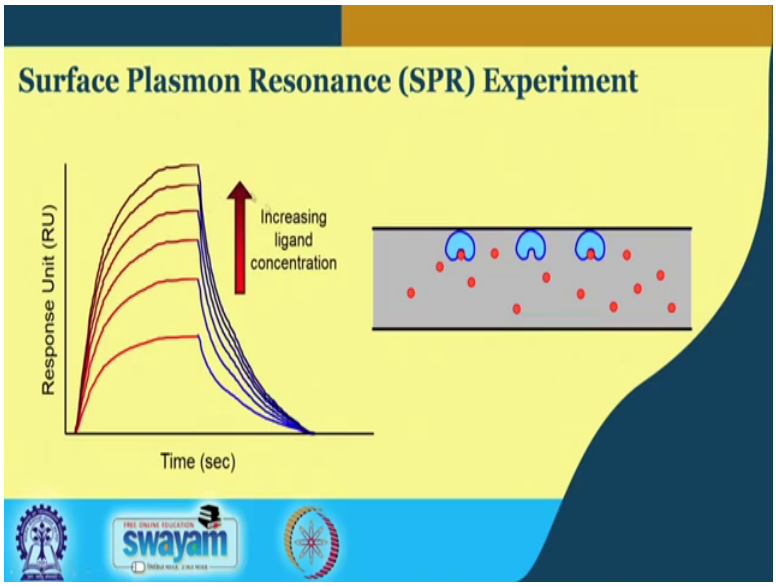

The way a typical experiment is done is what I have described is only one set, which is done using a particular ligand concentration. We can increase the ligand concentration. And in that case we will get a different response because the amount of proteins that are now bound will be higher. So, the response will also be higher if we keep on increasing the concentration of the ligand the response unit, where it gets saturated we also keep on increasing. So, here what I have shown is 6 different ligand concentrations, and you get this binding and dissociation curves. Now, we have ligand concentration and we have this saturated response unit.

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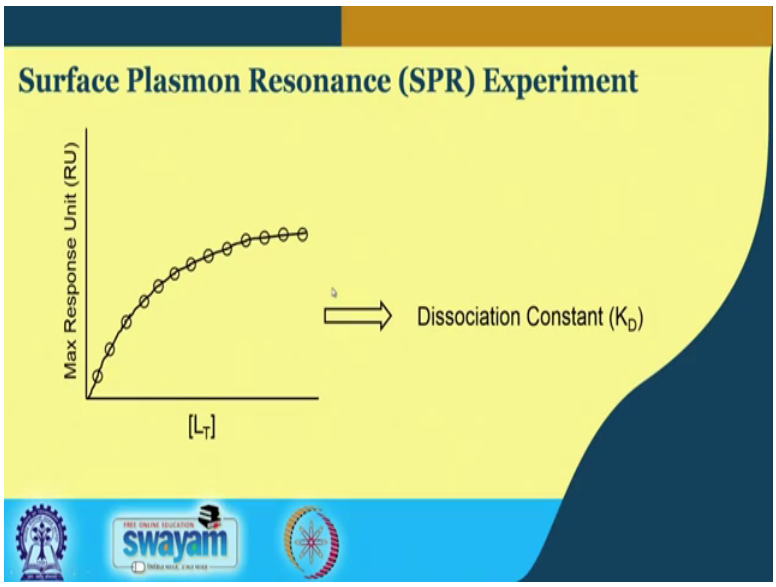

So, we have saturated response units. So, we can plot this two and we can get this curve which we have seen before. So, we can again fit this to the standard equations that I have described in the last lecture and get our dissociation constant.

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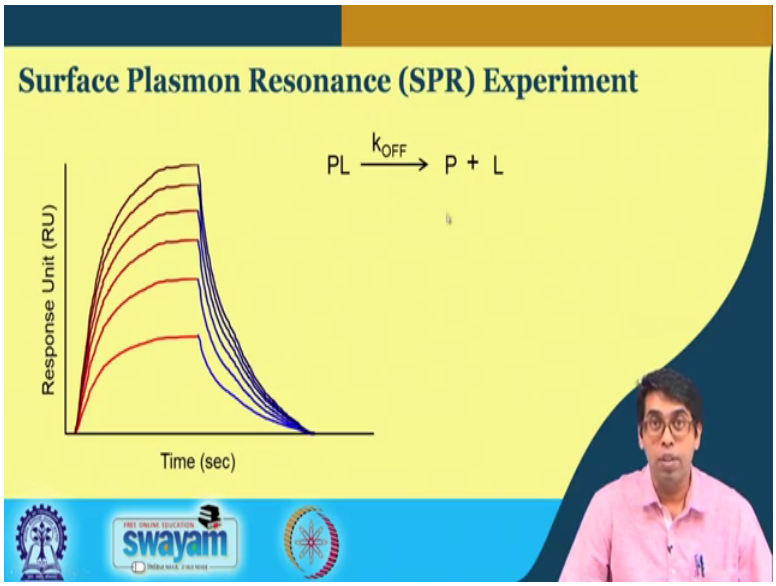

So, now we have K D and, but we still do not have the kinetics. So, SPR is something that can be used to get both kinetics and thermodynamics. So, now, we let us ignore this binding curve, and what we have is the dissociation curve. As I said before that just by flowing buffer what we have ensured is that this reversible this reverse reaction is minimal. So, the moment the ligand is coming off, it is carried out by the buffer trough the flow channel.

