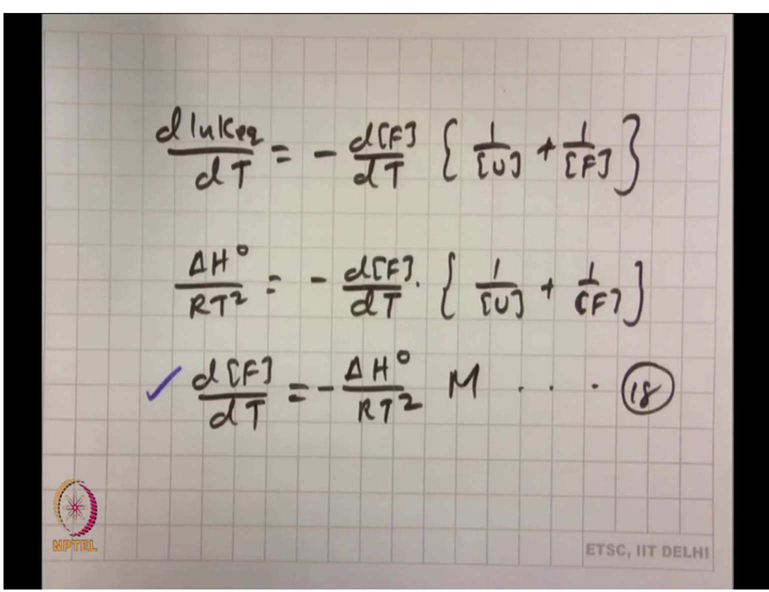
Bio-Physical Chemistry
Dr. Pramit Chowdhury
Department of Chemistry
Indian Institute of Technology, Delhi

Lecture - 34
Protein Folding Kinetics: Rapid Mixing and Relaxation Techniques (Contd.)

So, let us carry on with our discussions on the temperature jump technique. So, this is the last equation we cannot derived yesterday.

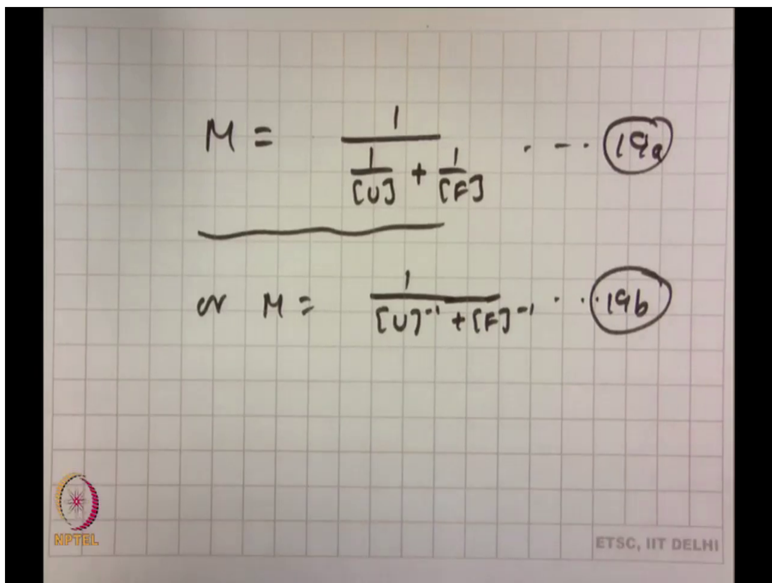
(Refer Slide Time: 00:30)



So, this is the one I am talking about equation number 18. So, where the change of the concentration of the folded state with temperature is given by this with the negative sign delta H naught over RT square times M. And then, the most important thing is that this change of

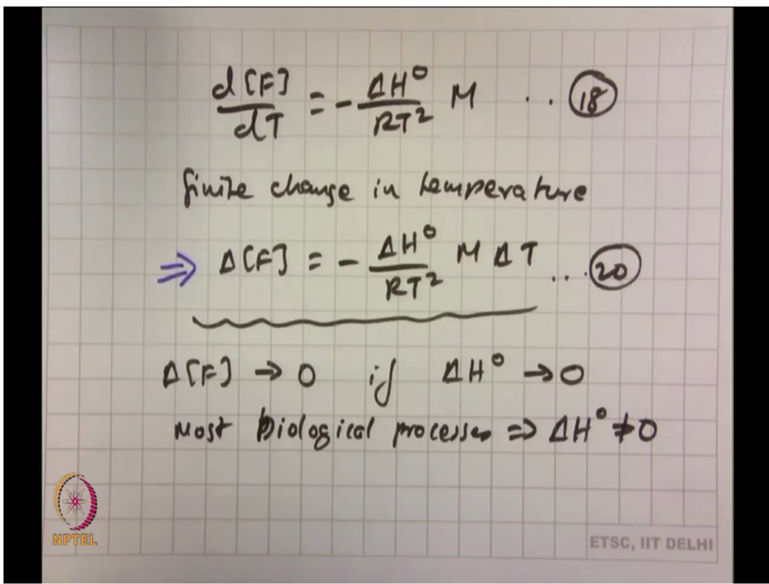
the folded state whether the concentration of the folded state is change or not will depend on two things; one is the delta H naught and the other one is the M right.

(Refer Slide Time: 01:06)



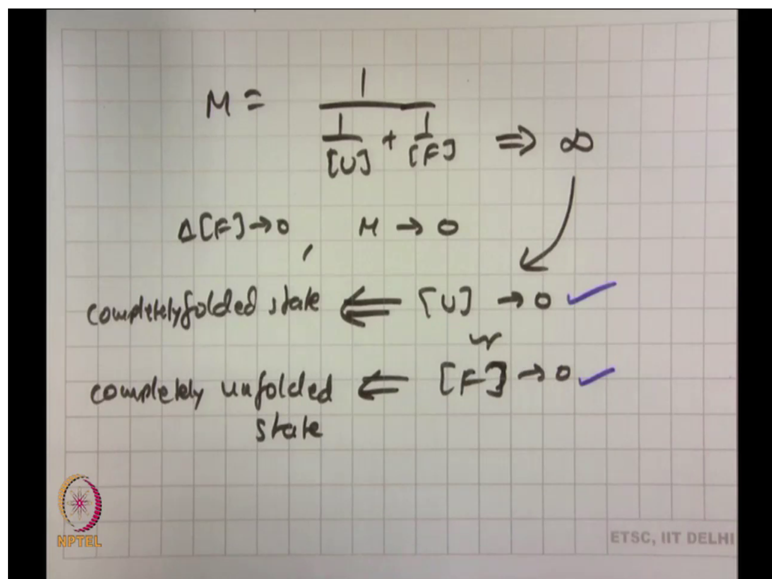
So, this is what M was. This is how we defined M; say it is 1 by 1 by concentration of U plus 1 by concentration of F.

(Refer Slide Time: 01:18)



And then, we went for a finite change; we went for a finite change and this is what we got right. This is one of the most important equations we have for lesser for temperature jump relaxation method. So, again as we see out here, the change in a folded state dependence upon 2 things delta H naught and M. See, if delta H naught is 0 then, the there would be no change in the concentration of the folded state. Which means is that if you are going to have a temperature jump, you better have a change in enthalpy right otherwise you are not going to see any change and good for us that most of the processes we are studying are accompanied by enthalpy changes and hence, that is taking care of.

