Bio-Physical Chemistry
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Lecture - 30
Protein Folding: The Chevron-Plot

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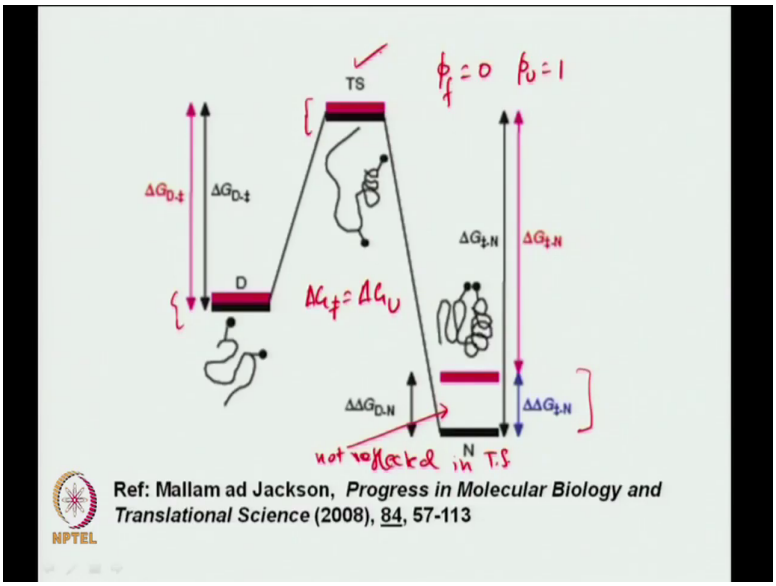

Last class, we were discussing phi value analysis right and these were the last couple of slides that I went through. So, one was this where phi f was equal to 0, phi u is equal to 1 and phi f is equal to 0 means that means, the phi folding is 0 because if you can see whatever difference you have in the native state which is this, the difference in the native state, this is not reflected in the transition state right. So that means, it does not matter what change you have in ready state; transition state does not care.

That means, transition state does not have any component or does not have a component of the native state. So, whatever structure you see in the native state is possibly not being found in the transition state for you to see any effect in the transition state per say right. So, then obviously, the other picture is the reverse of this. Where?

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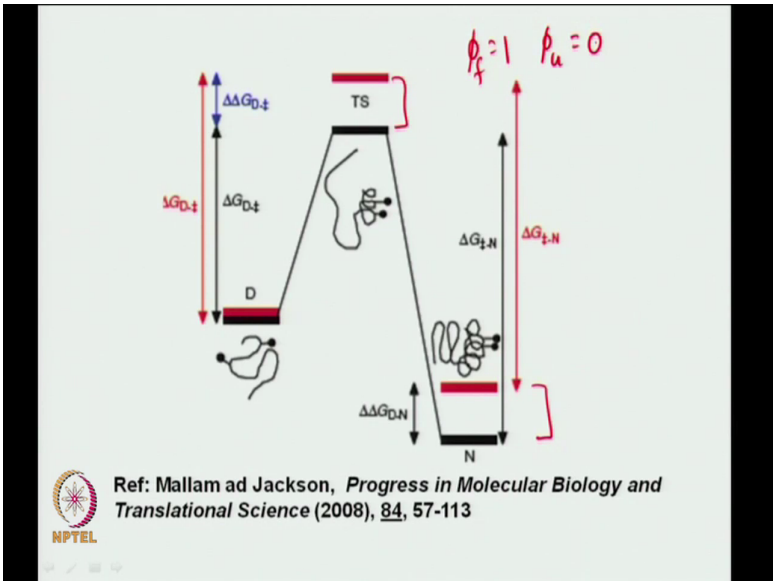

So, these are two extremes right; one is whatever we have in the native structure is not found in the transition state; the other one is what do we have in the native state is being found in the transition state.

So, in that case, what you will have is this and I can see this, these are very similar. In this case the same which means that if in the previous case phi f was 0, then in this case phi f is would

be 1 and obviously, phi u would be 0 and this is only for a two-state scenario, where phi f plus phi u goes to 1 ok.

So, this is how people would interpret if they would have energy free energy diagrams like this and as I as we were discussing last time, these are extremely important for you to figure out which amino acid affects to what extent; which amino acid is instrumental in the formation of the transition state, going to the native state; which amino acid in this transition state or rather I will let me go back and try to rephrase this discussion.

So, for example, you have a protein say two parts A and B ok. Say A part is the most important part which is the hydrophobic code and B part is the rest of the protein. Now, most of the surface residues.

Now, what will happen is say suppose you have A part which is the code, you would know that if I would make any changes in A part which is the code, then I would be having a change in the transition state and this would reflected here. Now, how much of a change you would have would depend upon what kind of a mutation you are doing, and we talked about these mutations before.

Now, go back and also think about this for the B part, where they are not really their responsible or protein stability. If you would be making some changes that those changes might or might not be reflected as such in the transition state because they per say are not that important for protein stability.

Now, what I am saying is not that important; it does not mean they are not important at all; they are important in their own ways right. So, these are some of the things that people have to take care of or people have to keep in mind, when they do these mutation analysis, these fibre analysis along with these assumptions that we discussed a little bit last time right.

You would not be undergoing a gross change in structure, there should not be a gross change in pathway and all these things; otherwise, these followed right ok.

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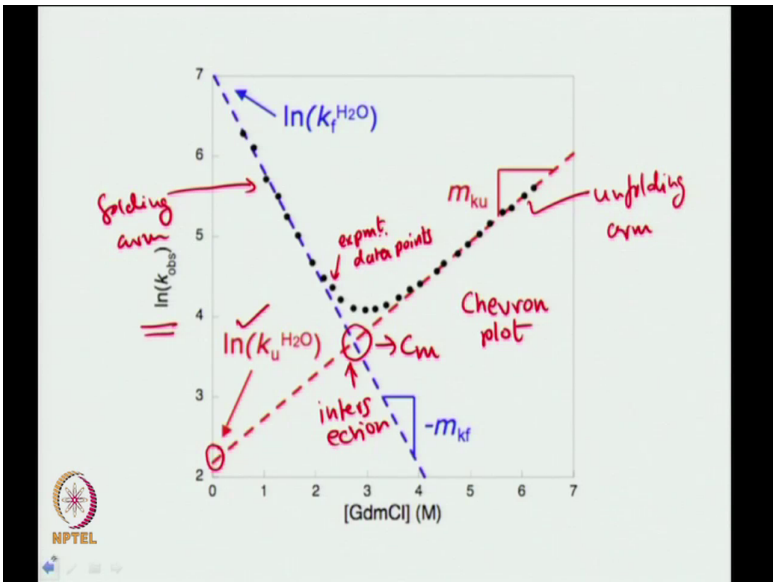

So, moving forward, let us look into kinetics. So, we have talked about the effect of amino acid on the transition state through the phi f for phi u values. Now, how do we measure those effects? The direct fallout of a change in the transition state is a kinetics right because that is the only way you can measure your changes in transition state. No other way, right now for proteins.

So, what are you looking at? You are looking at something known as the Chevron plot ok. So, let me write this here this is called a Chevron plot. Now, what are you seeing here? These black dots, these black dots are experimental data; that means, experimental data points ok. Now, what you have on the x axis is you have your concentration of denaturant. In this case, it is going to be hydrochloride. You can also have urea not a problem.

Now, what it tells you is if you look at say the higher concentrations of denaturant; say for example, from 4 to 7. If the concentration of denaturant is high, what would happen to the protein? Would it be in the folded or the unfolded state? It would be in the unfolded state.

So, for example, this is what you are doing, you are trying to measure kinetics right, you are trying to measure kinetics and you have done something to the protein, you have done something very faster. But you are trying to measure kinetics; that means, it has to be a function of time right. But for measuring kinetics, you have to do the change very fast and come to that later.