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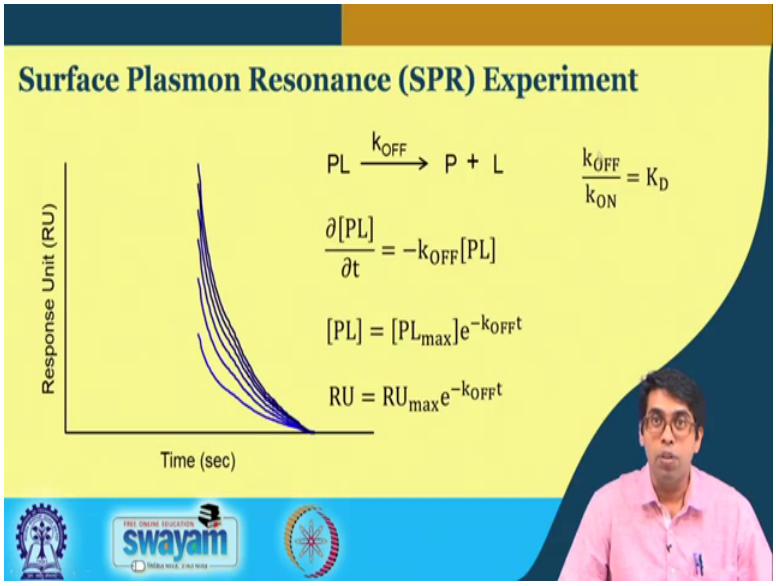

So, there is no rebinding to the protein. So, the concentration of this protein ligand complex depends on the concentration of the rate by which this protein ligand complex dissociates is given by the rate constant multiplied by the concentration of the protein ligand complex. So, we can simply integrate this equation and we will get a exponential decay.

Now, what we see is response unit, so the actual equation to which you will fit each of this curves will be something like this, where RU is the maximum response unit at each point and k OFF remains constant. So, you will get you should ideally get the same k OFF from all this 6 different experiments. So, by just by fitting these dissociation curves, we can get our OFF rate. So, at this point, we have our OFF rate, we have our k dissociation and we can simply calculate the ON rate. So, using SPR, you now have the thermodynamic constant and also the two kinetic parameters.

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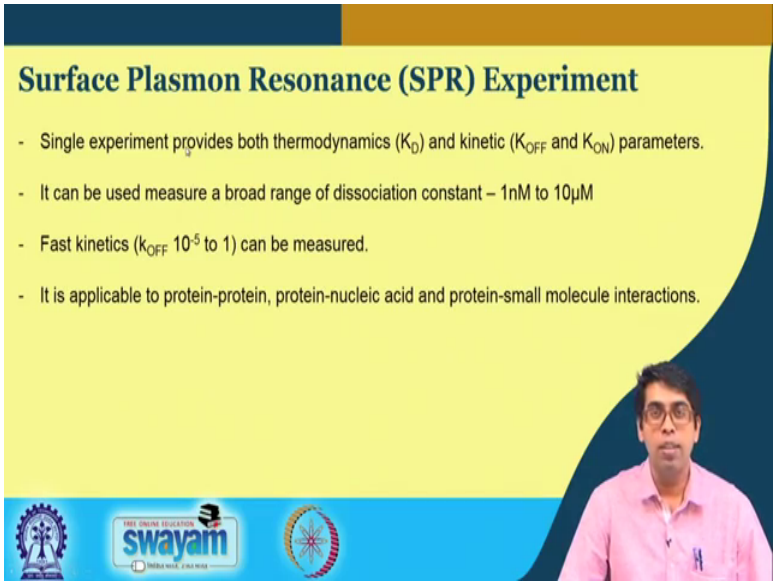

So, this is the summary of an SPR experiment. So, it is a single experiment which provides both thermodynamics that is K D and kinetic parameters that is k OFF and k ON. It can be used to measure a broad range of dissociation constants. So, it can be used to measure as low as 1 nano molar so very tight binding and as high as 10 micro molar, so the medium to weak range binding.

The kinetics that it can measure the OFF rates is again it can vary in this broad range 10 to the power minus 5 per second to 1 per second. And it is applicable to all source of

molecules. You can look at protein-protein interaction you can look at protein-nucleic acid interactions, and you can also look at protein small molecule interactions. So, these are the two experiments that I have discussed here, and but the and the principles are more or less the same. You have to somehow convert the reaction into a irreversible reaction and follow the formation of the ligand or the free protein as a function of time.

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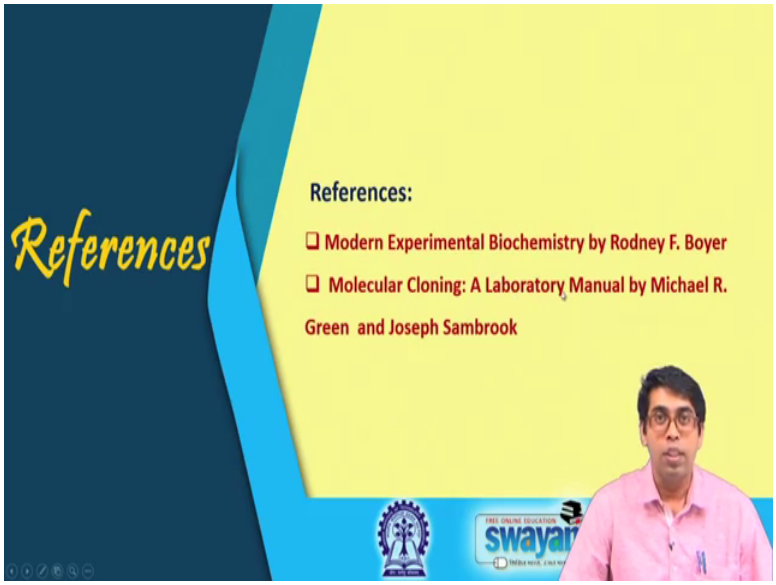

So, these are the references.

And thank you.