(Refer Slide Time: 02:04)



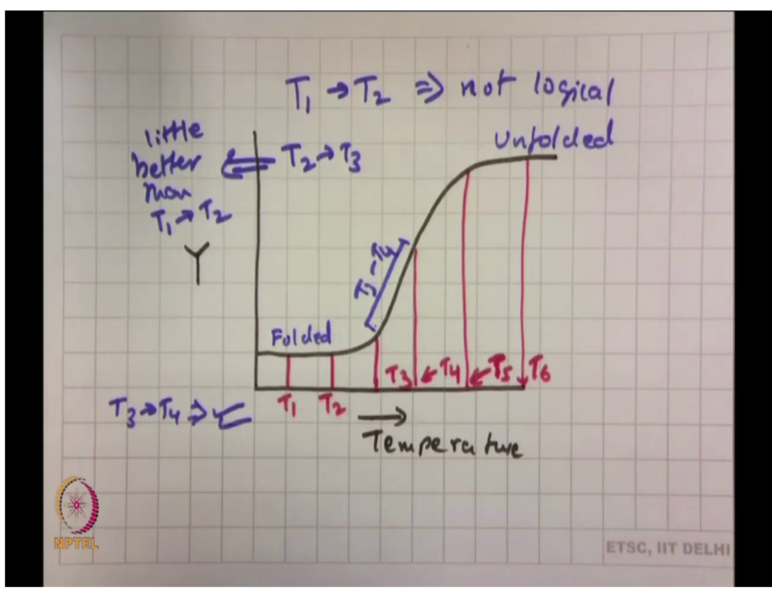
Now, the other important aspect was this, this was the last thing we were discussing. What about M? What is it dependence on M like? So, when can M be 0? M can be 0 if it is 1 by infinity that is the denominator is 0; rather denominator is infinity. So, the denominator is comprised of two terms, the reciprocal of the concentration of unfolded state and the reciprocal of the concentration of the folded state right.

So, what it means is that, for these one of these, even if one of these go to infinity; that means, if concentration U is equal to 0 or concentration of F is equal 0 isn't that denominator goes to infinity straight up right. So, then what it says is that, if concentration of U is equal to 0; that means, if concentration of U tends to 0 this we are talking about this one, then we are where we are completely at the folded side. And were the concentration of F is 0, the (Refer Time: 02:52) completely at the.

Student: Unfolded side.

Unfolded side. And hence, if you are at the two extremes, you better not use your temperature method because you are not going to make get much out of it why? Because M essentially will go to 0 for you right.

(Refer Slide Time: 03:28)



So, how does this translate into a measurement? The way it translates into a measurement is like this; say for example, consider a thermal denaturation profile right. So, let us look at a thermal denaturation profile of a protein. And suppose, you are looking at a certain change in a spectroscopic signal say its fluorescence its Y and this is how it looks like right. So, I can say this one is your folded state and this one is your unfolded ok. Now, on the x axis what we

have is we have temperature; that means, we are doing a temperature induced denature that is thermal denaturation.

So, now let us now think about this. We pick out few temperatures pick out say this is T 1, T 2, T 3, T 4, T 5, T 6. Now let us go interval by interval ok. Suppose, I making a temperature jump from T 1 to T 2 right; suppose I making a temperature jump from T 1 to T 2 to T 2. Now through out what your temperature jump is doing it is observing or what it is trying to give you an idea of the change in your concentration of the folded state ok. Now if you are jumping from T 1 to T 2, would this be a logical way of doing it based on the discussion we had before?

Student: No.

Why not?

Student: Completely folded.

Completely folded. So, you look at this where is T 1 to T 2? Even if you go from T 1 to T 2, you are still on your folded base is not it? There has been no unfolding so that means, even if there is any change; in delta F, what would that be very very small or almost close to 0 what is it mean? It goes back to M; so, because it is completely folded, then what happens to the concentration of U it is very small being close to 0. Hence, the denominator goes to what infinity right.

So, if you are on the folded base line; if you are on the folded base line, you would not be able to do that because you would not be able to do a temperature jump because even if you do a temperature jump, the change in the concentration of the folded state would be very small too small for you to detect anything even spectroscopically is that clear? Because you see on your y axis you have the spectroscopic signal Y right.

So, this folded base line from T 1 to T 2 because it is kind of parallel to the x axis what does it mean? That means, your Y is not changing; that means, there is almost no change in spectroscopic signal, you are not going to able to figure out anything right. And you are not supposed to because you are still in the folded base line your the folded state clear.

Now let us go to T 1 to T 3, it is a little better; it is a little better because when you go to T 3, you have just started unfolding the protein is not it ok? So obviously, T 1 the gap between so, if your if I write this one is not logical say T 1 to T 2 is not logical then, I can write say T 2 to T 3 ; T 2 to T 3 this would be better than T 1 to T 2 at least little better because you have a small change in the concentration of the folded state, but still keep in mind, look at the change in the spectroscopic signal from T 1 to T 3 or rather T 2 to T 3 it is not that high isn't it, it is still small ok.

Now suppose, you make a change from T 2 to T 4 is that a good one of doing it?

Student: No sir.

Look at T 2 to T 4 so, this is T 3 to T 4 right this is T 3 to T 4. Here you have a huge change in concentration of the folded state right. You have moved from a place where you have just started unfolding to a place where there is a huge amount of unfolded state present; that means, the concentration of unfolded state has decrease. So, then T 3 to T 4 would be a very good integral for you to do a temperature jump right. So, then T 3 to T 4 wants to try to do it because you have a huge change in the concentration of the folded state or unfolded state and your spectroscopic signal is also changing by this amount; by this amount your spectroscopic signal is also changing ok.

What about T 4 to T 5? There is still a change ok. Now see, T 4 to T 5 is also logical, so, T 4 to T 5 is almost like T 3 to T 4 right ok. Now, tell me this, the interval between T 3 to T 5 right the interval in T 3 to T 5 what does what does that signify you know what is what is significance of that interval?

Student: Transition region.

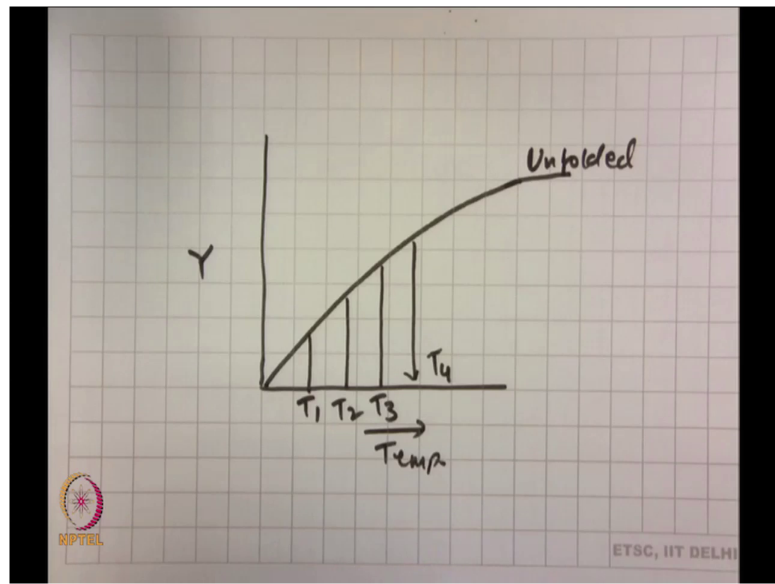
That is your transition region right and if you go back to your thermodynamics remember when we talked about delta G calculation, we said that we cannot do any delta G calculation based on your completely folded or completely unfolded state, you would you would be better of doing delta G calculation where?

Student: Transition region.

Where we have right in the transition region, where we have appraisal amounts of both present, the same thing is applying here. You can only have a decent change in spectroscopic signal if you are in the transition region only because in this case, the transition is sharp and you have a detectable change in the signals even you have a small temperature jump does not matter what your temperature jump is clear ok.

So obviously, then you can understand from T 3 to T 5, this would be the region where you would try to focus on your experiment because beneath T 3, you would not be getting much and above T 5 what would happen? Again, you are not be getting much why because now you are in the unfolded region where your concentration folded state is really low clear. So, that is why, when people typically do experiments remember when people typically do experiments, this this is the region they want to target based on this logic that we just discussed.