So, essentially what you are doing is say for example, you are trying to looking you are trying to look at the unfolding of a protein. So that means, you have to maintain a very high concentration of denaturant and you have to maintain that very fast because you have to observe kinetics right.

So, what you do is again, I will come to this, but just listen to this for the time being. What do you do is you take protein say in a syringe, protein ingest buffer no denaturant right and take a high concentration of buffer or rather high concentration a buffer having high concentration denaturant in another syringe right. Now, see the final concentration of denaturant, you would need to have is what? Maybe 5 molar, 6 molar does not matter ok.

So, if you are mixing it a high concentration of denaturant and a protein in buffer which is no denaturant; when you mix it because this is high, what will happen is the final concentration would be very high of denaturant, after you mixing it depending upon what ratio you mix it in.

But the way, you mix it in is making sure of the fact that the final concentration denaturant is pretty high say 5 to 6 mole or even more than that. Now, once you do it? What happens is you mix this, but you mix it very fast; then, just like that you mix it and there are ways of doing that these are called Rapid mixing techniques right.

The moment you have it, what will happen is the final concentration of the denaturant based on your mixing ratio and all this thing say is 6 molar. So, the what do you will see is that the final constant of the denaturant is 6 molar, then the once you mix it the protein starts unfolding and stops at whatever signal or whatever unfolded form it should be having at 6 molar.

Did you to get it or not? Again, so, you have a protein in buffer, you have high concentration of denaturant, you mix these two very fast in such a manner that the after mixing, the final concentration of the denaturant is 6 molar.

Now, if the final concentration, of the denaturant is 6 molar; then, the protein has to denature and it will stop at that confirmation unfold confirmation which corresponds to the 6 molar glucagon hydrochloride.

But you are mixing it very fast. Remember, you mix it; once you mix it, the protein sees the glucagon hydrochloride concentration and it starts responding to it. But cannot respond that fast because the protein is going to evolve into the unfolded form from the folded form that evolution in terms of time is a kinetics and that is what your k u is right. Because ku is unfolding and because you are keeping a high concentration of denaturant, again just listen to this, I will come back to this.

Because it is a high concentration of denaturant, you are looking at the protein being going or the protein being unfolded or going to the unfolded state. Is it a little more clear? Jenica still shakes her head; Jenica keep shaking, it is good exercise. We will come back to this right now.

So, the point was that if you are at the high concentration of denaturant, then this arm. So that means, this arm is essentially your unfolding arm; is not it? That means, if you have a high concentration of denaturant, essentially what you would be looking at is a protein unfolding. Hence, you would be looking at the rate constant of the protein unfolding that is ku right.

So that means, if you are looking at this figure, this unfolding arm; you can see all these data points are related to the ku and then, you extend it. What you get at this point is something which you have done before in terms of delta G. What is this? This is the unfolding in absence of any denaturant which is ln ku H2O ok.

Now, similarly, think about the reverse. You know, this is how people generally do it. What they do is if you are talking about a refolding; that means, you are talking about actually a protein folding or coming from the unfolded state to the folded state.

So, what you do is you take the protein in a very high concentration of buffer right, then you have you mix it with buffer which is no denaturant; you mix it with a certain ratio such that the denaturant concentration becomes very low after mixing.

So, you started with protein in a 6 molar, then you mix it with a high concentration of with buffer, no denaturant in such a ratio that the protein comes down to say 0.6 molar. Because it is 0.6 molar, then what would be the final state? Native state and then, what are you looking at? You are looking at the folding of the protein, then which arm I looking at? I looking at the unfolding arm or the folding arm.

Student: Folding.

You will be looking at the folding arm right.

So, then this one is your folding arm. So, then in the graph, you have two, you have two essential sides; one is the high concentration of denaturant corresponds to what? k u. The low concentration of denaturant, where the protein would essentially be folded is called what k f because that is your folding arm.

Now, you remember from delta G rather from our equivalent thermodynamics. These transitions that I does not matter, where I start from; say start from the folded state, I go to

the unfolded state, in between I will be having a state, what would happen, where k equilibrium would be equal to?

Student: 1.

1.

Now, if it is a two-state scenario, k equilibrium is equal to 1 means say you are going from the folded to the unfolded right. So that means, you are going, you are looking at k equilibrium is equal to k u over k f right. So, k u by k f would be equal to 1; that means, if you are very close to your midpoint of denaturation, then ku by kf would be very similar; only then, you can get k equilibrium to be 1.

So, if ku by k f would be very similar at your Cm; that means, the midpoint of the denaturation, they should be having the same value. They should that means, they should intersect at one point. So, then you can see this is the point of intersection right; this is the point of intersection and what does this point then correspond to?

Student: Cm.

This point corresponds to Cm right because that is where k u and k f are both comparable to each other. Is it clear or not? Right.

So, this is so now, you can realize this is another way for you to check, where my Cm is right. I just do kinetics on k u; I just do kinetics on the folding arm; I just do kinetics on unfolded arm; I extend those right and the place where they cross k u should be equal to k f k equivalent should be equal to 1, provided I am talking about two slit scenario and hence, I would be getting my Cm.

So, then essentially this would be my Cm. So, you can see if you look at the data points; now the black data points, they come like this from the left hand side; from the right hand side, they come like this and between they have a certain curvature.

So, that curvature that the minimum point of the curvature corresponds to what? The Cm value because that is where ku is equal to kf right. So, now, if you forget about all this and look at just the picture, does not it look like a V shape? So, this is called a Chevron plot. So, that V comes from the Chevron plot.

This is how it is named; that is why it is called a Chevron plot; it is a nice name ok. So, this is the essence of a Chevron plot right. So that means, by doing this, you can understand if there is a difference in k u and kf and hence, make out which arm; that means, whether the unfolded term or the folded arm is being affected right ok.

Now, before I go any further, realize this one is both of these would be having the respective slopes. So, for example, if you look at the ln k u H2O, this is the slope of m k u. Now, remember what m ku was?

We talked about this before right delta G is equal to delta G H2O minus m times concentration of denaturant right; where, m was related to something known as accessible surface area or surface exposure. So, the same thing you will be having here. The only difference is when you are doing delta G unfolding, you are talking about the total m; that means, the difference between u and f. But in this case, we are talking about two different nouns. What are you talking about?

One is you are talking about the difference between the transition state and the folded state or the transition state and the unfolded state. So, that is why if it is ln k u, then you are talking about m k u because it is a unfolding arm and if its ln k f that is you are looking at the folding arm; that means, you are going from the unfolded to the transition state and then, to the folded state, then you are talking about m k f ok..

Second point is you look at this x axis, what is it? It is not ln k or ln kf, what is it? It is ln k observed. Do you know what k observed is? Here is equal to there has to be relation between ku or kf right; others you will not be able to use this or let us try to figure that out. Now, that is the next point of contention ok.

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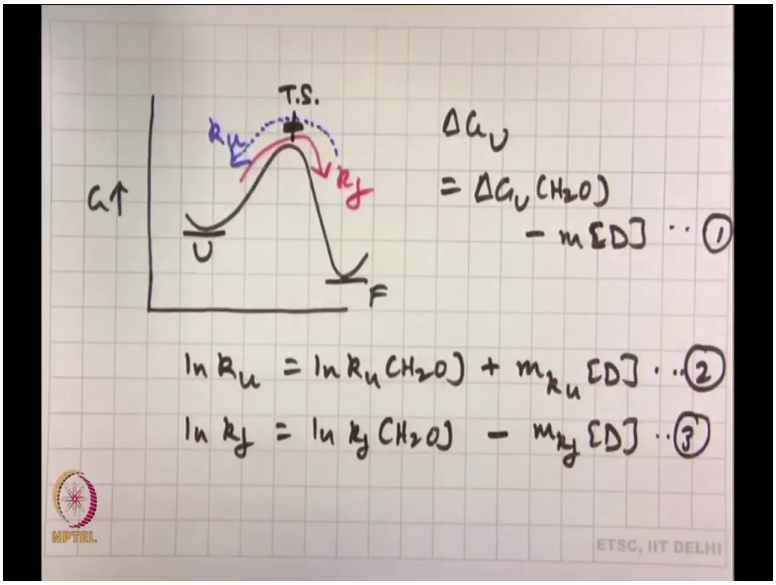

So, let us look at a two-state kinetics. So that means, we are essential again looking at a free energy profile. So that means, this is F, this is U and this is your transition state and this is G. So, if you are going from here to here; that means, if you are moving over the barrier like this that means, from the U to F, what are you looking at?

