Bio-Physical Chemistry
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Lecture - 31
Protein Folding Kinetics: Rapid Mixing and Relaxation Techniques

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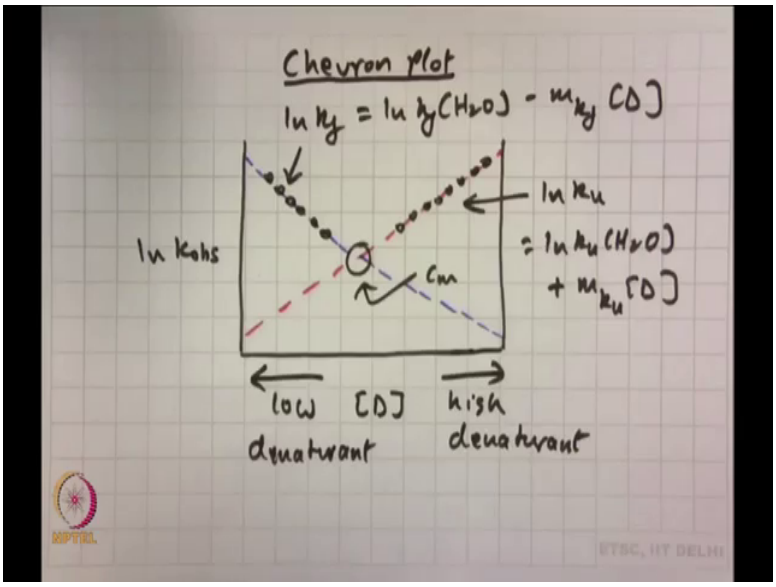

So, last class we were discussing Chevron Plot. So, this is what we were looking at. So, if you have if you are trying to look at the kinetics of folding experiment, so, this is say your y-axis is the natural log of K observed, the observed rate constant and this is your concentration of denaturant on the x-axis. And, then what do you what we had was like this say we have it is coming from here.

So, this is the unfolding arm and this is the folding arm I will just label those really quick. The points where they cross what is that point known as? This is your c m isn't it and there were

black dots. So, these black dots are your experimental points remember these were experimental points right. So, this was what k u and this was k f.

So, remember what we had what was ln k f equal to? k f H 2 O minus m k f times concentration of D, isn't it? Right and then k u was equal to? k u H 2 O plus m k u times concentration of D right and then we discussed why they would be following these slopes right ok. So, that means, if you go to a very high denaturant side so, for example, if you are going to a very high denaturant side here then; that means, the unfolding rate constant would be highest because you already on the denatured side it is easy to denature right.

Similarly, for the folding if you are going to go to the folded side, then what will happen is the folding rate constant would increase with decrease in concentration of denaturant or would increase with or rather would increase with decreasing concentration denaturant or decrease with increasing concentration of denaturant. Because, when you increase the concentration of denaturant you are on what the unfolded side when you decrease the concentration of denaturant you are on the folded side.

So, that is why this is your low denaturant and this is your high denaturant ok. So, this was your typical chevron plot. Guys, please go through this carefully right. You have to be able to get used to chevron analysis if you would carry on biophysical studies of protein folding because this is one of the ways where people look at what is happening to the transition state.

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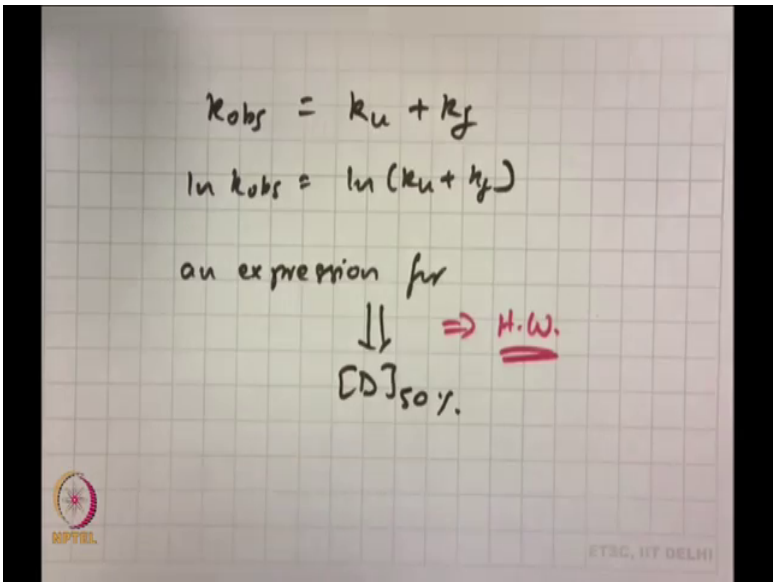

Now, from here what we said is that on the y-axis we have k observed and we said that k observed is equal to what? k u plus k of f right. It is the sum of both and then we can take the natural log on both sides. So, then we can write k observed is equal to natural log of k u plus k f I wrote this down last time. And, now you can I will not write the next one down, but you can see what I can replace with by see k u. See k u and look at k you from this equation right.

So, what is k u equal to? k u is equal to?

Student: (Refer Time: 04:43).

k u H 2 O times?

Student: (Refer Time: 04:45).

e to the power.

Student: m k u.

mk u D right and similarly what is k f? k f H 2 O times e to the power minus m k of D and that is what we wrote down last time good.

Now, let us some take some examples and see what happens, but before doing that just let me tell you one thing you can also do is if you are given these two equations this equation for ln k f and this equation for ln k u this is what I wanted to figure out. I want to figure out I wanted to figure out an expression I wanted to figure out an expression for concentration of D; that means, what is its 50 percent?

Student: c m.

c m right. This is what I wanted to figure out. I wanted to figure out an expression for c m which is the concentration of D at 50 percent denaturation right. So, based on these two equations you are going to figure it out right. This is a homework problem for you right. So, this is a homework problem for you. Please do that.

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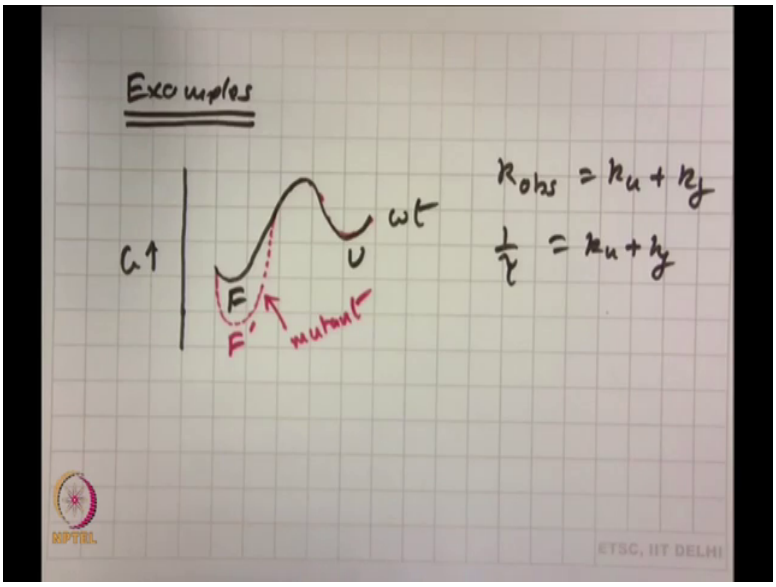

Now, let us take few examples. Let us consider a simple two state scenario ok. We are always talking about two state to keep matters very simple. So, we have a free energy profile. So, this is G for you. So, suppose I am I have something like this. So, this is the folded state, this is the unfolded state right and this is your wild type, remember this is your wild type.

Now, what you do is you do a mutation. You do a mutation and then this is how it changes the free energy profile change like this ok. So, then the red one is your mutant. So, the red one is your mutant and then this one is F prime the folded state of the mutant. Now, tell me looking at this figure what have you done?