(Refer Slide Time: 10:45)



Now please keep in mind, every protein; every protein depending upon what type of protein you are taking would not be giving this amount of or this cooperative a transition; different proteins would be having different transitions. For example, let me just give you a quick plot say for a protein, this is the spectroscopic signal and this is temperature right and its transition looks something like this say its transition is looking something like this right. So, this is your unfolded base line.

So, at least on the unfolded base line, you can see its kind of tapering of not much of a change, but do you have a folded base line? You actually do not have a folded base line right. Now tell me this, if I have here T 1, here T 2, here T 3 throughout this temperature intervals would not be having changes? You would be having changes right. So, if you would be having changes then, you can easily monitor or you can easily go for your temperature jump technique within

this interval because that is your transition region ok. So, then this is T 4, T 4 and so on is it clear?

So, again you take any protein, but before you do a temperature jump, what you do what would you rather do tell me? Before you would you would do a kinetic measurement assuming the fact that you have a temperature jump instrument with you right, I have not talked about the instrumentation I will just go over it briefly in a couple of slides. But again the question is if you have a temperature jump a instrument with you right you can do a temperature jump, you are given a protein which you not know much about would you go for a temperature jump straight away? Would you start with that temperature jump yes or no?

Student: No sir.

No, what would you rather do first?

Student: (Refer time: 12:28).

Yes, you would do a?

Student: Thermal denaturation.

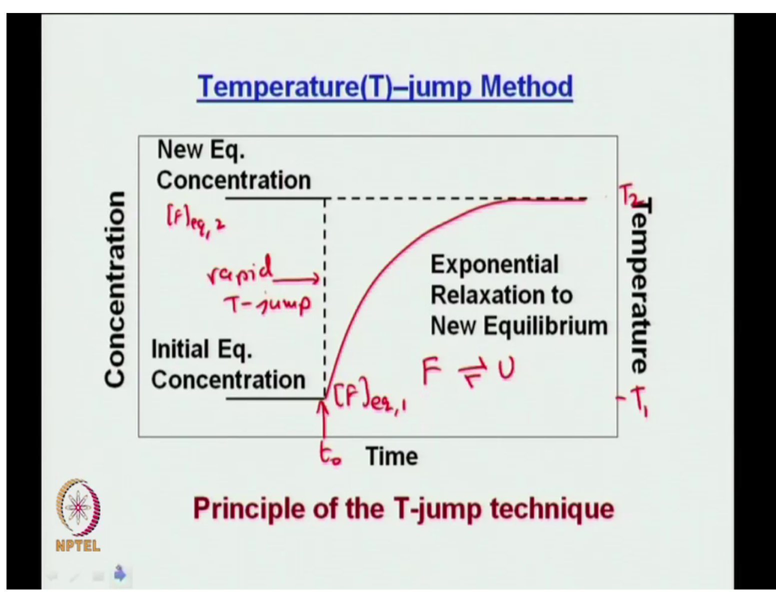
You would do a thermal denaturation; that means, that is why equilibrium is so important. You first do a thermal denaturation right just based on cd or florescence or whatever to see where the transition is. Because blindly if you start from any particular temperature, you might not be getting any signal, which will tell you that may be there is not much happening out there, may be either you are in the very folded base line or you are in the very much unfolded base line ok.

So, that is why you first take a protein and before you do any experiment like this, the first thing you do is you go for a equilibrium denaturation experiment, if you are doing a

temperature dependence, then you do go a thermal denaturation, if you are doing a rapid mixing, then you go for a chemical denaturation, but you have to have that thing in hand ok.

So, that is typically what your temperature jump is all about right and again you cannot just take any arbitrary temperature, you have to make sure that you are in a region where there appraisal amounts of both present that is the bottom line. Only then, would M be significant remember otherwise M would be close to 0 and hence, you change would also be very small right. So, this was about the theory of the temperature jump.

(Refer Slide Time: 13:56)



Now going back to the slides, we was talking about relaxation technique this was the temperature jump technique we are talked about yesterday. Are you guys clear about the concept behind how the temperature jump is done? How you have to go from one equilibrium to another equilibrium and so on? What relaxation looking at?

(Refer Slide Time: 14:09)

- Protein is initially at equilibrium ($T = T_1$) --- Initial Eq Concentration
- At time $t = t_0$, a pulse, i.e. a rapid change in temperature is brought about in the solution; the temperature rises to T_2
- Shift in temperature signifies that equilibrium constant has changed
- Concentration of folded protein has to change
- Assumption: rate of equilibration is slow as compared to the temperature rise

So, this we had completed, this we had seen yesterday and this is where I stopped looking at the slide.

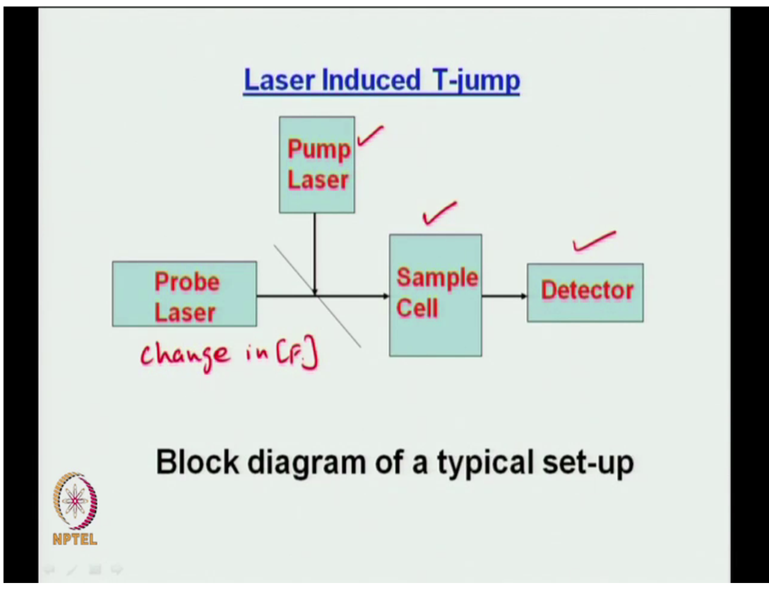
(Refer Slide Time: 14:17)

- The principle is to make a rapid change in temperature (ΔT) assuming $\Delta H^o \neq 0$ and $M \neq 0$
- As a result of this rapid change, the folded protein finds itself in a non-equilibrium situation (since temperature increase has shifted the equilibrium constant)
- Thus the folded protein adjusts itself to the new equilibrium situation at the higher temperature T_2
- The time it (folded protein) takes to adjust is known as the relaxation time

So, the principle is to make a rapid change in temperature delta T assuming delta H naught is not equal to 0 and M is not equal to 0 that is what we just discussed. As a result of this rapid change, the folded protein finds itself in a non equilibrium situation its repetition of what we did yesterday. Since, temperature increase has shifted the equilibrium constant good.

Thus the folded protein adjust itself to the new equilibrium situation at the higher temperature T 2; that is why we always write concentration of F equilibrium comma 2, 2 means at temperature T 2 right. And, the time it takes that is the folded protein takes to adjust to this new equilibrium situation is known as your relaxation time that is why it is called relaxation method. Because you are changing something and then, because of that change the protein is relaxing to the new change that is why it is called relaxation method.

(Refer Slide Time: 15:17)



So, this is about instrumentation how do you actually do it right. One of the, there are two ways of doing it, two popular ways; one is using a capacitor discharge you suddenly discharge a capacitor right you have a temperature increase and then you do it, the other way of doing it is using a laser right now this is how you do it. So, look at the block diagram, it is just a schematic and try to understand it.

So, in the sample cell, you have a sample right so, this is this is the sample cell you have your sample. So, your protein in buffer right. It is at the certain temperature right. Now, you are trying to increase the temperature from T 1 to T 2 making sure that T 1 to T 2 are kind of in the transition region at least the spectroscopic signal change between T 1 to T 2 is decent enough for you to get something out.