You are looking at a folding process. So, the corresponding rate constant would be k f, similarly if we are looking at, similarly if you are looking at the reverse process that is going

from the folded to the unfolded state, what are you looking at? The rate constant is you are looking at k u right because you are looking at the unfolding process, simple.

Now, before you knew from thermodynamics, delta G unfolding; that means, I am going from F to U can be written as delta G unfolding in neat water; that means, no denaturant minus m into concentration of denaturant. Do you remember this? We had done this ages ago right ok.

But this was from equilibrium thermodynamics, there was no kinetics involved here. Now, how do we relate this or is there any similarity between this one and the kinetics, we are looking at? So, what people found is, what people found is this say suppose you are looking at the unfolding; that means, you are going from folded to the unfolded state.

So, then what you can write is natural log of k u is equal to ln k u H2O plus m k u into concentration of denaturant. So, if this is one, now let this be equation number two ok. So, this is a linear relation. See this is very similar to delta G u. But only in this case, you are not having delta G u.

What do you instead have? You have ln k u; but remember, can you convert this to a free energy form? Can you or can you not? You can right, remember through this transient state theory. Say k is equal to nu times kappa e to the power minus delta G, whichever you looking at by rt right.

So, you can easily get the delta G, but you remember in that case the delta G is always the difference. So, if you are looking at unfolding is the difference between the transition state and the folded state, but in this case the difference is between what? The unfold and the folded state, it is not the transition state.

So, this is why kinetics is so important here ok. Coming back to this again, you look at this equation very carefully, what does it say? If I am taking ku or the natural log of it, it is equal to the ku; there is a rate constant in absence of a denaturant plus m times d; where, m is m k u because you are looking at the unfolding process ok.

So, what it means is what it means is with increase in concentration of d, what happens to ln k u? Does it increase or decrease? Keeping in mind, the fact that m is always algebraically positive, it should increase right ok. See what is the essence of that? Think about this, if you are going to increase concentration of denaturant, if you are going to increase concentration of denaturant, would it be more difficult or less difficult for you to unfold?

Student: Less difficult.

Less difficult. So, if it is less difficult for you to unfold, then would not the rate constant be higher that is what we are looking at, very simple ok; nothing hidden. As this is just by sheer logic and obviously, people found that it was valid experimentally and that is what we also saw in the previous slide. I will come back to that again ok.

So, what it means is that the more denaturant concentration increase which is D here; the more denaturant concentration increase D, then my unfolding rate would become rate constant would become higher or unfolding would become faster because I am pushing the protein more and more towards the unfolded state and becomes easy for me to denature it right.

Now, if this is the case then you can understand for the folded form, I would be having something like this ln k f is equal to ln k f H2O D. I am going to have m k f, but I will be having a minus here. Now, tell me is this equation logical or not? The previous equation was I go to a higher concentration of denaturant, my unfolding rate constant increases ok. Remember both m k u and m k f are algebraically positive right.

Now, in the second case, what does it say? What it says is if I increase the concentration of denaturant, what happens to kf or ln kf? It decrease; make sense right. Because if I am moving more and more to the unfolded state, it will become that much harder for me to come back to the native state right.

So, that is what it means. So, in other words, the lower is the concentration of denaturant, the higher or the easier it is for me to go to the folded state and hence, higher is the rate constant

which is ku and then, ln k u ok. So, now if I just come back to this slide. So, if you look at the slide, can you see the slide?

Student: Yes.

So, now, you can understand this a little more details. So, if you look at the right side which is the unfolded round, you can look at all the black points. So, the black points are fit to ln k is equal to ln k u H2O plus m times D. On the left side you have the folded form and that is being fit to what it being fit to ln k f H2O minus m times D and that is why m ku is positive in this case. There is the slope is positive and m k f, it is minus m k f; that means, for the folding arm the slope is negative because k f decreases if concentration of D is increasing ok.

This essentially what your Chevron plot is. So, nothing big. What you are just doing is you are combining two things. You are combining two forward stuff; one is the forward reaction, the other one is a backward reaction which are your forward or backward, you decide; does not matter, where you start from right.

Now, this is very similar to something I think you guys have done already and let us do it really quick, you did it in kinetics. In kinetics, what you did was. Did you do this reversible reaction? Did you do this reversible reaction? Only one voice says yes.

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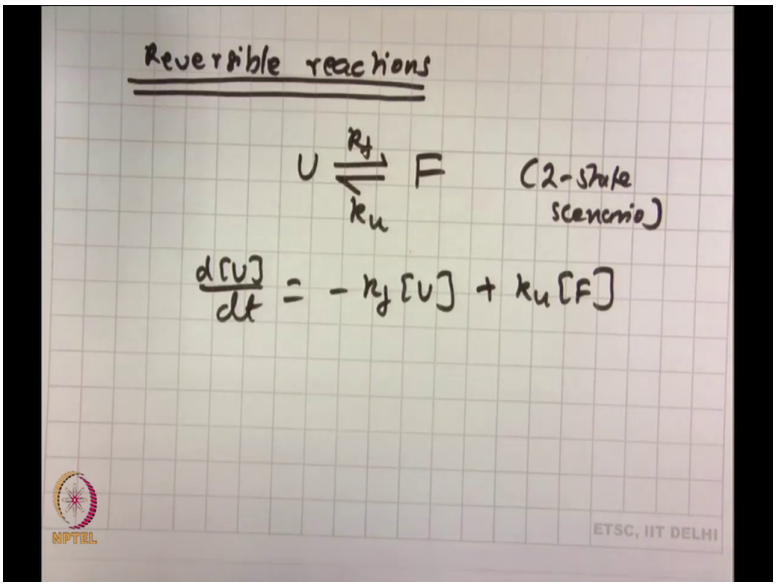

Second, I am sure you have done it right. Take it from me. If not from you, take it from me. So, remember when we are talking about this folding and unfolding, we are not talking about irreversible, it is not a one way process; why? Because at each and every point, we are maintaining what an equilibrium between these two states and the more we talk about equilibrium, in this case obviously, it we are trying to focus on an or rather on a reversible equilibrium.

Now, in that case, what I can write is say I am going from the unfolded to the folded state right. Does not matter [FL]. See if I do this U to F, then this one would be k f for me and this one would be k u and please remember, again I am talking about a two-state scenario, only two states right.

So, now, what I can do is, then I can write d of U over d of t would be minus k of f into U plus have k of u into consideration of F; is not it? U is the unfolded form, F is a folded form and both of these can end up convert because an equilibrium, also what I have is also, what I say this is equation.