Student: (Refer Time: 07:10).

You have stabilized the native state right by introducing this mutation. Remember mutation is also referred to as protein engineering ok; that means, you are engineering some sort of stabilization or destabilization in the protein or stability or a stability or the reverse. But, having done the native state what you have done is you can see from the figure the way it is drawn you have not affected the.

Student: (Refer Time: 07:35).

Transition state neither of you affected the unfolded state ok. The only thing that is stabilized is the native state; that means, the only free energy change is in the native state. Now, is the protein being stabilized or destabilized what do you think.

Student: Stabilized.

Stabilized. Why is it so?

Student: Barrier increases.

Barrier increases right; that means, barrier increase and also what you can see is the absolute delta G between U and F also.

Student: Increases.

Increases right. So, both good; so, that means, this protein should be more stable the mutant should be more stable in the wild type right.

Now, let us go back to your old equation k observed is equal to k u plus k of f right. So, this remember k observed can be written as 1 by tau and we said tau was tau relaxation or tau observed whatever; k of u plus k of f. Now, think about this; think about this under highly

unfolding conditions; that means, highly denaturing conditions where conditions are really unfolding type; that means, high concentrations of denaturant right.

So, keep this figure in mind; keep this figure in mind and let us talk about.

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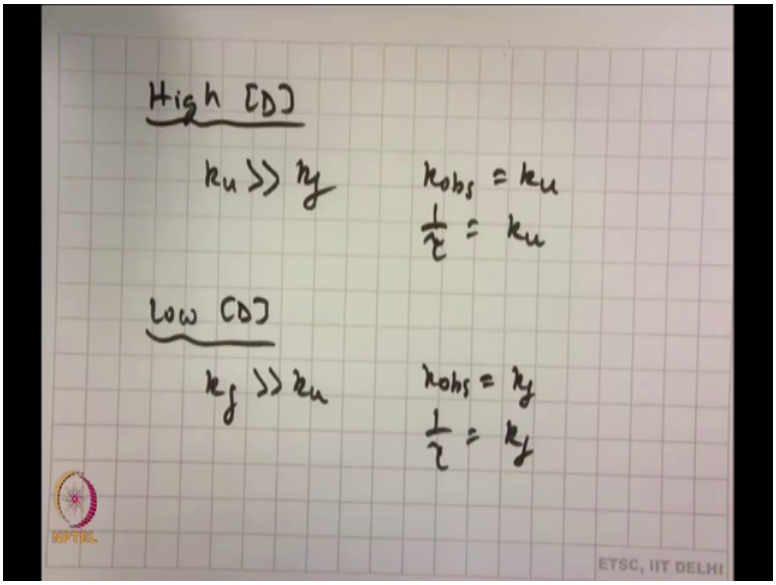

Let us move to the next page high concentration of denaturant. Now, under high concentration of denaturant tell me this which one would predominate? Would k u predominate would k f predominate?

Student: k u.

k u would predominate right because it is already on the denature side ok. So, they are now here I can write then k u is much greater than k f, isn't it? If k u is much greater than k f, then

what is k observed equal to k u or 1 by tau is equal to k u, isn't it? So, similarly if I would ever go to the highly non-denaturing conditions; that means, highly native like conditions what would happen?

Student: k u.

For highly native like conditions; that means, for low D, what would happen? k f is much greater than k u know, isn't it? Because we are already on the native side and then k observed is equal to k f or 1 by tau is equal to k f keep these things in mind. These things become come in very handy. Are you guys clear up to this point right?

Now, go back to the figure; go back to the figure and this is what I will ask you. What I will ask you is how do you think the chevron plot is going to look like in this case? Tell me two very important things that are going to change in the chevron plot two very important things. What is the first one? Based on the; based on the difference in free energy what is the first one that is going to change?

Student: (Refer Time: 10:37).

Let us go ahead step by step right.

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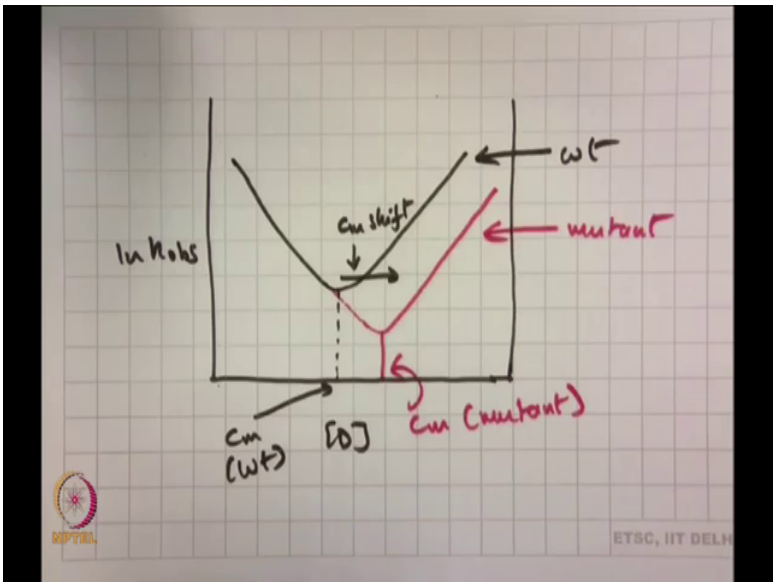

Say, suppose let us draw the chevron plot. Suppose this is the one. So, looks like a V, right? This is the one which is for the wild type ok, where this is natural log of k observed and this is the concentration of denaturant and this corresponds to what c m, isn't it? So, this is corresponding to c m for wild type, is it clear? Very good.

Now, tell me help me in drawing the chevron plot for the mutant. Tell me what would happen? Look at this the first thing because of the increase in stability of the mutant what would happen to the c m, would it increase or decrease?

Student: Increase.

It would increase because you would need because it is more stable the mutant is more stable you would need more denaturant to denature it. That means, what would happen is this would

shift to the right higher denaturant, very good that would be one. So, this how you go trying to analyze the chevron plot.

The next is which rate constant would be affected and this you have to say which rate constant would be affected?

Student: k u.

k u?

Student: Yes.

Would k f be affected?

Student: No.

See, in this case k f is not affected why because you coming from when you are coming from U to F, the barrier has not changed. So, k f remains the same, but when you are going from F to U, what has changed?

Student: Barriers.

The barriers changed. So, what will happen now k u would change because looking at an unfolding ok. Now, good so, k u would change. How would it change? Give me a qualitative idea. Would it would k u increase or would k u decrease?

Student: Decrease.

k u decrease why?

Student: (Refer Time: 12:52).

Energy barrier is higher good. So, then should the plot not be looking like this? Can I draw a plot like this? So, this is c m for mutant. So, this is the chevron plot for the mutant and this one is for the wild type. So, is it clear how the chevron plot would change?

So, you can see one thing. The first one is the c m has shifted by this amount. So, this is your c m shift right because you have stabilized the mutant stabilized protein by mutation. Now, initially your k was here, now k has come down here why because k u is decreasing you have increased the barrier.

So, it has become harder for you to unfold as compared to the wild type, but also k f which is on this side is not being affected at all, right? So, that means, k f is not affected, but k u is being affected. So, this is one way of looking at it. At least this is what the picture is the simplest picture you can draw based on the example we started with. Is everybody clear with interpretation? Good.

So, now let us move on. Let us look at the second case. So, this was example 1.