Now, what is the what does this pump laser do? So, look at this pump laser, what does this pump laser do? I will show you a better diagram in the next slide but let me tell you this ok. You have to do a temperature jump; that means, you have to increase a temperature the pump laser is such; the pump laser is such that you have a protein in buffer the sample I am not telling you which part, but the sample absorbs the laser.;

The sample absorbs the laser radiation of the pump laser; now because the laser is appearing in if the laser light is appearing in pulses. That means, like this, like this it is not a continuous light, it is a varying impulses like this you have a laser light, again you have a laser light, again you have a laser light right it is called a laser pulse. And this pulse is very small you have a huge amount of energy being thrown into the sample. Now, do not worry about the actual details of it right.

So, now, the sample absorbs it. The moment the sample absorbs your laser, what will happen is because the laser is putting a lot of energy, the sample will undergo increase in energy so, how would the sample reacts respond? It will undergo increase in.

Student: Temperature.

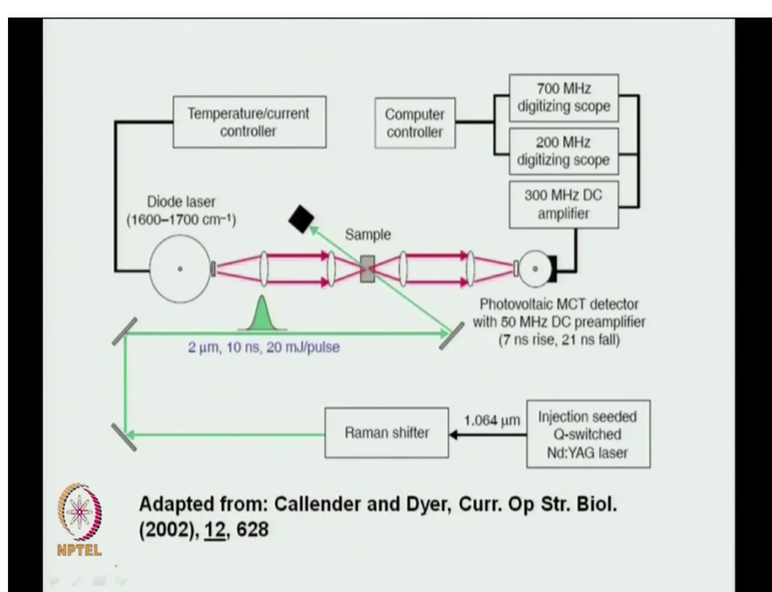
Temperature. The moment you undergo increase in temperature, the protein now will also respond right and then, you look at the change using a detector using a detector, but what is the probe laser doing? This is; this is what the probe laser is doing, you are pumping at a different wave length it is called the pump laser because your pumping energy into the system that is why it is called a pump laser your system is absorbing the pump laser and undergoing increase in temperature.

But now, you are probing. Probing means you are probing the change; you are probing the change in the concentration of the folded state of the protein, but how is it coming out in terms of a certain spectroscopic signal right and that signal, you are probing with a different laser called a probe laser. So, this means this is the one you are using to monitor the change in the concentration of the folded state. Just hold on, I will tell you in little more clear terms what

is the difference between the pump and the probe laser, what am I referring to, its actually very interesting ok.

So, do you understand now, the difference of pump and probe laser? So, pump laser what it does is, it helps to increase the temperature that is it, it does not help you to detect. The probe laser comes in because you are now probing right, you are probing so, the pump is pumping in energy increase in the temperature. The probe laser as the name suggests is its helping you to probe the change in spectroscopic signal that is why it is called a probe laser. So, you are probing, you are searching, you are probing for the change in spectroscopic signal ok.

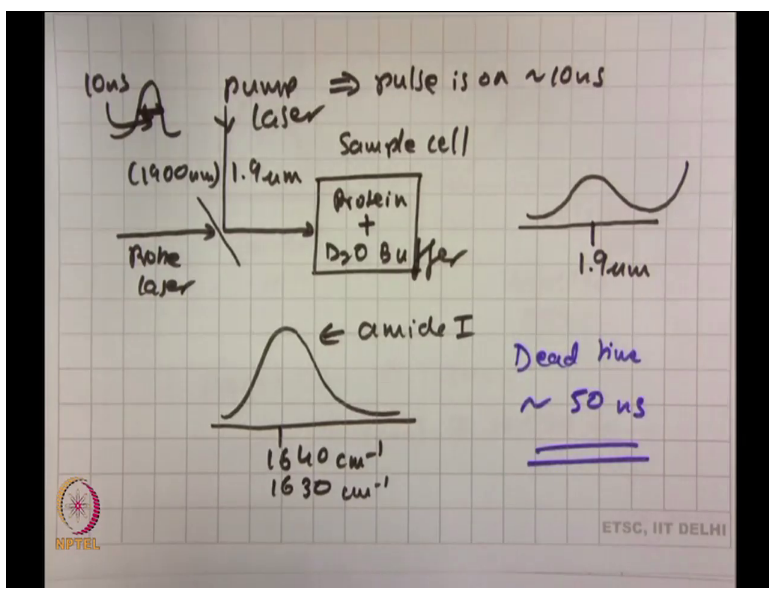
(Refer Slide Time: 19:02)



Now, this is a very simplistic diagram, this is how it happens, but how does it actually take place? Ok, this is an actual set u p ok; this is an actual set up not that you have to know it by

heart, but let us try to understand what happens. First of all, let me go back to the sheet and tell you exactly what is trying to take place.

(Refer Slide Time: 19:24)



So, you have a sample cell. So, this is your sample cell ok; this is your sample cell, in this what you have is your protein in buffer but I am taking D 2 O buffer I am not taking H 2 O buffer. I will tell you reason for that right, I am taking a D 2 O buffer. That means, instead of H 2 I am taking what? Heavy water right you know D 2 O is available ok.

Now, my laser is coming in, if you remember my laser is coming in. So, based on that block diagram, the pump laser comes in like this and goes into this. So, this is my pump laser. Now listen to this very carefully, what do you think which component; which component in this sample cell do you think is absorbing the pump laser?

Student: D 2 O.

D 2 O excellent. See the concentration of the protein is typically depending upon this size of the protein is typically in the order of higher micro molars to some milli molar right, but what is the concentration of D 2 O? It is much higher, isn't it? You look at the concentration of water, what is the concentration of water neat water? Neat water is 55 molar if you would remember. So, then accordingly with the density, you can easily get the concentrated D 2 O it would also be similar ok.

So, the concentration of D 2 O is much higher. So, the more D 2 O you have, the more you would be able to absorb the energy because you are more D 2 O molecules as simple as that. Now, the pump laser; the pump laser has a wave length of 1.9 micron; that means, 1900 nanometers; that means, 1900 nanometers ok. Why am I using 1.9 micron?

I am using 1.9 micron because if you ever, if would ever look at the IR spectrum of D 2 O, if you would ever look at the IR spectrum of D 2 O and I am only going to show you that, the IR spectrum of D 2 O has a small absorption band at 1.9 micron; has a small absorption band at 1.9 micron and it has a much bigger absorption band elsewhere. But, there are reasons why you always go for the small absorption band to maintain uniformity in heating, but you know that is separate do not worry about it.

Again so, at 1.9 micron your pump laser is coming in because your D 2 O has an absorbance out there; because your D 2 O has an absorbance out there it would absorb the 1.9 micron is it clear ok. Then the moment it absorbs the 1.9 micron, I will tell you what happens is the pump laser; the pump laser each pulse; each pulse is on for about say 10 nano seconds; each pulse is on for about 10 nano seconds and with pretty high energy.

So, the D 2 O absorbs this pump laser ah that means, this wavelength because it absorbs what happens is it has got thermally excited because it is absorb the laser energy right, the moment it is thermally excited, it is at a excited state right. Now what happens is, it will relax because it cannot stay at the excited state forever it will relax. Now on relaxation what would it do?

Student: (Refer time: 23:14).