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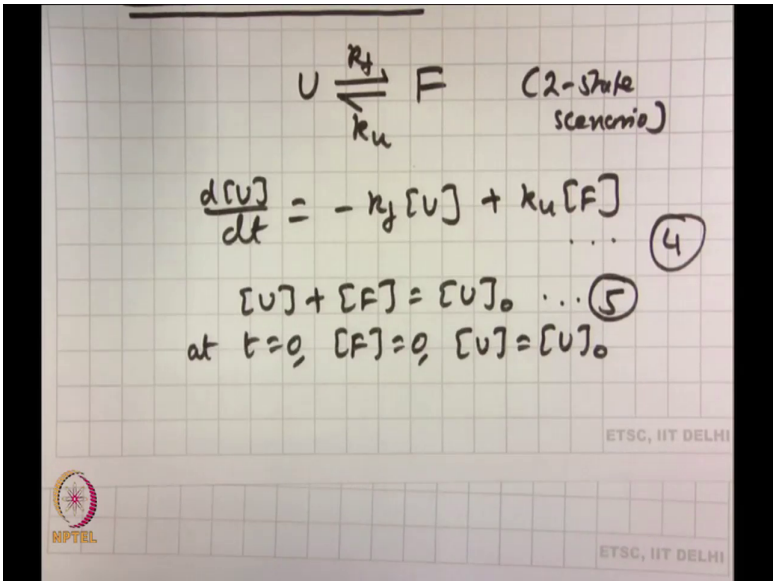

Student: 4.

4. Also, what I have is concentration of U plus concentration of F is equal to concentration of at the initial time t is equal to 0 which is U naught. So, my assumption is that when I start, concentration of F is equal to 0; so that means, at t is equal to 0, concentration of F is equal to 0 and concentration of U is equal to U naught.

So that means, if concentration of U is equal to U naught, maintaining the fact that nothing is really lost or gained. Then, U naught should be equal to concentration of U plus conservation of F. At any given time, does not matter what it is.

So, then I can use 5 and put it in 4, see my rate is in terms of d over d of t right. So, I would try to replace what I try I would try to replace F by U and U naught, that is essentially what I am trying to do ok.

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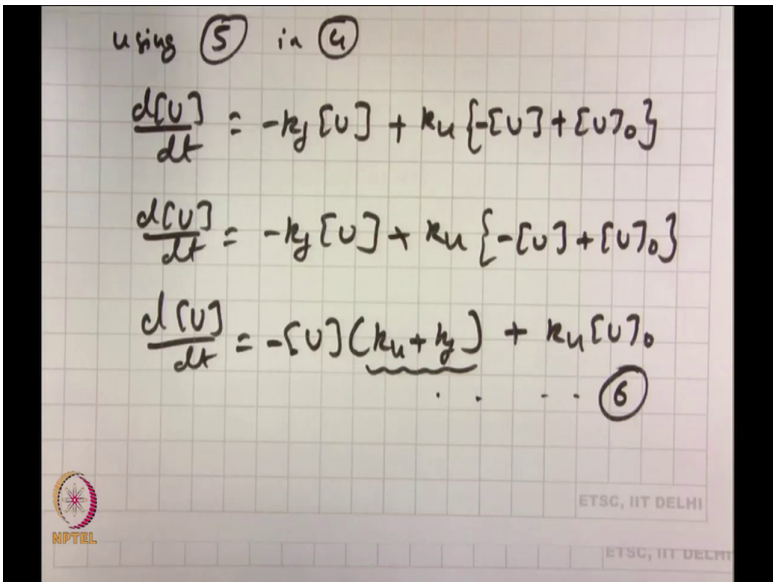

So that means, using 5 in 4, we can write d of U over d of t is equal to minus k f times concentration of U plus k u. Now, what I do is I replace concentration of F by concentration of U minus concentration of U naught right ok. So, now if this is what I am having, I just

replaced F. I have not done nothing, I have not done much right, I have just replaced F, that is it ok. Now, I splayed out.

So, what I have is d of U of d of t is equal to minus k f times concentration of U ok, then plus k u. You did not tell me; I had a made a mistake. But anyway, I will correct the mistake here ok. So, what is F is equal to? So, this tell me, where is the sign mistake? So, this is minus and this is?

Student: Plus.

Plus right? So, then U, let me write it again U naught. So, what I can right now is d of U over d of t is equal to concentration of U minus k u plus k of f plus k u times U naught. Say this is equation number 6. I always make sign mistakes ok. Equation number 6.

Now, look at one thing. There is a very important thing that has cropped up. The thing that has cropped up is this guy, k u plus k f. This is a parameter which tells you what? It is not one rate constant, but it is the sum of the rate constants you are looking at ok. It is a sum of the rate constants; you are looking at. Now, what we are going to do is we are going to solve this equation ok. So, to try to solve this equation, what we do is we just integrate.

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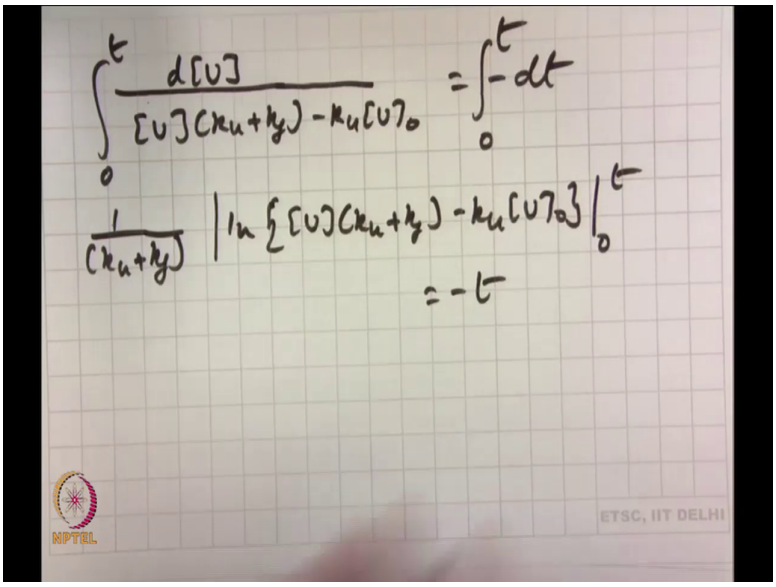

So that means, what I do is I take d of U over concentration of U k u plus k of f k u U naught is equal to minus d of t and I will integrate. What will I integrate between? I will integrate between t is equal to 0 to t is equal to t; t is equal to 0 to t is equal to t, you know that is integration I am going to follow as simple.

Now, on the left hand side, what are you going to have? On the left hand side, see this is just 1 by k of u plus k of f ok, then let me write this; natural log concentration of U k of u plus k of f minus k u U naught and this has to be evaluated from 0 to t. This is equal to minus t (Refer Time: 27:55) right; a simple integration.

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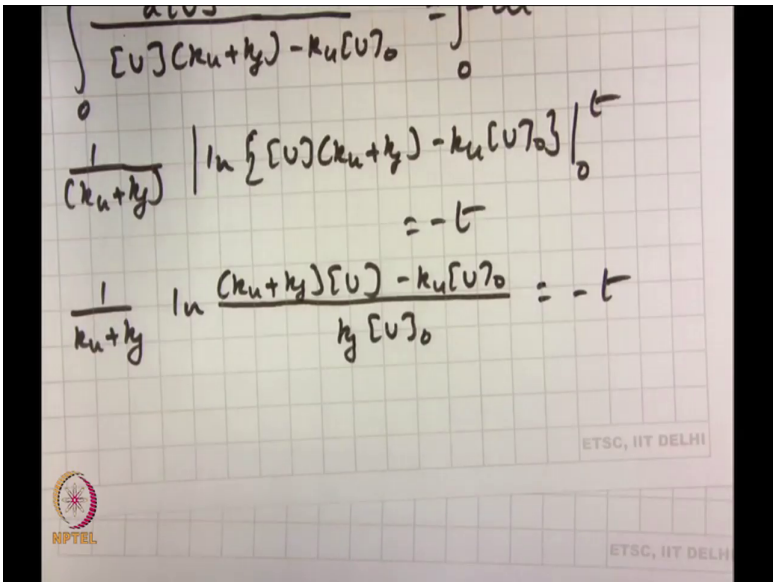

Now, how would this simplify? Can you tell me? So, can I write this. So, 1 by k of u plus k of f natural log; see the top one. The top one is t. So, everything remains the same. So that means, I can write k of u plus k of f times u minus k of u times U naught. Now, what comes at the bottom; tell me quick?