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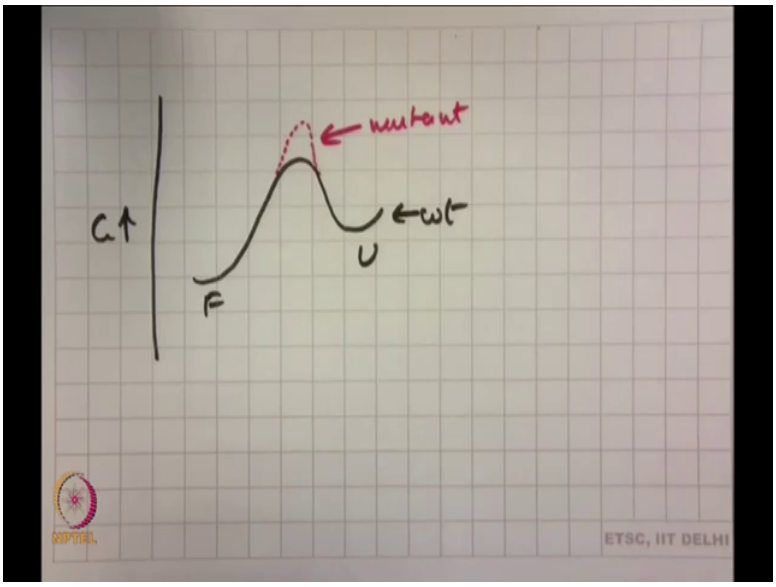

Let us look at this example where this is G ok, this is again F, this is again U. Wild type we do a mutation, now because of the mutation; because of the mutation this is what happens. Now, for you what has changed in this case?

Student: (Refer Time: 15:38).

Has the U changed? No. Has the F changed? No. Has the T s changed?

Student: Yes.

Yes. So, the barrier two folding or unfolding both.

Student: (Refer Time: 15:52).

Have increased, right? Guys, so, then in this case how would my chevron plot look like?

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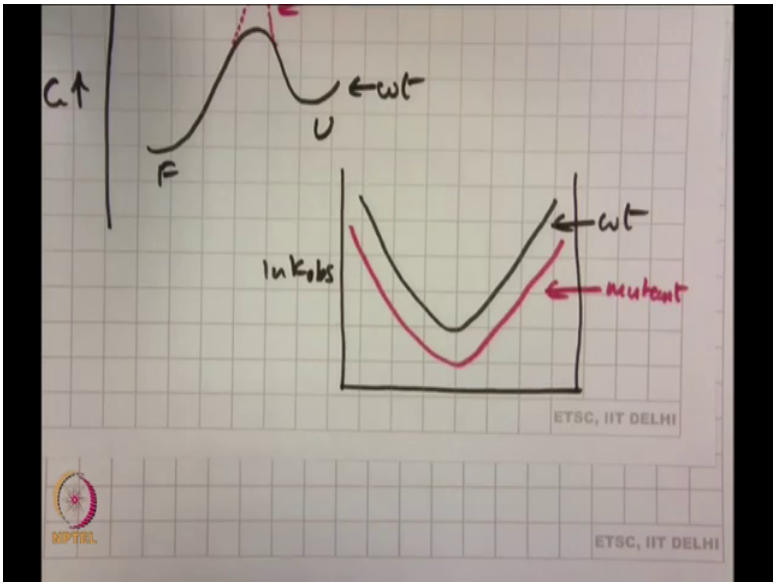

So, if I am if I am trying to draw my chevron plot say if this is my initial chevron plot. This is my wild type looks like a V again this is ln k observed. Now, tell me how the chevron plot look like?

Student: (Refer Time: 16:28).

Good, both would decrease right. Will c m change?

Student: Will not change.

c m will not change because stability has not changed. Stability is defined by what? The difference between the free folded and the unfolded state. Stability is not changed, but your free energy barrier has changed. So, this would be your mutant clear? So, that means, both k u and k f are decreasing.

In the previous case, k f no effect; k u decreased , but because of the increase in the free energy whether to whether in the forward direction or the reverse direction whichever directions you are looking at, this is the picture, clear? Is this example with you?.

So, in the exam if you are given a free energy diagram you should be able to draw a chevron plot or if you are given a chevron plot you should be able to interpret what changes you are having in a free energy diagram, using this line good.

See, so that you know pretty much brings us to the end of our theory section in the sense that these are the changes we look for if we are doing an experiments ok. Obviously, this kinetic I mean this kinetic data are taken from experiments right. Let us try to look at what experiments are.

So, if you remember one thing what I said was if you are going to talk about protein kinetics; that means, you have to follow the protein folding or unfolding as a function of time and hence one of the major things of doing is you have to do whatever change you have to do you have to do it very fast and then allow the protein to evolve or respond to that specific change.

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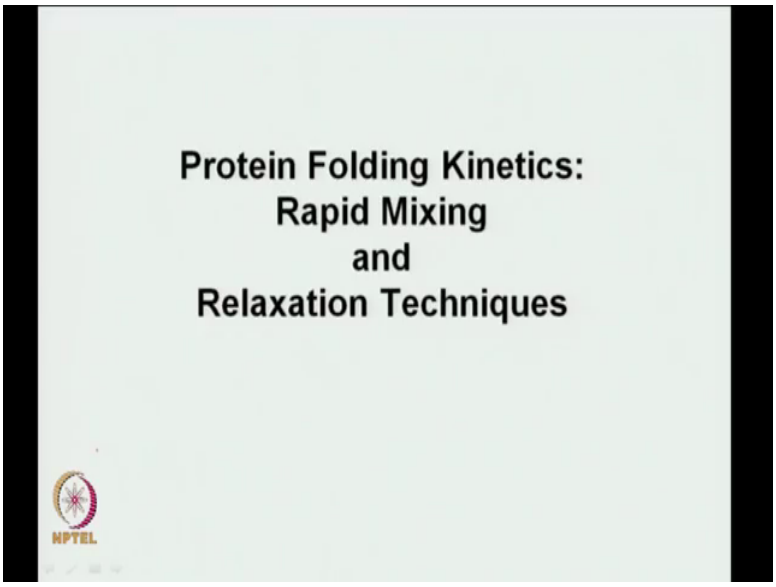

So, then this brings us to one of the most important topics of this course which is protein folding kinetics, rapid mixing and relaxation techniques. So, we are going back to the slides now right. So, what are the different techniques we are looking at? This we are going to go step by step.

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The first one is a rapid mixing technique right. The first one is a rapid mixing technique. So, rapid mixing as the name suggests is that you have to mix two things very rapidly as simple as that. Once you mix them very rapidly what you got to make sure is that the protein does not unfold or fold or does not change too much while you are mixing right. Sorry, the sound was too loud. Does not does not change too much while you are mixing because if it changes too much then there is no use following the kinetics; the protein has already changed to a huge extent.

So, our goal is to try to mix it as fast as possible and what can we do for that. But, before going into the actual technique, let me tell you what are the tools, I will just tell you the tools and then we will discuss those later, you would use to detect the changes. See changes can be many things and one of the major things is an optical signal right. Say either absorbance,

fluorescence, circular dichroism or IR which is also an absorbance, the vibration spectroscopy right.

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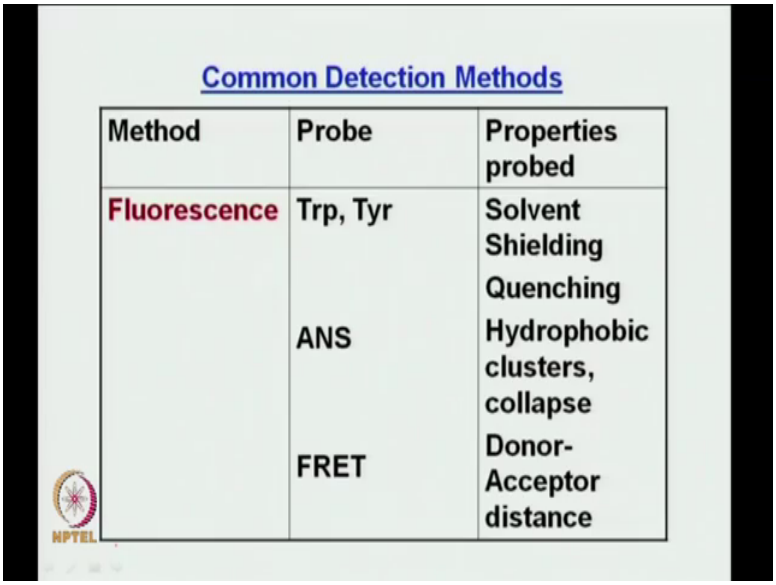

So, let us look at the next slide this kind of summarizes the common detection methods; methods means the tools. These are not the techniques, but the tools. The tools are the first one is fluorescence ok. What can you probe? I am sure you guys know about this proteins have three intrinsic fluorescent probes, what are those?