It would get rid of the excess energy, now tell me who is going to pick that excess energy up?

Student: (Refer time: 23:21).

The protein would; the protein would be picking the excess energy up. So, you can realize one thing, you are not directly heating the protein, what are you directly heating? You are actually directly heating the solvent which is D 2 O. D 2 O is getting excited to an excited state, then it relaxes, it gets rid of the excess energy and that energy is taken as the taken up by the protein and the protein also undergoes a?

Student: Rise in.

Rise in temperature that is how we bring about a change in temperature in the protein molecules ok. Now this can happen very fast; this can happen very fast, much faster then what we have encountered in the rapid mixing techniques even with the microfluidic mixer.

This can happen in the order of this D 2 O; this D 2 O can let its energy go to the protein in the order of say 100 picoseconds or so. That means, the faster a protein takes it up depending upon the size of the food and all those things, the faster essentially would be able to monitor the change ok, but there is a limitation I will tell you what the limitation is right.

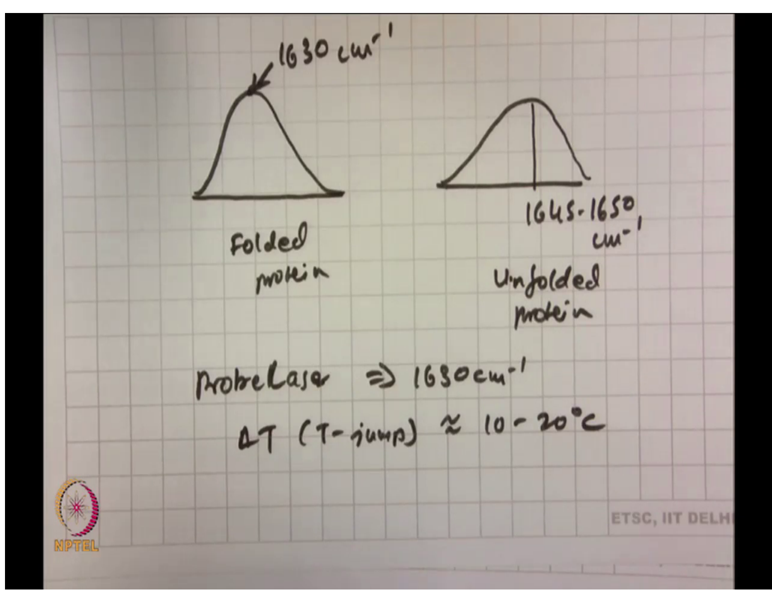
So, this was about the pumping; that means, now you have done what you have done is, you have increased the temperature, the protein has undergone a temperature change and the temperature and the protein is now at a higher temperature T 2 from T 1 because of absorption of this laser pump or laser wave length. Now, how would you prove?

Now what is known is; what is known is we will talk about IR what is known is that proteins typically have well developed absorption bands this is called a amide I band; amide I band

depending upon the structure in the region of 1640 centimeter inverse say 1635, 1640 centimeter inverse right now you can write 1640, 1630 either way is fine.

Now remember there was another laser what was that laser? That laser was the probe laser ok. So, the probe laser was coming from this side; the probe laser was coming from this side now what happens is there is a reason why I have written 1630 and 1640 see this is what happens typically right.

(Refer Slide Time: 25:59)



If we take an alpha helical protein, if you would take an alpha helical protein I am just going beyond what I showed right now, but let me give you the significance. If you take an alpha helical protein what happens is, at the folded state the alpha helical protein typically has an higher absorption at 1630 centimeter inverse ok.

Now when you unfold the protein so, this is folded protein, when you unfold the protein, the same absorption band; the same absorption band that this amide I band shifts to about 1645 to 1650 centimeter inverse ok. So, this is your unfolded protein. Now, this is what the probe laser are, probe laser does for you. The probe laser; the probe laser; the probe laser has a wavelength or frequency corresponding to 1630 centimeter inverse corresponding 1630 centimeter inverse.

Now, tell me the concept is very simple. Your probe laser is always probing what at the peak of it folded state which is 1630 that is the absorption peak of a folded state, this is IR right. Now, what is going to happen if I am going to decrease the amount of folded state and increase the amount of unfolded state; what is going to happen to the intensity of this IR band?

Student: Decrease.

It is going to decrease? So that means, the probe laser is actually probing the change in intensity of this band at 1630 centimeter inverse as a function of time. Did you get my point? So, the probe laser has a fixed lasing frequency at what? 1630 centimeter inverse just one, see if the probe laser is part is fixed at 1630 and you are doing a temperature jump because the protein is unfolding. That means, the intensity which was there at T 1 at 1630, when you go to T 2 it would decrease because it would decrease the probe would pick it up because it is always fixed at 1630.

So, it sees that as you have increase the temperature and the protein is responding, the probe what is what does the probe see; at every time point it slowly sees that my intensity at 1630 slowly decreasing, decreasing, decreasing, decreasing as a function of time. And then, you pick the kinetics up to the detector that is what your probe laser is doing, that is why you have two different lasers; is it clear how the experiment is done? This is how the experiment is done ok.

Now, what about using D 2 O? This is why we use D 2 O. The reason we use D 2 O is that if you; if you would use H 2 O not D 2 O if you would use H 2 O a life would have been much

simpler if you would have used H_2O. But the problem is remember where are you probing the protein? At 1630, H_2O has a huge absorption band at that place then, what about change you have in the protein would be masked by that absorption of H_2O because it is a huge absorption, but the moment you do an isotropic substitution, what you do is remember your vibrational frequency depends upon what?

Student: Mu.

1 by 2 pi root over k by mu and keeping force constant the same because mu is changing there would be shift in a vibration frequency. So, you so, you move; you move a masking band out of the way and just look at the protein itself that is the function of D_2O that is why you do it in D_2O clear.

Student: Yes sir.

So, there are few aspects you have to keep in mind, one is you have two lasers; one is the pump laser, one is the probe laser, second is you are not directly heating the protein instead what are you heating? You are heating D_2O. D_2O has a small absorption band at about 1.9 micron that is the wavelength of your pump laser, the probe laser say as a fix absorption wavelength of 1630 centimeter inverse. What is it probing? It is probing the change in intensity of the alpha helical band of the folded state at 1630 centimeter inverse based on your temperature jump.

Now typically, the magnitude of temperature jump you do is between like it can be anywhere I mean to get a decent change, if it can be say from 8 to 15 degree Celsius or even more than that. So, your delta T there is a temperature jump is about 8 to 15 degree Celsius or more than that ok. So, let me write that down. So, your delta T; delta T that is your T jump is typically between 10 to 20 degree Celsius its typically like that and if you are getting a change, then using all these things together you are getting the change ok.

Now, just to tell you the significance of one more thing, remember I told you that the pump laser has a pulse of 10 nanoseconds; that means, when the pump laser is coming in. That

means, when the pump laser is coming in, it is coming in with the pulse of light which is looking like this and the width of this is 10 nanoseconds. It is called something referred to as full width at half maximum, but do not worry about it, it is the width of your pulse laser pulse.