Remember, it is t is equal to what? 0. If t is equal to 0, what happens to U? U is usually u 0. So, what am I left with? So, see this is U 0 k u plus u 0 k f minus U 0 k u. So, what am I left with? I am left with k f into U naught and this would be equal to minus t right.

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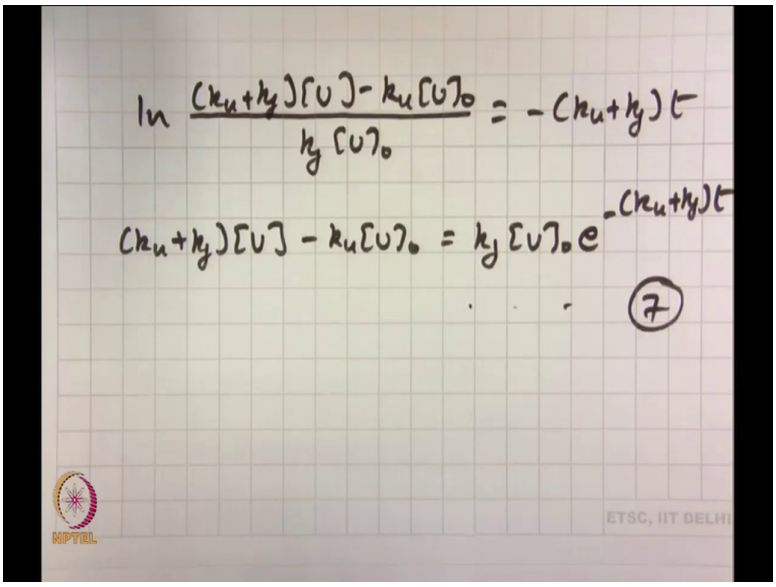

So, if I do that, if I do that, what I will have quickly is natural log k u plus k f U minus k u concentration of U naught over k f U naught is equal to minus ku plus k f times t or finally, I can write this k u plus k f to concentration of U minus k u concentration of U naught is equal to k f U naught. Then, what should I write e to the power minus k of u plus k of f times t.

And this would be which equation equation number 7, this would be equation number 7 right. Now, guys keep this in mind, on the Chevron plot on the y axis what was the thing you had? You had natural log of k observed and I told you k observed was just not ku or kf. Now, do you understand what k observed is? It is ku plus k f ok; k observed is equal to ku plus k f.

Say if this is the equation I have, I am still not reached the final form. If this is the equation I have, now tell me when I am reaching equilibrium, when do I reach equilibrium, what is t at the time? When do I say that a system has reached equilibrium, what can I take t to be? If the

initial part is 0, then what is my t at equilibrium? Can I take it to be infinity? Because what is equilibrium? Equilibrium means I have given it enough time for it to equilibrate right.

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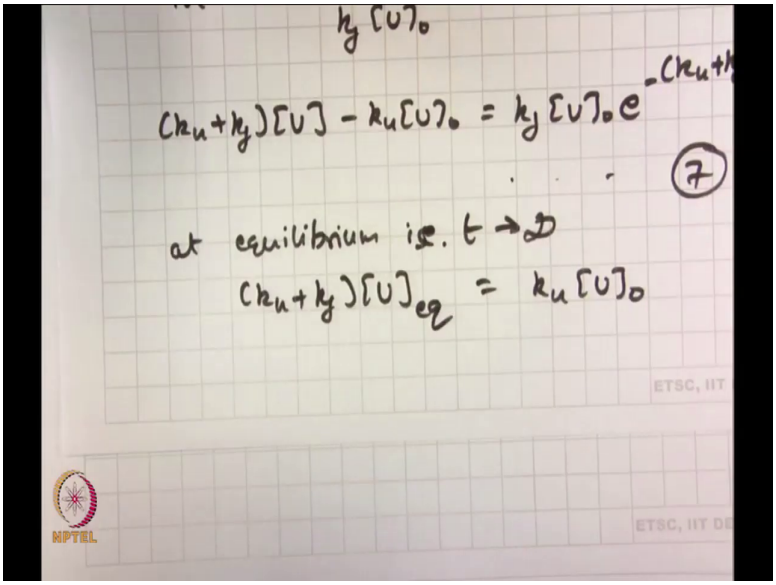

So that means, at equilibrium, that is t tends to infinity; what happens to equation number 7? What happens to equation number 7 to the right side? Where does it go? It goes to 0 right. So, that means, the right side of equation 7 goes to 0. Because e to the power minus infinity is what? 0 right.

So, hence, what I can write is k u plus k f concentration of U is equal to k u concentration of U naught. But this is at t tends to infinity, that means, I have already reached equilibrium. Then, what should the concentration of U be branded as now? It should be labelled as?

Student: Equilibrium.

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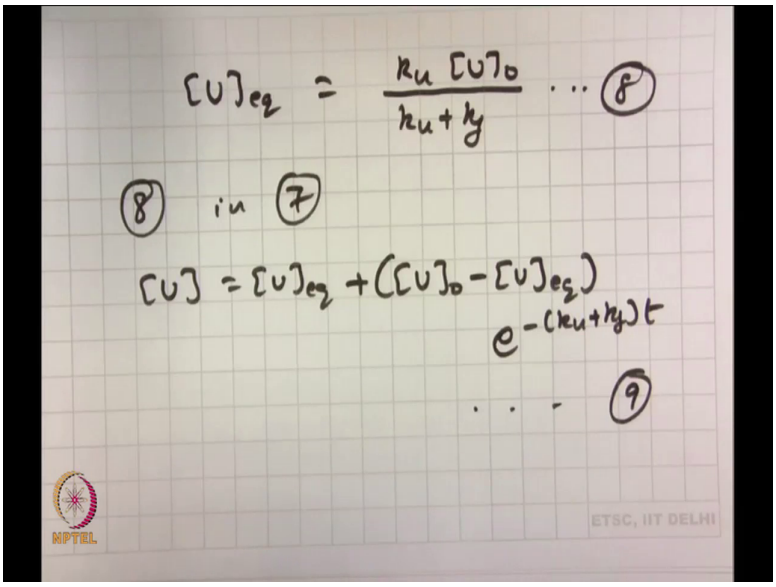

Equilibrium. So, quickly, then I have U equilibrium is equal to k u concentration of U naught over k u plus k f and let this be equation number 8. See why need why do I need k equilibrium or rather why do I need U equilibrium? I need U equilibrium because after I do mixing, after I do mixing where does my chain stop? My chain stops at that point beyond which there is no further change.

And what is that point? The point is the equilibrium right and that is why that U equilibrium or if I had gone the other way around, the F equilibrium is so important to for me because the change would stop, once I have reached equilibrium. Because you know at equilibrium, what happens? There is no further change du by dt is equal to 0 or df by dt is equal to 0, there is no further change. Is not it?

Hence, I can use this 8 in 7; do some algebra and finally, what you will get is I will give you the equation. So, the equation will look like this; concentration of U is equal to concentration of U equilibrium plus in brackets U naught minus U equilibrium, then this times.

So, this bracket times; so, the last bracket times e to the power minus ku plus kf t and let this be number 9. So, this is the final equation, you have and this is the equation you are looking at. You can do one more simplification, let me tell you, what you can do is you can, you can take U equilibrium to the left hand side right.

And then, take log on both sides or natural log on both sides. So that means, you would be having is ln U minus U equilibrium would be equal to ln U naught minus U equilibrium minus ku plus kf times t, you can easily do that right ok.

Now, tell me, what is the significance of this equation? How do you interpret it or how do you look at it logically? So, this is what it means, means if I am going to look at my kinetics, the concentration of U, the concentration of U at any given time after I had done this should be that means, where it stops; so that means, where the change stops has to be U equilibrium under those conditions right.

Now, how did the change take place that means, how did you come from initial condition of U naught to U equilibrium, that is what you are looking at. So, you the way you came from an initial condition of U naught to U equilibrium was by doing this, by this exponential relaxation factor which is U e to the minus k u plus k f.