Student: (Refer Time: 20:14).

One is tryptophan; one is tyrosine and the other one?

Student: Phenyl alanine.

Phenyl alanine ok.

So, you can see two of these are written tryptophan and tyrosine. So, if you would ever look at the changes of these of the signal coming from these residues when you go from either the unfold to the folded state, the folded to the unfolded state as a function of time you would be getting a kinetics. So, what do these what does the signal depend upon? It depends upon solvent, shielding, quenching and all these things. So, these we will talk about later, but just for you to have the idea.

The next one is ANS. ANS is a certain dye ANS is the dye which combines or which adheres or binds to hydrophobic patches in proteins. So, you can actually look at the evolution of the fluorescence of ANS as a function of time and see how that happens, it is number 2.

And, then one thing we discussed at the very beginning of the class which was FRET; that means, if you have two fluorophores, a donor and a acceptor see when the protein is unfolded because the protein is unfolded these donors are very far apart from each other. Now, as the protein will fold these two would come close. So, essentially what you can do is if you can look at the FRET signal and find out the FRET signal as a function of time that will give you the kinetics ok.

So, all these we will take individually.

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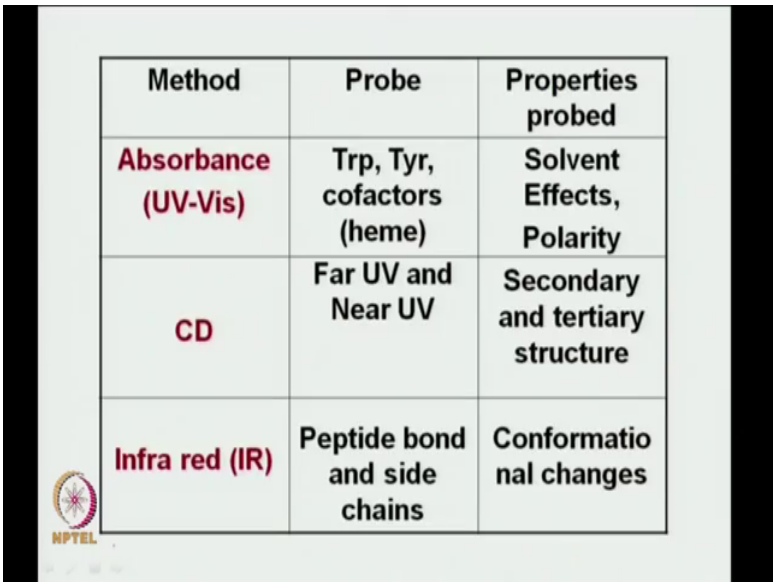

Another method is absorbance, UV visible: you know tryptophan, tyrosine, there are cofactors known as heme cofactors – if you go for the heme proteins like myoglobin, porphine moieties and all these things. So, they have also their own absorbances and these absorbances change as the proteins fold and unfold. So, you can also look at that right.

Next one is circular dichroism: circular dichroism is essentially going to tell you about the changes in secondary and tertiary structure of the proteins. So, does not matter which one you pick whether it is the secondary structure or the tertiary structure, you can plot certain changes in the secondary structure as a function of time again whether the protein is folding unfolding and then again get the kinetic information right. See all these we will discuss. So, do not worry.

The last one is infrared. So, this is also an absorbance method in the top we had absorbance which was UV-Vis which is in the UV and the visible region. The last one is absorbance in the infrared. So, we are talking about vibration spectroscopy and proteins have very well developed vibrational bands; one of the most popular bands being known as an amide one band, right. And, if you would ever look at the amide one band changing as a function of you know denaturation or refolding, then you can also plot the kinetics.

And, these are some of the most common tools or methods that people use to follow kinetics.

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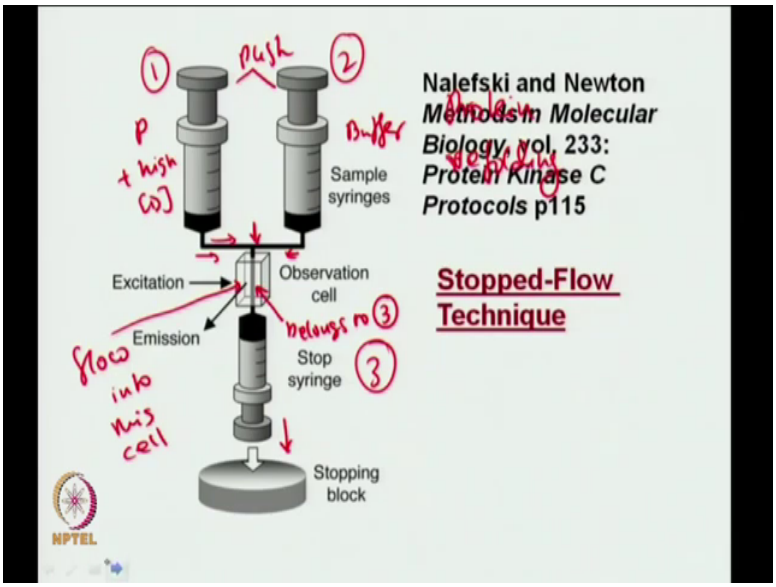

So, having said that let us look at our first technique. This technique is referred to as a stopped-flow technique. So, as the technique tells you stopped-flow means you have to stop the flow somehow right. So, this is what is done. Remember we are talking about a rapid mixing technique right. So, you have two syringes right. So, you have two syringes, say this is

syringe 1 and this is syringe 2 and there is also another syringe here. This is a stop syringe, say this is syringe 3.

Now, what you do is because you are going to have to do a rapid mixing see your whole system or technique should be having a few components. One is you should be able to mix two solutions very rapidly, number 1; then, you should be able to have a cell that means, a cell in the system where you can observe the protein unfolding or refolding; then, to observe that you have to have a detector.

So, you will be having a detector that is essentially the principal components and of you know and going into the details you would be having many other things. But, let us just stick to the very basics the bare minimum basics that is what we need to know.

So, here what you do is you are going to do a rapid mixing right. So, what means is say suppose I am looking at protein unfolding, so, that means, rather protein say I am looking at protein refolding ok. So, this is r sorry it looks like a v this is r it is protein refolding. So, what I am trying to do is this. What I am trying to do is I am going to start with a protein which is already in a denatured state. See, am going to start with protein which are denatured state; that means, a protein has to be in high concentration of denature.

So, what I write here is protein plus high concentration of D ok. This is coming in one syringe right one of the syringes has this. Now, how can bring the denaturant concentration down? The only way I can bring it down is if I mix it with a very low concentration denaturant or essentially buffer; if I mix it with buffer and depending upon the amount of buffer you are throwing in, the amount of dilution you are having you would be having your final denaturant concentration.

So, this one is say just buffer ok. Now, what you do is you take these two syringes, you push these two syringes together. So, one syringe has the protein with a high denaturant, the other one is going to have what the protein in buffer sorry this is the buffer right. These two samples are there for you in that two syringes to start ok.

Now, the technique or the instrument is so set up that, if you click on a computer on the software then these two syringes would be push together. The moment you put the push the two syringes together; that means, you push these two together right you push these two together. The moment you push these two together, what will happen? These two would flow out, right? From one you have the protein and the denaturant and the other one you have the buffer.