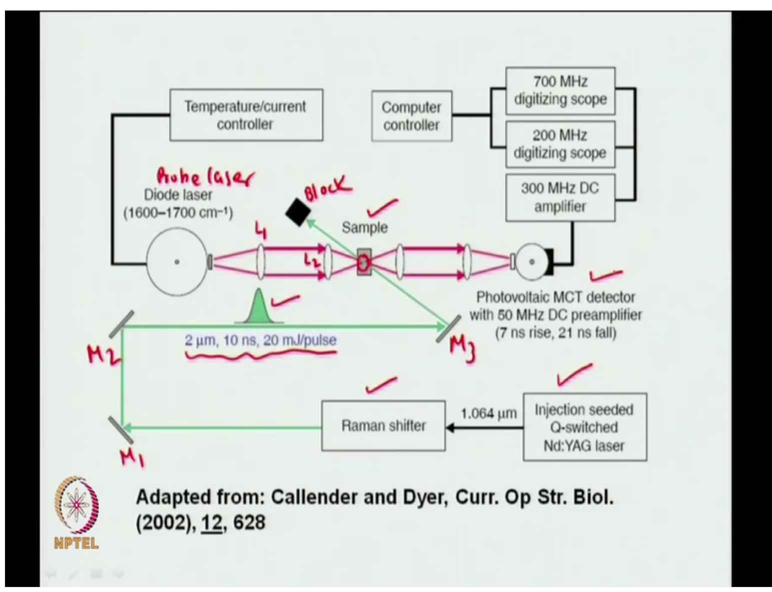
So, you can understand one thing, if the laser pulse is on for 10 nanoseconds; that means, for 10 nanoseconds it is on and then it goes off and then it comes on again it comes in pulses. So, within 10 nanoseconds, it actually not being able to detect any changes because the laser pulse is always on those (Refer Time: 31:41) 10 nanoseconds. You can only detect the change when? You can only detect the change when your laser pulse is off and your stuff is relaxing because of the new temperature jump.

So, that is why typically here in temperature jump technique, your fastest time scale is in the order of the one I am talking about right now is in the order of say 30 to 40 nanoseconds or more; that means, anything greater than 40 to 50 nanoseconds you can easily probe using a temperature jump technique. But there is also a limitation on the higher side, if I start going into it that is will be too much of details.

But, what I am trying to tell you is that, you started from a stop flow which was dead time was 1 milli second, you went a continuous flow with the dead time was 45 micro second, you made a further improvement by making a micro fluidic with dead time was 5 microsecond. Now, using a temperature jump technique you are coming to a dead time which is about 50 nanoseconds.

So, in this case I can write the dead time is about 50 nanoseconds, but people have been able to go much faster than that you know, but this is in general what happens right.

(Refer Slide Time: 33:16)



Now let us come back to the slide and tell you something about the instrumentation just follow me, not that you have to remember it by heart, but just follow me ok. So, this is a laser, this is your starting laser, this laser has a wavelength of you can see its 1.064 micron that is 1 0 6 4 nanometers ok. It goes through something known as a Raman shifter. A Raman shifter, you know if you go through a Raman, if a Raman spectroscopy there are two kinds of lines, what are the two kinds of lines you get apart from the Rayleigh one?

Student: Strokes and anti-strokes.

Strokes and anti-strokes right ok. Now remember where does D 2 O absorb? I said 1.9 micron so that means, I have to go from a wavelength of 1.1 micron essentially which is 1.064 to a wavelength of what? 1.9 micron. So, I would do a Raman shifting and take the Stokes line

which what which Stoke line is that the that Stokes line which shifts or decreases its wavelength or rather increases wavelength that is decrease the frequency.

Increases its wavelength from 1.11 micron to 1.9 micron that is the essence of having a Raman shifter there to shift your wavelength to that place there is that value 1.9 micron where D 2 O can absorb that is a use of the Raman shifter right good. Now, you can see you are going like this so, this is the mirror see this is M 1, this is M 2 then what are the mirrors? The mirror mirrors are just like guiding say they just guiding the propagation of the laser beam after coming out of the Raman shifter so that means, now you can see this is very important what is the wavelength? 2 micron like 1.9 micron.

What is the pulse duration you see this pulse what is the pulse duration is 10 nanosecond and this is the energy 20 milli joules per pulse, this is the decent enough energy again do not worry about the actual value of the energy, but let me take it from me, this is the decent enough energy to bring about a you know a increasing temperature jump detectable increase in temperature jump.

Now, here is another mirror say M 3. So, this is your sample you can see now this this green line is your 2 micron right this now goes in and what happens look at this point this this is the sample; this is the sample in this you have protein and D 2 O buffer ok, but the laser is not illuminating your whole portion it is the laser is a fine focus beam, it is going through only one point so that means, at that point, you are having a temperature change isn't it because the other points where you do not have laser illumination you are not doing anything good.

So, this is your sample and this is this is kind of a block which blocks your laser beam because laser beams can be very harmful at high energy so, you would take every precaution if ever you are working with the laser later to we able to block it so, that there are no extraneous lights coming out good. Now that is your; that is your what should I say that is your pump laser right, this was your pump laser. Now we still have what? The probe laser ok. So, this is what a probe laser is so, this is your probe laser. See what is the frequency of the probe laser? The frequency of the probe laser can be tuned between 1600 to 1700 centimeter inverse and

let me tell you this, if you are going for alpha helix what frequency would be go for as you just said.

Student: 1630.

1630 so, again take it from me, you can tune the laser diode frequency laser diode in such a way the current and all those things, then it gives out gives out typically that frequency which corresponds to 1630 centimeter inverse you can do that. Now once you do that, again you have a set of lenses. So, this is L 1, this is L 2 a set of lenses; now guys tell me where should this probe laser focus?

Student: Same point.

Should it not focus at the same point through which the pump laser passed.

Student: Yes.

Do you understand that?

Student: (Refer Time: 37:29).

See the pump laser is not illuminating your whole sample, it is only illuminating a certain area, certain point. See if you have to get a signal change, where would you probe? You would not probe any arbitrary area of the sample where would you rather probe? You would rather probe at that place where your pump laser is passing through because that is where you having the change.

So, the probe that is why you can see you have this arrangement and finally, where is the probe focused? The probe is focused at the same point where the pump is. So, this is called over lapping between your pump and probe beams that you overlap these two beams. So, that

you get a detectable signal and after that, this is your detector and you have a oscilloscope and all these things so, that is a secondary right now and you get your change.

Now, also let me tell you this, this is a very very good instrument if you know how to maintain it, if you know how to work with it. It is not that many people have it in the volt, it is actually select instruments I mean, if you compare with the number of research with the number of groups doing research, the number of groups have actually have actually having this instrument is very very small mainly a handful or more than that ok.

So, in that sense, it is a rare instrument to be in position of let me tell you that and if people are looking at protein folding unfolding. This is one of the better instruments that people can actually think of having if they are really looking at all these kinetic changes and all these things ok. So, guys this essentially is your laser induced temperature jump; because your inducing the temperature jump.

And even your even probing the change using lasers, one is the pump laser and the other is a probe laser ok. I do not want you to remember all these things, I do not want you to remember this one. But, at least guys remember the block diagram, remember the essence of what a pump beam does, what a probe beam does and the principle of this technique what I told you is, it is one of the ways it is actually being done in practice good.

(Refer Slide Time: 39:57)

So, this then we done with relaxation method and the last technique; the last technique that I am going to discuss with you an experimental technique is known as optical triggering: a flash photolysis ok.

So, optical triggering means you trigger using some sort of light, some sort of optical thing. What is the flash photolysis? Flash means it happens in a flash, it is like a camera flash you see a flash it happens in a flash. What is photolysis? What is photo stand for?

Student: Photon.

Photons right, what is lysis?

Student: Breakage.

Breakage; that means, in a flash, using photons you are breaking a bond and that is how or this would help you to monitor a certain change in folding or unfolding how do we do that? So, for this again we go back to your dear old friend cytochrome c right.

(Refer Slide Time: 41:01)

Principle of the method

- In absence of CO (carbon monoxide), the unfolding transition of cytochrome c begins at ~ 4.5 M GdmCl and gets completed by 6 M (c_m = 5.1 M)
- In presence of CO equilibrium is shifted towards the unfolded state due to preferential binding of CO to the unfolded state; here unfolding occurs at a much lower denturant concentration with c_m = 3.7 M GdmCl

This is the principle of the method. The principle is cytochrome c in the folded state right does not absorb or does not ligate or does not bind carbon monoxide. Instead, if you have to have cytochrome c bind carbon monoxide where would you have to go? You have to go to an unfolded state right.