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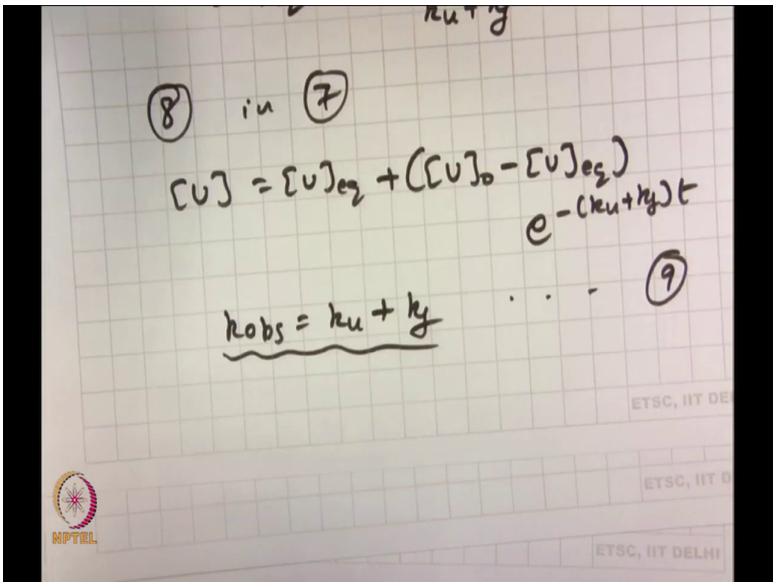

So, what I can write now is I can write then k observed is equal to ku plus kf and this is what you are plotting on the y axis of your Chevron plot ok. This is what you are plotting on the y axis of a Chevron plot, please remember that. Is it clear or no? Is it clear to everybody?

This is extremely important. See this is why, this is why this rate constant or this method is called a relaxation method. It is called a relaxation method because it is called a relaxation method because you are looking at relaxation vary at an equal rate between 2 species.

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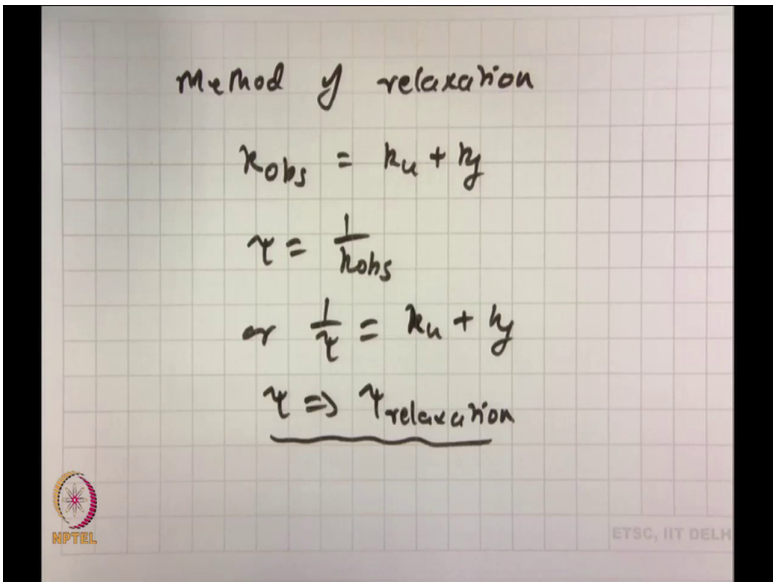

So, this is a method of relaxation. Relaxation means not that you are relaxing right. What relaxation means is that means, you have started from a non equilibrium situation and you have allowed the system to relax to what? An equilibrium situation that is what relaxation is and what is the rate constant of the relaxation? So, k relaxation or k observed is equal to k u plus k of f or I can write what is the relation between k and tau?

Student: (Refer Time: 36:13).

So that means, tau is equal to 1 by k observed or 1 by tau is equal to k observed is equal to k u plus k of f and this tau, this tau is referred to as tau relaxation ok. So, this is k u and this tau is referred to as tau relaxation and that is what you do. There are ways of measuring this relaxation time constant and we will discuss those very soon.

But whenever you are talking about a two-state scenario, whenever you are talking about a Chevron plot, it is always talking about k observed and that is what k observed is and that is what we derived ok. So, that is what your relaxation method is ok.

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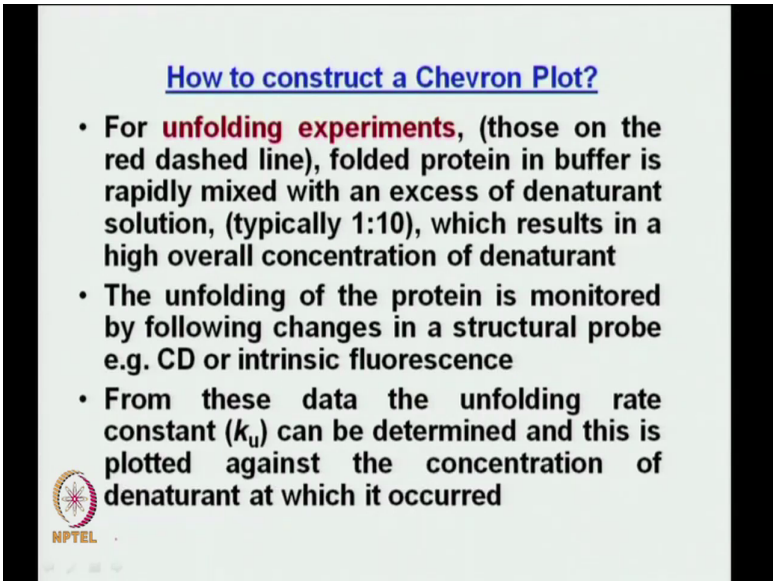

Now, how would you construct a Chevron plot right? We have seen the Chevron plot; how would you construct a Chevron plot? That means, how would you actually give rise to Chevron plot, given any protein? Now, this is what you would do.

The first thing is if you are going to do an unfolding experiment that means, you are trying to unfold the protein and get k u. What you would do is as those on the red dashed line which was to the right of the curve right.

So, you see the red dashed line, this is the unfolding ok, that is what you are trying to do. So, for unfolding experiments, what you do is you take the folded protein in buffer and it is rapidly mixed. Remember, I told you have to mix it very rapidly.

After it is rapidly mixed with an excess of denaturant solution; typically, it says 1 is to 10 which results in a high overall concentration of denaturant and once you given a high overall concentration denaturant, then you are sure that the product is going to the unfolded state as defined by that final denaturant concentration ok, as defined by that final denaturant concentration.

So, remember when we were writing ln k u was equal to ln k u observed plus m k u times d, that d is what?

Student: (Refer Time: 38:17).

Which denaturant concentration is that? That d is that d is the final denaturant concentration, where the protein is relaxing to. Is not it? Because that is what defines, what your final equilibrium state of the protein is, that is where the relaxation should stop or that is where the process should stop.

That you have to make clear; is that is it with you guys? So, that d, what we talked about in the equation, I forgot was it equation 1 or 2 I guess. 2 and 3 that d is your final denaturant concentration because that is what you are defining to be the final state, where I want my protein to come to ok.

So, then if this is an unfolding experiment, how would you do a folding experiment? Or the folding experiment would just reverse, but before going there what it says is how would you monitor this.

You will be monitored by some time by some type of signal, say in optical signal, it can be fluorescence, it can be CD, it can be just simple absorbance something else. So, from these

data, the unfolding ku can be determined and this is plot plotted against the concentration denaturant at which it occurred right.

So, this concentration of denaturant is a final denaturant concentration. So, now, you can realize how to get so many data points. Remember all those data points here, remember all this black data points, you can see right. This black data points, how do you get this black data points? You get these black data points by doing what? By taking different final denaturant concentrations.