Then, what would happen is you can see these, these places. So, this solution would come like this solution would come in this direction and they would mix isn't it? They would mix and then they would finally, flow into this observation cell. Are you guys with me up till this point?

Again, you have two syringes remember you have to push these two syringes simultaneously. So, these are two syringes you have to push these two syringes simultaneously like this and because you are pushing these two syringes the solutions are being thrown out.

Then once they meet here say once they meet here, this is the meeting point the solutions mix, but remember I told you the solutions have to be in a certain cuvette or a container because if you are doing an absorbance or whatever you have to have a sample cell. So, this is the observation cell where the solution comes in. Are you clear up till this point? Right.

Now, the next stage is see I have started mixing isn't it? But, the technique is called a stopped flow; that means, you have started the flow, but you have to stop the flow now because how long do you want it to mix right. So, now, what it does is again this is a very nice thing. See, the solution comes in, right? You can see here this is belonging to belongs to syringe 3, ok. It is connected to syringe 3.

So, what would happen is the moment you mix and the solutions come in the observation cell where you have this connection to the syringe, the syringe starts filling up, isn't it? Because solutions are coming into that syringe which is the stop syringe. Now, once the stop syringe starts filling up what will happen is you see this is being pushed back now, because initially the

stop syringe are nothing. Now, you are pushing in solution the mix solutions and the solution is coming in and this one is being this plunger is being pushed back.

Now, how far will it be pushed back? It will be pushed back until it hits this stopping block. The moment it hits the stopping block what it does is the stopping block says ok, that is it. I have enough solution for me to do the measurement because see, I cannot just do with any small volume of solution. I have to have that much solution with which I can reliably do a measurement ok.

So, essentially what the stopping syringe tells you is that the stopping syringe is so designed that it should be having that much of say 300 micro litres or 400 micro litres of volume which would come into the stopping syringe. When that comes in, the stopping syringe hits the stopping block, the stopping block asks it to stop; that means, your mixing is done, clear? Once the mixing is done now, what will you do?

Your mixing is over your mixing is over means what does it mean? Your mixing is over means that your protein which started with a high concentration of denaturant has mixed with buffer in a certain ratio and it sees suddenly a very low concentration of denaturant because you have mix it very fast. I will tell you how fast it is. You have mix it very fast; typically you can do this in order of millisecond or so ok. You can do this very fast or few hundred micro seconds.

Once it is mixed now depending upon the protein because the protein now suddenly sees a very low concentration of denaturant it understands that it is a unfold so, it responds to that sorry, it understands there is a fold. So, it response to that folding condition and then starts folding and that change whether it is in absorbance, whether it is in fluorescence, whether it is in IR, whether it is CD that means, secondary or tertiary structure you measure as a function of time.

And, this technique is referred to as a stopped-flow method. Is it clear or not? Is the technique clear or not? Guys have any doubts, let me know right now. So, you understand why it is called the stopped-flow right. It is called the stopped-flow because you have to stop the

mixing and the mixing is stopped by what? The third syringe which hits a stopping block and that is where the name comes from, the stopped-flow.

So, that means, you are stopping the flow only after you stop the flow are you ready to take your first measurement before this thing happens; that means, before you are stopping the flow you cannot do any measurement right because this instrument is not yet ready for you to collect a measurement because the observation volume or observation cell is not yet prepared or not yet primed for a kinetic measurement. Is this clear or not? Any doubts? No doubts? Fine.

Think about it, but please let me know if you have any doubts, right? Here we are actually talking about techniques which are being used in present day research. There are quite a few people who have this stopped-flow technique and they used it in various ways to monitor protein folding if that is what they are interested in or that is what the research is based on ok, right.

So, you can understand then this mixing has to be done rapidly right because I cannot just you cannot just sit and just push these two syringes like this, right? And, wait for ages because by the time you push these two syringes, the protein has already changed. So, you have to do this mixing pretty fast.

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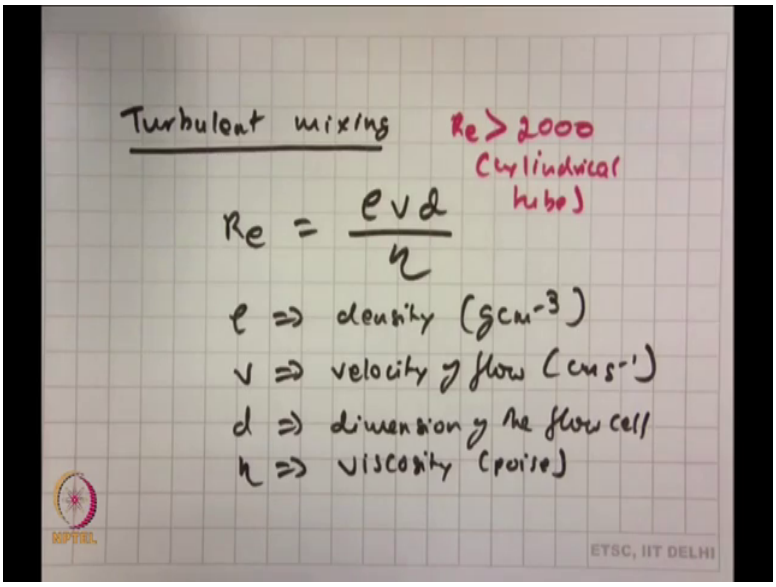

So, because you do this mixing pretty fast, this brings us to an important technique or important concept which is referred to as turbulent mixing which is referred to as turbulent mixing. That means, mixing is very turbulent right like turbulent weather, it is turbulent mixing. Now, turbulent mixing turbulent mixing is characterized by a certain number. Do you know what number is that and viscosity?

Student: Reynolds number.

Reynolds number, good. So, Reynolds number; that means, the type of mixing we have whether it is laminar, whether it is turbulent is given by Reynolds number. The Reynolds number is given by rho times v times d over eta ok, where rho is density in grams per centimetre cube; v is the velocity of flow say centimetre per second; d is the dimension of the flow cell; that means, the tubes through which the solution flows and lastly what is eta, can

you say? The viscosity which is in (Refer Time: 33:31) of poise (Refer Time: 33:24) of poise right.

So, three factors or rather four factors which would determine your Reynolds final Reynolds number and this Reynolds number for a turbulent flow; for a turbulent flow this Reynolds number R e has to be greater than 2000 for a cylindrical tube; for a cylindrical tube ok.

So, then what it means is that if you have to have fast mixing you have to have turbulent flow ok; that means, you have to have a huge amount of pressure going in. Now, keep this in mind what is the expression for pressure? What is pressure equal to?

Student: Force per (Refer Time: 34:19).

Force per unit area. So, you now you understand. So, for people who you know like for soccer players or athletes they have very pointed studs all right, spike boots, studs. Why do the why are they pointed? They are pointed because at their point of pressure or at their point of contact with the ground the pressure is really high. Why because the area is small this force per unit area. The same thing happens here.

So, think about this how can you increase say for a defined flow rate, how can you increase the pressure? You can increase the pressure by decrease the dimensions of the?

Student: (Refer Time: 34:55).

Tubes, right. If the tube is this large then obviously, the pressure will be low; if the tube is this small for the very for the same flow rate or the same force you are putting in the pressure would be high, but remember you cannot. So, one of the ways you can say is this is this is Reynolds number, I can easily play with the dimensions right, but then you cannot decrease the dimensions too much.

You cannot decrease the dimensions too much because if you decrease the dimensions too much, then your pressure would be so high that you might actually end up damaging the instrument because pressures can be really high under the small dimensions right ok. So, that is typically what is done right. So, whatever rapid mixing technique you are using it has to be based on turbulent flow which is defined by your Reynolds number and that is what the number tells you right that is what the number you are going to supposed to have.

So, coming back to the slides again, let us look at another version of this same thing you are looking at I have taken it from this.