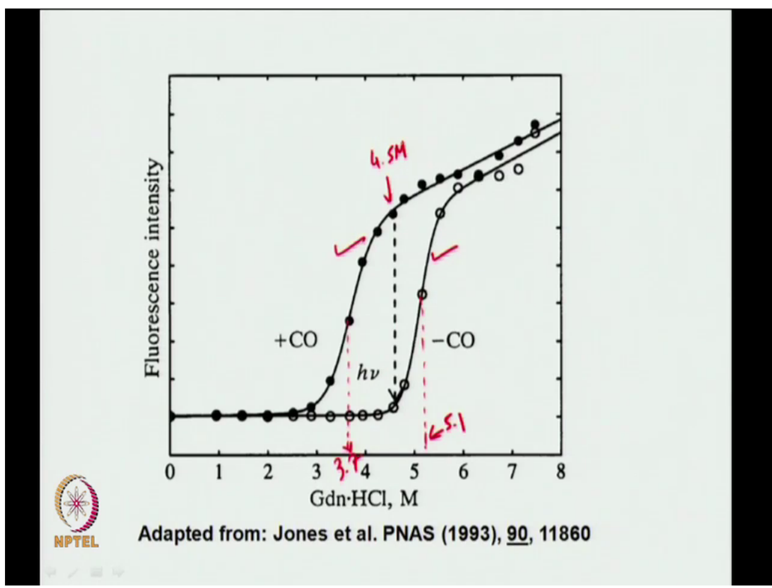
Now, suppose what you have done is, you have unfolded cytochrome c with guanidine hydrochloride and you have you are passing carbon monoxide and guanidine hydrochloride

and cytochrome c binds guanidine hydrochloride ok. Now this is what the method says. In the absence of carbon monoxide, that means, you have not bound any carbon monoxide, the unfolding transition of cytochrome c begins that 4.5 molar guanidine hydrochloride and gets completed by 6 molar. So, the c m which is remember what the c m is right it is 5.1 molar ok.

Now, if it if this point was about the absence of carbon monoxide, the next one would always be about what the presence. So, in the presence of carbon monoxide, the equilibrium is shifted towards the unfolded state due to preferential binding of CO to the unfolded state, that is what I just said CO does not bind to the folded state, it preferentially binds to the unfolded state; that means, the moment you binds CO what you do is, you shift the equilibrium towards the unfolded state.

Now here, unfolding occurs at a much lower denaturant concentration with c m being 3.7 molar. So, you can see what is happened? What is happened is, in absence of CO the c m was 5.1, in the presence of CO, the c m was come down to what? 3.7. So, this different from 1.4 now that is pretty high ok.

(Refer Slide Time: 42:44)



Now how do you see that in experiment see, this is what you are doing, this is the fluorescence intensity, remember that fluorescence intensity the trip intensity we are looking at and on the x axis what you have? You have guanidine hydrochloride concentration. There are two curves you are looking at; there are two curves you are looking at, first look at this curve this curve, this curve is the one where guanidine hydrochloride is bound to CO ok.

Now, as was showed in the previous slide, see its unfolding transition is about 3.7 so, this 3.7 would be somewhere here, this is 3.7 molar this is the c m right and then it unfolds. Now this one is the one without CO; that means, guanidine; that means, cytochrome c knows CO; obviously, it is more stable right and the c m is much higher so, this is if this is the c m, say this would be what was it? 5.1 I guess 5.1 ok.

Now, look at the dotted line what does it say? The dotted line; the dotted line at this dotted line, you have a concentration of 4.5 molar guanidine hydrochloride ok. Now, just forget everything else, focus on the dotted line and the two curves. For the upper curve which is the (Refer Time: 44:12) CO curve 4.5 molar lies in which part of the curve? Does it lie in the folded part or the unfolded part?

Student: (Refer Time: 44:19).

It lies in the unfolded part right; that means, your transition is already over good ok. Now, if you draw a vertical line now; that means, you have not changed the guano hydrochloride concentration right, if you come to the other curve where there is no CO where does this 4.5 molar now lie?

Student: Folded.

It lies in the folded baseline ok, now do you understand the difference now. So, what you can do now is, now this is what the flash trigger is going to do or optical trigger is going to do. You take guanidine hydrochloride with CO rather you take cytochrome c with CO at 4.5 molar guanidine hydrochloride. So, under those conditions based on the forced curve, it is already unfolded clear.

Now what you do is, you know that if I remove CO, the protein from the unfolded state would immediately come to the what? The folded state. So, what you do is, you shine it with the laser right, that laser is absorbed by cytochrome c and that laser helps in breaking the bond between.

Student: (Refer Time: 45:27).

This CO and cytochrome c and remember when the CO bound CO bind where does the CO bind you think.

Student: Iron.

To iron so, very simple so, the bond breaks. The moment the bond breaks what happens? No CO. How would the protein respond now?

Student: (Refer time: 45:43).

The protein would respond by going to the folded state and then you monitor that relaxation. So, that is what an optical triggering is all about is it clear to you guys I would not go more than this, but what he says is You take a bound a CO bound cytochrome c in a high concentration guano hydrochloride 4.5 molar, for the CO bound cytochrome c you are in the unfolded regime. You do a fission of your bond, you do a section of you bond with light, the moment this CO is cleaved the bond between CO and Fe is cleaved what happens?

The protein now realizes that I have to fold because I am more stable at 4.5 molar without CO I have to go to the folded state. So, its started going to the folded state and then, you some how monitor that relaxation using this technique and that relaxation was triggered by what was triggered by that photolysis and hence, it is called optical trigger based on a flash photolysis method is the principle clear right based on this diagram is principle clear ok.

So that means, you have to understand that if I am going to do this, I better have a laser where cytochrome c absorbs that means, cytochrome c has absorption band at my laser frequency because it has to absorb the laser. See the point is you can have a very high intensity laser does not matter, but if your sample does not absorb the laser light, it does not matter right just straight pass through because isn’t it is fully transversal there is no absorption at all.

The only way you can excite a sample or heat a sample lap is, you make sure that the sample actually absorbs the light you are passing through right that is the minimum you can do ok.

(Refer Slide Time: 47:32)

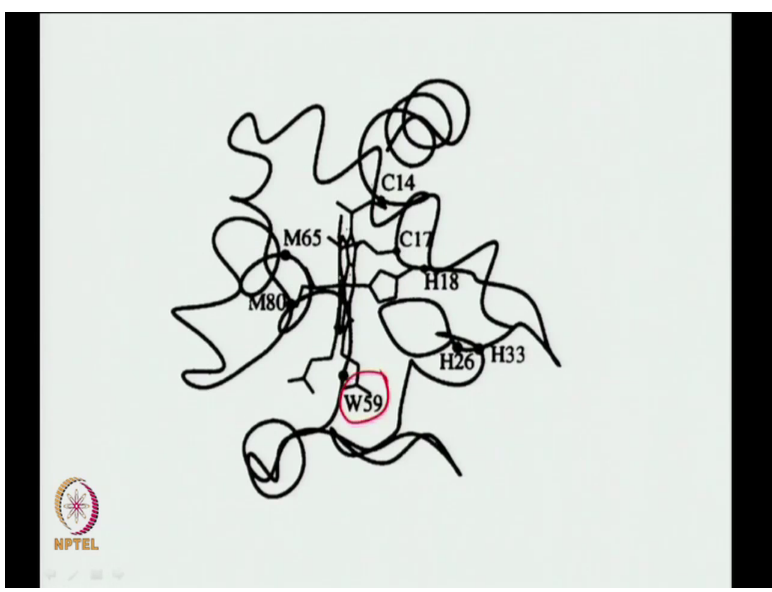
- Thus at a GdmCl concentration of ~ 4.6 M, photodissociation of CO will initiate the transition from the unfolded to the folded state
- Since CO is known to photodissociate from the heme in < 1 ps (picoseconds), thus one in principle can observe the protein dynamics at a very fast time scale

So, thus this is what it says at a guanine hydrochloride concentration of 4.6 molar, the photodissociation of CO will initiate the transition from the unfolded to the folded state ok. Since CO is known to photodissociate from the heme in less than 1 picosecond. Now, please look at this, less than 1 picoseconds, thus one in principle can observe the protein dynamics at a very fast timescale ok.