So, different final denaturant concentrations will be giving you what? Different values right and then, you plot those and you get your ku because remember with a denaturant concentration, your ku is going to change too and this, I am only talking about the what the unfolding arm.

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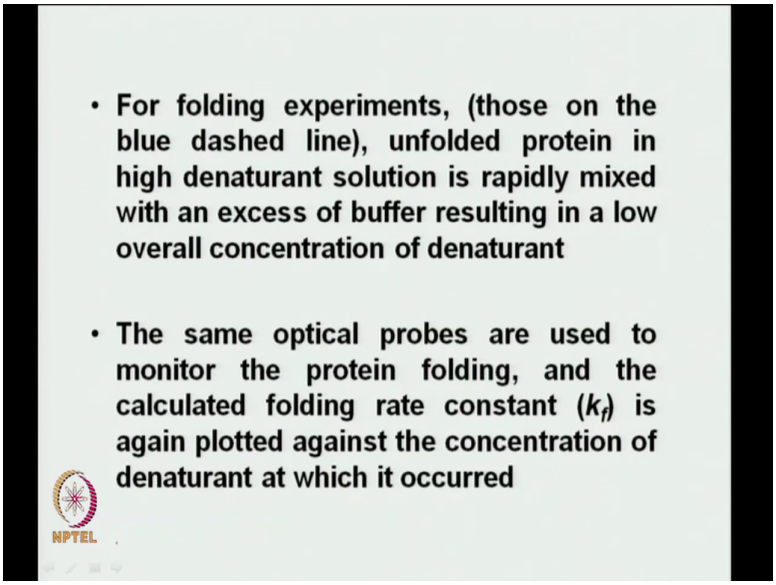

So, the folding arm, you do the same thing right. So, in the folding arm what you would do is for folding experiments those to the left that means, the blue dashed line; unfolded protein in high denaturant solution, you have already unfolded, but you are looking at a folding experiment, it has to be rapidly mixed with buffer which is very low concentration denaturant, no denaturant and then, because it results in an overall low concentration of denaturant and the protein would start folding and essentially, that is what you are going to follow ok.

So, then the same optical probes can be used the same thing like CD, fluorescence, you know absorbance whatever you use there, you can also use the same thing and then, draw the left half of the figure and finally, you fit those that means, you join those and what you get is your Chevron plot.

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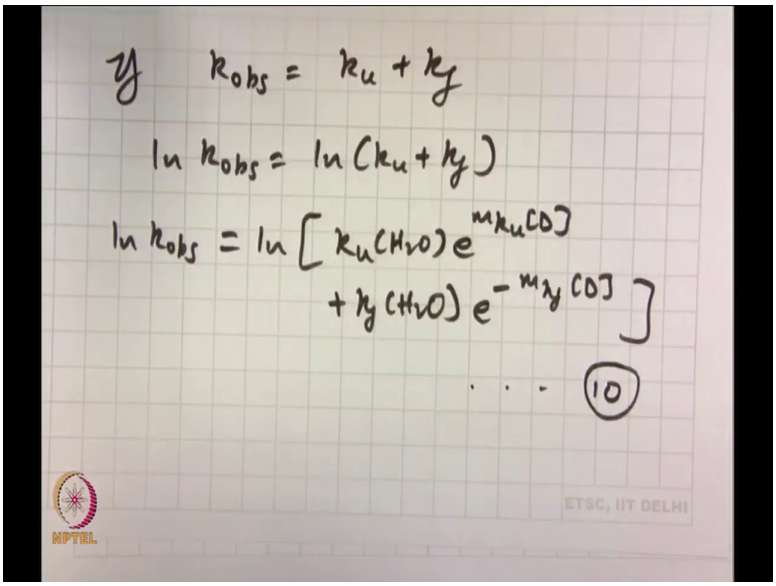

So, then what I can now write is if k observed, if k observed is equal to k of u plus k of f, then ln k observed is equal to ln k of u plus k of f or I can write ln k observed would be equal to ln. Now, what is k u equal to? What is k u equal to? Can I write k u H2O, then e to the power what m k u?

What was it? This what we are going to have right. Remember what did we have, ln k u was equal to what? ln k u H2O plus m k u times D. So, then if you take ln k u minus ln k H2O, what do you, what would you have? ku over k u H2O would be m k u times concentration of D.

So, then m k u or ln k would or k u be equal to k f times e to the power m k u concentration of D ok, look at that. This comes this comes from equation number 2 and for, k f what it would be? So, k f would be k f H2O e to the power minus m k f concentration of D right; D is denaturant.

So, what do you have now is it is very simple that is what why said it was a fitting thing. So, you have k u from your series of experiments, unfolding experiments, you have k f from a series of folding experiments.

Now, you know that your ln k observed is equal to, where k observed is equal to k u plus kf; then, you just use this equation, final equation to draw that Chevron plot that fit through your data points and that is what you have. As such the concept wise, it is very simple; experiment wise, you can understand it is tedious right because you have to do so many of those experiments.

You have to give each time, you have to give rise to different final denaturant concentrations whether high or low whether you are looking at the unfolding or the folding arm. But people have been doing this regularly right and remember, all these things are related to your (Refer Time: 43:37) analysis. So, what were the ok, let this be equation number 10 ok.

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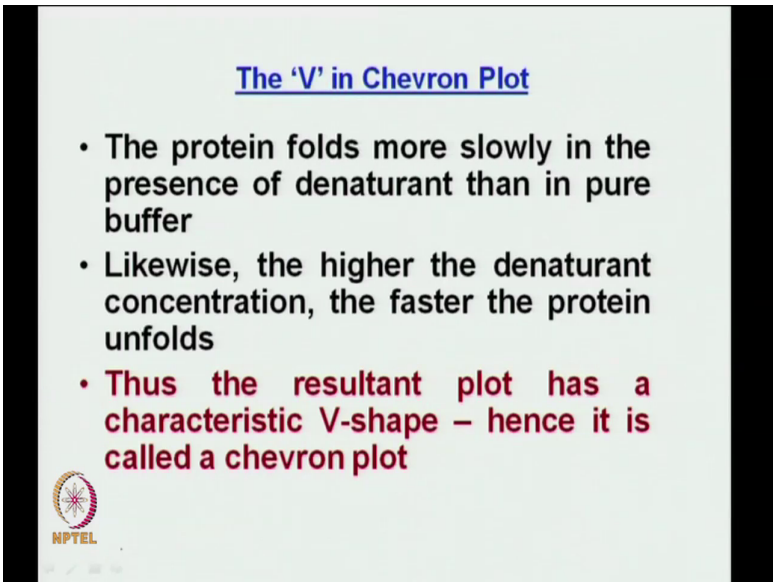

So, then as I said the V in the Chevron plot. So, what is the V in a Chevron plot? It means that the protein folds more slowly in the presence of denaturant than in pure buffer that is understandable. It has to be right because remember what ln k f was? ln k f was equal to ln k H2O minus m k f times d right.

So, in presence of denaturant, it would fold more slowly, likewise the higher the denaturant concentration the faster the protein would fold right. Thus, the resultant plot would be looking like a V and hence, it is called a Chevron plot. Hence, this is a V shape.

So, you can understand, so this is how it comes right. This is how it comes. You are looking at k u, you are looking at k f and that is typically or essentially, this tells you why you are getting a V – shape. Remember the discussion or whatever we discussed just now was based

on the assumption of what? There is a very stringent assumption, what was it? Even we derived that kinetic, what was what was it?

Student: (Refer Time: 45:05) two state.

The two state. There was a very stringent assumption. So, possibly if you would see ever any deviations from the Chevron plot, you would understand that whatever I am looking at is not strictly a two-state scenario, that is also one of the ways of checking right.