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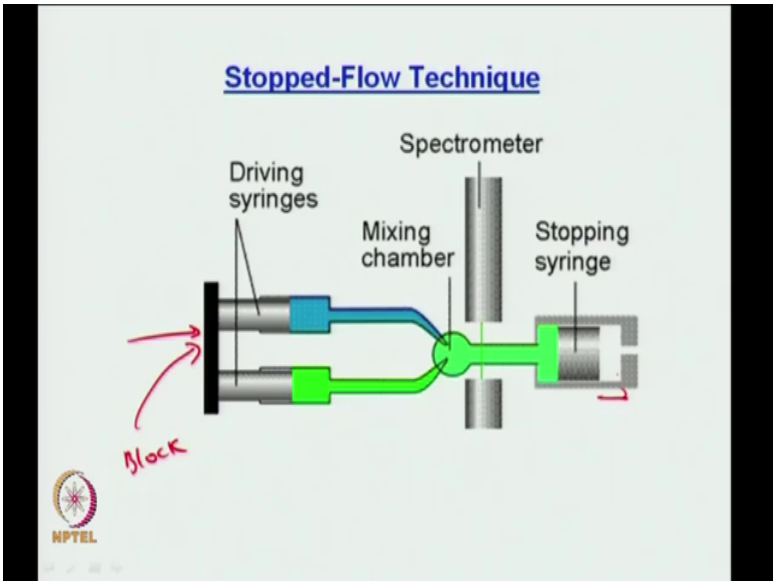

Again, this is the same thing. So, here this is what I was saying you see this drive this block you see this block, what does this block do? You see this block is uniformly connecting the two syringes. So, this instrument is so build for you that when you click on the software when

you click on the software what does it do is, it pushes the block. The moment you push the block what will happen? It will squeeze the solutions out of these two syringes right.

Now, this one is a mixing chamber cum say observation chamber, this is a just a little different thing. See this is your stopping syringe and the behind the stopping syringe you have another stopping block, isn't it? So, the stopping syringe can only move to this extent it cannot move further. The block stops it and then your actual data collection starts.

So, guys now you can understand one thing, there is a limitation to this instrument. What is the limitation? The limitation is first of all how faster mixing you are doing, but not only that the limitation is the moment the mixing is done you are not being able to start the data collection.

When are you starting the data collection? You are starting the data collection only when your observation cell has the required amount of volume of solution, the stopping block has stopped the flow only then are you starting to observe, is it clear?

So, that means, there is a time delay between the moment you mix and the moment you start your data collection. During this time you are not being able to observe any conformational events, right? take it from me, during this time you are not being able to observe any confirmation events and because during this time you are not being able to observe any confirmation events this lag time between the mixing and your forced data point is referred to as the dead time ok.

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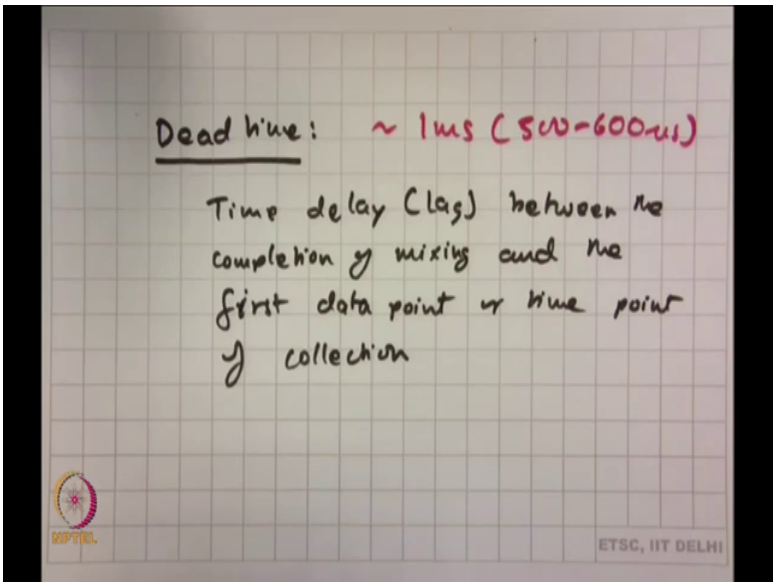

So, this brings us to the next topic this lag. So, what is the dead time? So, the dead time is a time delay is a time delay or time lag between the completion of mixing and the first data point or time point of collection. This is the dead time because this typically is dead for you, you cannot observe anything during this time ok. So, this is a limitation.

Typically in stopped-flow apparatus the this dead time is of the order of 1 millisecond you can even I think go to 500 to 600 microseconds; that means, below 600 microseconds or 1 millisecond depending upon the stopped-flow instrument you cannot go right. Because 1 millisecond is typically the time which is a dead time only after which the instrument allows you to start recording your data. Is this clear or not?

So, this is typically what goes on and that is one of the disadvantages of this stopped-flow method right, but then I also come to another method and then I will tell you why stopped-flow also has its own advantages, right. I will tell you that later ok.

Now, one of the ways of trying to improve it is this. Obviously, you can improve your mixing efficiency that is one, but that can also be improved here. The other thing is see remember in the stopped-flow what we had was there was a third syringe which was stopping the flow.

So, suppose you can say I want to do this what I want to do is I would remove the third syringe, instead I would let the solution flow from the moment it mixes and then I will observe it from the moment it starts flowing; that means, throughout the flow I will observe it. I will not stop its flow ok, that flow is referred to as continuous flow.

So, that is the next technique which is referred to as a continuous flow technique I will discuss that very soon, but just trying to make the connection between these two different methods. One is the stopped-flow because it stops the flow and the other one is continuous flow because it never stops the flow it allows a flow to go through.

Now, before going over to the continuous flow let me show you. So, the same thing. So, now, you understand right if I am going to observe protein refolding; that means, protein folding in one syringe going to have protein in high denature and the other case I would be having buffer ok. If I am going to observe protein what unfolding what would I have now?

Student: Low denaturant.

Low denaturant or no denaturant; that means, protein in buffer in one syringe and the other syringe I will be having a high concentration denaturant ok, simple. So, that means, think about the chevron plot now, depending upon what do you want if you have very high denaturant you would get k u; if you have low denaturant you would get k f and that is how you start going out by the chevron plot.

Remember, chevron plot when we are talking about the equation ln k u is equal to ln k u H 2 O plus n q times concentration of D; that concentration of D was what? Your final denaturant concentration.

On the x-axis what do you have is your denaturant concentration from 0 to 7 or 8 or 9 depending upon what denaturant you are taking. So, if you are say if you are monitoring folding say if you are monitoring folding say; that means, you are going from the unfold to the folded state, you can come back to different final denaturant concentrations; that means, you can come back to 0.5, 1, 1.5 and so.

And, hence you give get those data points for every denaturant concentration. The same thing happens for the k u if you are going the other way around and this is how you build a chevron plot. Guys, is it clear? This is how you actually construct a chevron plot right.