So, what it means is, you can break the bond between Fe and CO in a timescale as fast as 1 picosecond now let me tell you it is much lesser than that, it is like 100 femtosecond and so it is like point 1 picoseconds and so right. So that means, essentially the moment you have broken the bond so, let us take 1 picosecond, beyond 1 picosecond you have the full time window for you to monitor the change. So, is not this the one which you gives the fastest time of whatever techniques you have discussed?

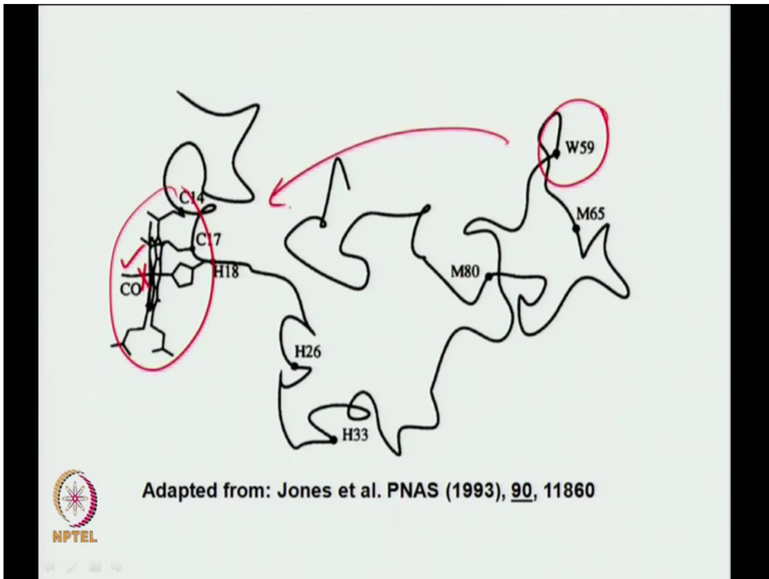
So, here therefore, this is the one which gives you the fastest time scale for you to monitor protein folding and that is what it says, thus one in principle can observe the protein dynamics at the very very fast time scale; that means, from 1 picosecond in higher. Let us not worry about how people observe that, you know that is the different issue, but will not be concerned about that. But, this is what the principle of the method is you trigger folding from an unfolded situation by breaking a bond between Fe and CO the carbon ok.

(Refer Slide Time: 49:13)



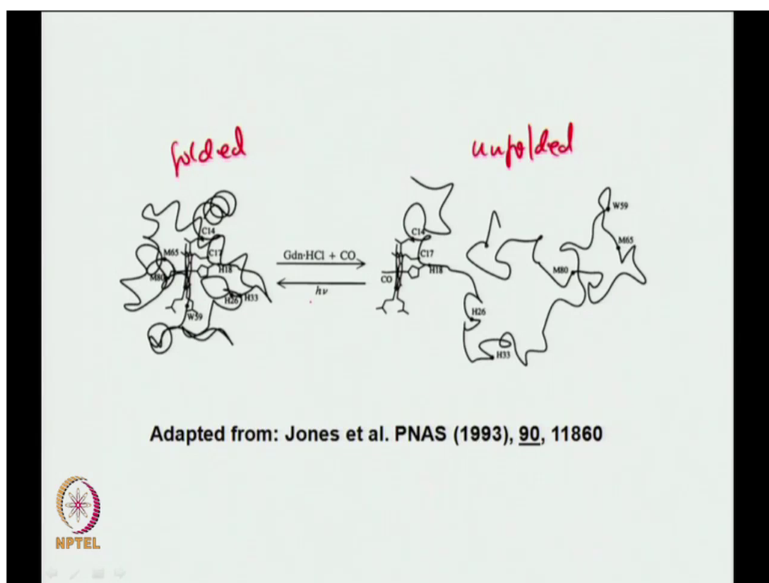
So, essentially this is what you are doing then, if this is your folded state there is a folded state, you have the trip; this is the tryptophan and you have the heme in the middle.

(Refer Slide Time: 49:22)



This is the unfolded state where you can see. Where is tryptophan? Tryptophan is here, the heme is here right and this is your CO ok; that means, if you would now break the bond; if you would now break this bond; if you now break this bond what would happen? It would go back to the folded state and this tryptophan would come closer to the heme in the folded state right.

(Refer Slide Time: 49:47)



So, this is in a nutshell, this is what it shows. So, you can see, when you add guanine hydrochloride and CO to the folded state so, this is the folded

Student: Yes sir.

It goes to the unfolded. Now, you do a H nu what is H nu mean? That means, you shine light.

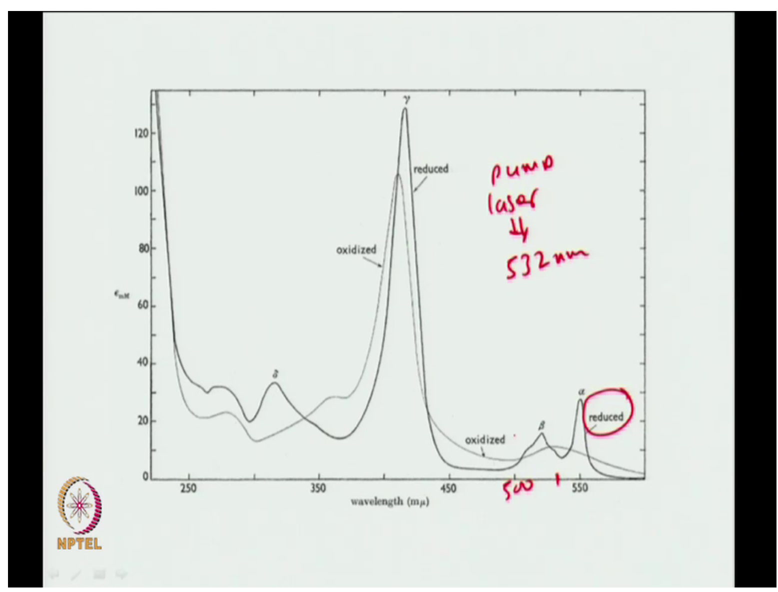
Student: Light.

The moment you shine light, CO goes out bond is broken and it comes back to the folded state and you monitor the rate at which it does ok. That means, here you are monitoring what you are monitoring not unfolding you are monitoring what

Student: Folding.

Folding.

(Refer Slide Time: 50:22)



And I was telling you the absorption right so, this is the last slide you can see the lasing wavelength; the lasing wavelength was so, the laser the pump laser this is the pump laser, these are the wavelength of 532 nano meters where does 532 nanometers lie? So, this is 500 so, 530 would be somewhere here say or may be somewhere there.

So, I am talking here please remember I am talking with the reduced one; that means, the ones with the alpha and beta not the other one, this is the reduced cytochrome c. I am talking about not that is the different thing, but anyway at the point of 532 nanometer, your sample does

have an absorbance see would absorb the light and then, your bond fission would occur and after that you can do your experiment as you are supposed to; good.

So, this brings us to the end of the techniques, that at least I planned to discuss with you. You know to tell you the truth, more or less these are the techniques, more or less these are the most general techniques that are been used nowadays. You know that apart from few other ones which are kind of little more advanced for by people to study proteins right.

And, if you would ever encounter one of these, principles are always the same is just how you do the experiment or how you do the final detection based on what. Like rapid mixing both were mixing one was the stop flow, one was the continuous flow then you had a relaxation by doing a temperature jump. In this case, you are not doing a temperature jump, you are doing a bond fission and that is itself triggering your change in conformation of the protein.