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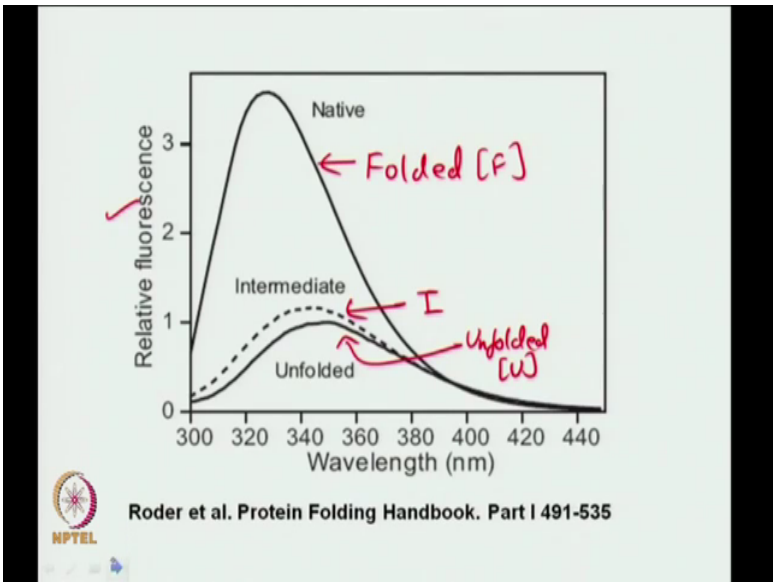

So, going ahead I told you right we are going to look at a change. So, let us see what this change is. So, this is a protein a certain protein and this we are looking at florescence, you see it is looking at florescence. Now, this one is for your folded protein. This florescence is for your folded protein ok. This is a fluorescent spectrum typically of your folded protein ok. This is coming for tryptophan, say we will discuss this later, but just take it. There is an optical signal for you fluorescence.

Now, this protein might be have an intermediate which is given by this dotted line this is an intermediate I , but what you can observe is the this one this one is your unfolded state which is U and this is F ok. Now, what is the difference in the fluorescence intensity between the folded and the unfolded state which one has more?

Student: Unfolded.

Unfolded state has less, right? folded state has more. So, what does it mean? What it means is if you would be if you would be looking at a protein folding; that means, this protein folding from a high denaturant concentration and if you are going to look at the tryptophan signal, what would happen? Or if you are going to look at the florescence signal, what would happen would increase or decrease?

Student: Increase.

It would increase right because unfolded state is low and then goes to the folded state. So, this is the change you are going to look at and this is the change you are going to monitor as a function of time. You are with me right?

Now, let us look at; let us look at an actual example ok.

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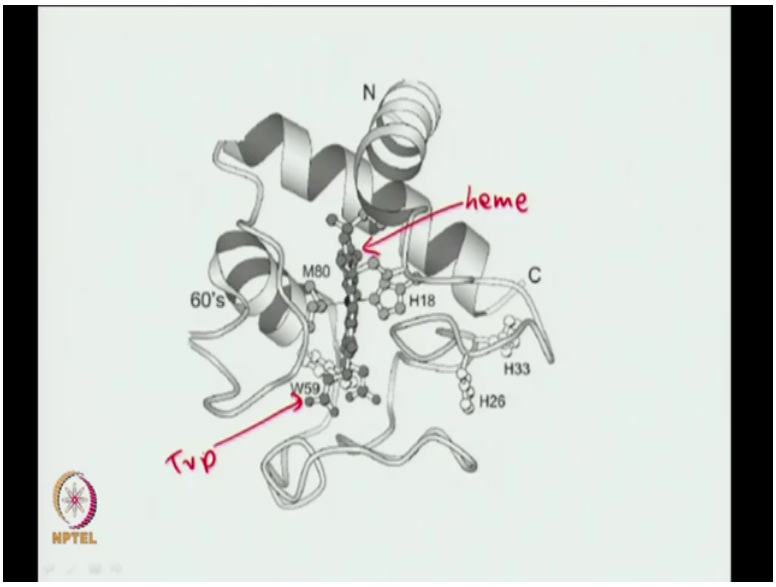

So, this example is the protein cytochrome C all of you guys have heard about this protein cytochrome C? You must have right in the respiratory chain and all these things the electron transfer right? Now, the cytochrome C is primarily alpha helical you can see this N is the N terminus and this C is the C terminus. Now, this is what is most important. You can see in the middle this is heme right and here this is a tryptophan W59; that is your tryptophan that means, that is tryptophan which is at position number 59 in the chain.

Now, what happens is; what happens is in the folded state remember in the folded state or before going there the tryptophan and the heme, they constitute a FRET pair; that means, the tryptophan is the donor and the heme is the acceptor just take it from me for the time being. So, what will happen is remember what I told you before, if they are very close to each other then they are going to talk; that means, tryptophan is going to transfer energy to the acceptor.

If we are going to go; if they are going to go far apart then that energy transfer would decrease. So, you can understand when the when the protein is folded, Trp and heme because they are very close, the Trp intensity would be very low the florescence intensity. Why? Because it has transferred most of its energy to where?

Student: (Refer Time: 45:26).

The heme. The moment you unfold it because then I am moving far apart because of unfolding what will happen is a tryptophan intensity would.

Student: (Refer Time: 45:35).

Go on increasing. Now, this is just the reverse of what we just discussed. In the last one we just discussed that it should be the reverse that in the folded state should be having a higher intensity unfolded state lower, but that was not a heme protein, now this is a heme protein we are talking about.

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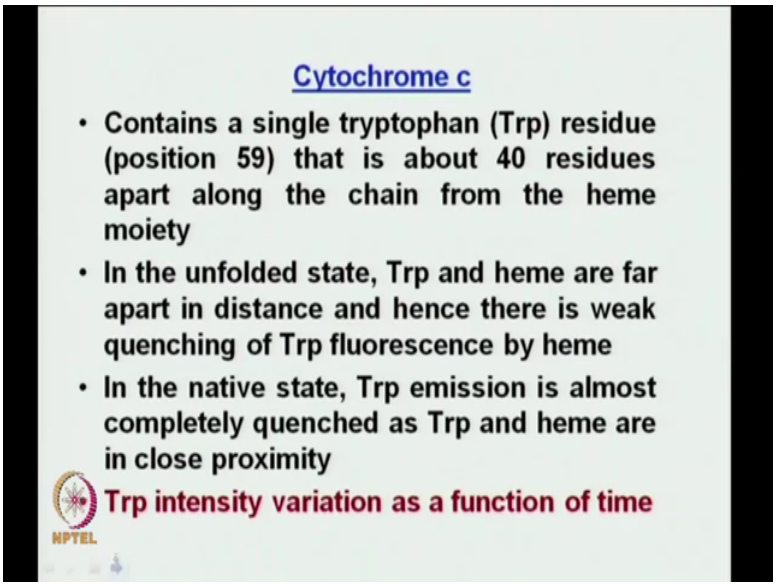

Now, see this is what happens then. So, some facts about cytochrome c it contains a single tryptophan residue that is about 40 residues apart or along the chain from the heme moiety ok. In the unfolded state, Trp and heme are far apart in distance and hence there is a weak quenching of Trp fluorescence by heme.

In the native state, Trp emission is almost completely quenched as Trp and heme are in very close proximity. The distance is about 2 nanometers or 20 angstroms that is very close and hence most of the energy from Trp is transferred to the heme good. So, now, you can understand what will happen is that if you are going to follow a folding experiment, what would happen to the Trp intensity now? Would it increase or decrease?

Student: Would decrease.

It would decrease, why? Because in the folded state it has the lowest intensity, in the unfolded state it has the?

Student: Highest intensity.

Highest intensity, right ok. So, that is what you see here.

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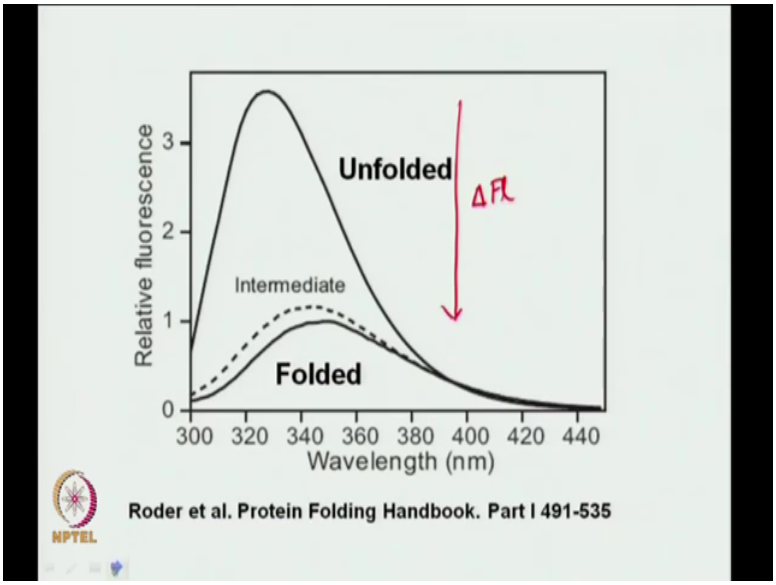

So, Trp and then you look at the Trp intensity variation as a function of time. So, this what you this what we see now. Do not worry about the; do not worry about the shape or the emission maxima of the figures spectra do not worry about that. This is what you just consider. In the unfolded state the Trp has a very high intensity; in the folded state the cytochrome c the folded state has a very low Trp intensity.

So, if you would monitor this change, if you would monitor this change this change in florescence say delta F delta Fl where Fl is florescence right if you would monitor this change as a function of time you would getting a kinetics that is what people have done right and that is what I am going to show you right now ok.

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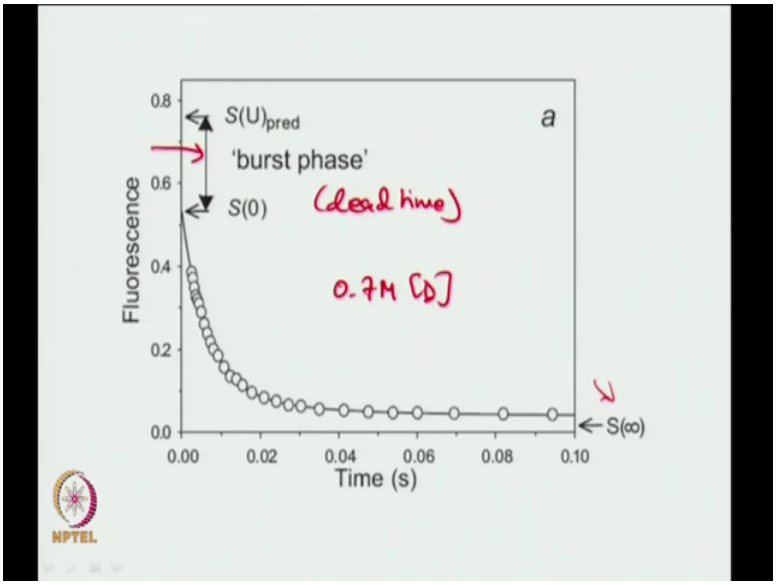

So, look at this. This is a typical this is the typical kinetics you are looking at. Now, do not worry about this burst phase first. Let me tell you what you are seeing. On the x-axis what do you have? You have time. On the y-axis what do you have? You have fluorescence right.

Now, see there is something known as S infinity. Can you see this something known as f S infinity out here? What does S infinity mean? S is the signal; signal at finite time, what does that mean to you? What do we mean by infinite time?

Student: (Refer Time: 48:14).

Ha?

Student: Reaction completed.

Reaction completed ok. So, tell me a thermodynamic term for infinite time?

Student: Equilibrium.

Equilibrium, good. Equilibrium, so, that means, infinity means that after you have refolded the protein, you have allowed it to come to a certain point beyond which there will be no further change because it has already reached equilibrium. So, S infinity refers to it is finite time infinite time is not naturally that you sit there and wait for infinity right literally. You just wait for the reaction to over, what you see is that in two consecutive or three consecutive time points there is not much of a change in florescence; that means, you know whatever change has had to happen did happen good.

Now, what is S 0, can someone tell me? If S infinity is monitoring the signal at infinite time what is S 0.

Student: (Refer Time: 49:11).

Monitoring the intensity at time is equal to 0 ok. Now, in a stopped-flow technique what is the time 0? Tell me when does your time 0 start?

Student: (Refer Time: 49:22).

Yes, then when it hits the block right. So, you can understand now, I have already told you by the time; by the time you start looking at the first point; that means, by the time you have hit

the block or the syringe 3 has hit the block that solution has already mixed and it is already aged; that means, it is already there for some time during which you cannot collect the data.

But, some changes has already happened during that time. So, then your S 0; that means, your time 0 starts from when you the stopping block hits the syringe 3, but that is not time 0 for the reaction, is it? The reaction has already started before. Your time 0 is defined by the instrument. Your time 0 is not defined by the reaction. The reaction time 0 is far earlier. The only reason your time 0 is later because that is what the technique offers you. You cannot go faster than that. So, then you can understand.

So, now, you can see this difference, what is this difference correspond to now? A certain time what is the time?

Student: Dead time.

The dead time. So, this essentially is your corresponding to dead time. Why is it called the burst phase? It is called the burst phase because there is a certain burst in conformational change in the protein which are not being able to collect. Burst happens always very fast right and because it is occurring so fast much faster, than when you can start collecting it is called the burst phase with respect to your instrument. Is that clear? That is why it is called the burst phase here comes in a burst right.

Now, you can; now you can ask me what is S U predicted, right? What is S U predicted? See how can you give how can you get S U predicted?

Student: When we pressed it?

The moment you pressed?

Student: Yes.

But, then when the moment you pressed right you are not being able to collect? Right. So, how will you get S U predicted, tell me? Is there any other way? Tell me, so, this is what we will end our class with. Let us not talk about S U predicted now, let us talk about the signal at in finite time.

Suppose, you would predict the signal at in finite time ok, what would you do? You are not doing a kinetics, I am telling you are looking at an equilibrium situation where you are predicting the signal at infinite time. How would you predict the signal, come on?

Student: (Refer Time: 52:15).

Ah?

Student: (Refer Time: 52:19).

No, but you are not doing an kinetic experiment. I am telling you that how would you predict your signal at infinite time? Come on guys, we have done this. Apart from kinetics, what have we done?

Student: Thermodynamics.

Good, we have done thermodynamics. Now, suppose I am doing this change; suppose I am doing this change; suppose I am doing this change to a final concentration of 0.7 molar denaturant this is (Refer Time: 52:45). But, you always know if you would do a transition; that means, if you would do a denaturing transition of this protein cytochrome c as a function of mono hydrochloride you will be getting certain points certain values of fluorescence as a function of denaturant, do you remember this or not?

These are thermodynamics. Remember these transitions, the sigmoidal transitions? One was thermal, the other one was what?

Student: Chemical.

Chemical. Now, from chemical it is the equilibrium; that means, that is in finite time. So, when you doing an equilibrium at each and every point whether it is 0.5, 0.7, 1 molar mono hydrochloride you exactly know what your equilibrium value of fluorescence is in this case. So, you can always trace back and look at the predicted value and look at the ratio. I will talk about this more in the next class ok.

So, that is how you can do because you always have equilibrium experiments at your hand. Now, tell me what about S U predicted? How would you do that?

Student: (Refer Time: 53:58).

Huh?

Student: By extrapolation.

By extrapolation from where?

Student: (Refer Time: 54:09).

Student: Linear graphs (Refer Time: 54:10).

I can. Extrapolation is only applied to linear graphs? Listen, one way is if you are talking about S U predicted that is time 0, think about a negative time; that means, before the mixing is actually started, what is it S U predicted? It should be equivalent to your thermodynamic variable or thermodynamic value at that high concentration of denaturant like S infinity. The moment you mix it, you cannot see.

What you said is right, you extrapolate. So, what you do is you do your final denaturant concentration is varied like say 1 molar, 0.7 molar, 0.5 molar, 1.5 molar ok. You draw these I mean you have this kinetic traces, the circles are your points data points, the black curve is an exponential fit and then what you do is you can see out here there is no data point out there. So, you can have different denaturant concentrations final, extrapolate the exponential thing and then try to get S of U that is one way of doing it right, but it needs a series of experiments right.

We will stop here. I have already gone beyond time.