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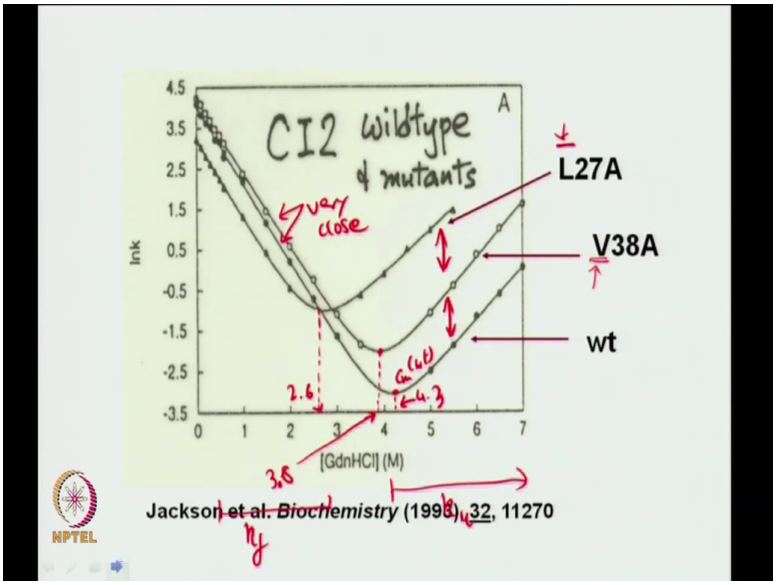

So, let me give you an example. This is for Chymotrypsin Inhibitors 2. Remember, we talked about this with regards to that nucleation condensation mechanism and the fibre level analysis. So, see what happens. This is the wild type ok. So that means, wild type means, you have not done any mutation.

Then, do you understand, there is these are three different Chevron plots of three proteins; one protein is your wild type wt; the other protein is L27A. These this one, L27A. What you have done is you have replaced the leucine at the 27th position by alanine right and the other one is your?

Student: V38A.

V38A; that means, you replaced a valine 38 position by alanine. Now, look at what has happened? What has happened is to the right of the figure, first of all look at the wild type, the wild type, remember from if this is a wild type, where does the denaturation midpoint come in? It comes close to about 4.2, 4.3 right.

This is Cm for the wild type; is not it? If this is Cm for the wild type. So, this say this is what should I write? So, this is Cm for the wild type ok. Now, when you have gone to this mutant V38A, what has happened? What has happened to the Cm?

Student: It has decreased.

It has decreased ok; that means, this is the new Cm now. So, if this Cm was say 4.3 or 2 or 3, this Cm say would be what? 3.8 molar no hydrochloride. Then, what has happened? As a result of mutation, what has happened to the protein? In one word, destabilized ok. Now, now you go to L27A, where does the Cm shift? You can see now, the Cm shifts to somewhere here. So, this is would be what? 2.6. It is more destabilized right, straight away you have this.

So, what do you understand this, all these both these mutations are destabilizing in nature. Now, if they are destabilizing in nature, tell me what happens? What do you mean by their destabilizing nature? If their destabilizing in nature, I do not think I have space here, but I will try to draw. If they are destabilizing in nature, now this what is going to happen right. So, I will use the slide, I will rather use the page.

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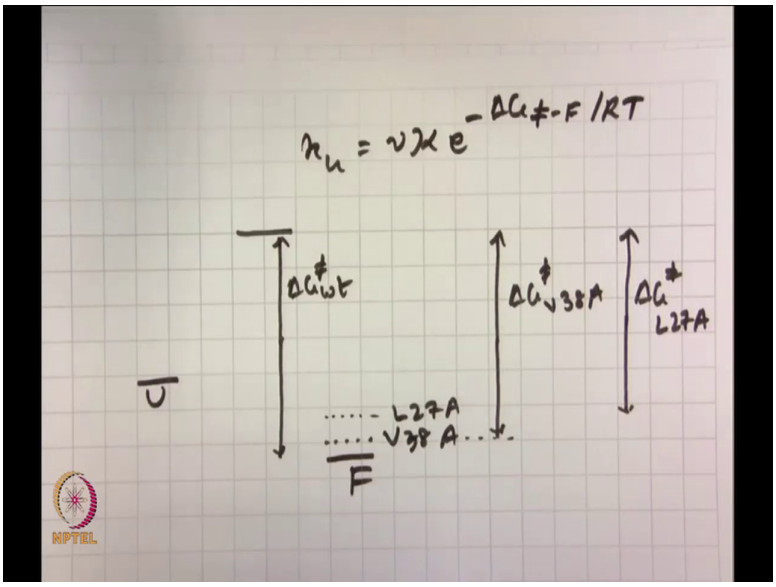

If they are destabilizing in nature of say for example, this is your transition state; this is your U, this is your F. If it is destabilizing; destabilizing means what? Destabilizing means that the free energy difference you have between the folded and the unfolded state has decreased ok.

So, maybe this is V28. So, sorry V39; is it 38? 38A ok. Now, now what has happened is with l, it is more destabilized. So, then it would go here L27A right ok. Now, we do not know anything about the transition state here right. But let us look at the kinetics. Let us look at the kinetic traces. If you, base your assumptions on this ok, we do not know anything else.

See what happens as we as we progressively destabilize, what happens? What happens to the barrier? So, initially the barrier was this right; the free energy barrier. You are looking at

unfolding remember k u; that means, you are going from F to U. So, this was your initial for delta G wild type.

So, delta G dagger ok. Now, when you are going to V38A. So, V38A say if this is V38A, this would be delta G dagger V38A. So, what has happened? Already that energy difference between your trying to state an native state as decreased ok. So, now, if you go for L27A, what will happen? It would be a further decrease.

So, delta G double dagger L27A ok. What is k u in terms of your transition state theory? k u is equal to remember nu times kappa that transmission coefficient e to the power minus delta G hash F by RT because I am looking at unfolding right ok. Now, you tell me, if you are destabilizing the protein, if you are destabilizing the protein; that means, you are destabilizing the native state with respect to the unfolded state, will it be easier for you to unfold or less easy for you to unfold?

Student: (Refer Time: 51:05) less.

Student: Easy, easy.

Think again, you are destabilizing the native state with respect to the unfolded state. That means, the unfolded state says stays like here, where your native state is going up right. So, now, is the energy barrier decreasing or increasing? Decreasing as you destabilized. So, if we decrease at destabilized, what will happen ku? Will it increase or decrease?

Student: Increase; increase.

It will increase right. Sure with me on that page; good same page then. Now, let us go back to the slide and see what we have. If you go back to the slide, you see this portion talks about ku; is not it? Because this is my unfolded arm, anything beyond talks about ku right that means to the right. So, at wild type, look at k u; when you go to V38A, look at k u; when you go to L27A, look at ku. Which has the highest k u? L27A.

Student: L27A.

And it makes sense right because you have destabilized so much that the energy (Refer Time: 52:14) is decreased and if the energy (Refer Time: 52:17) is decreased; that means, it is easier for you to unfold. It will unfold at a faster rate and hence, the rate constant has increased and what you are plotting here is ln k and because you are in the unfolding side, you are essentially plotting k u and that is why you can see these gaps coming in ok.

Now, contrary to this, look at the folding side. Tell me do you see that much of an effect on the folding side? The left side is a folding side right. So, this is now this is your folding side right. So, this is your folding side; this is your folding side kf ok. Do you see that much of a difference between the kinetic between the k f's as you saw difference between k u's ok? Tell me in one word what does it mean? See.

Student: (Refer Time: 53:16).

Ha.

Student: The energy between is not changed.

Energy between U and T S has not changed; that means, no matter what mutations you have done out there, possibly the difference between U and T.S has not been affected that much and as a result the difference between the corresponding k f's is not that huge. Specially, if you look at the wild type and V38A, if you look at these two curves, you see these two curves very close; is not it? Very close, but obviously, L27 is a little farther off, L27 is a little farther off.

So, this is how you would try to interpret a Chevron plot ok. This is one way of looking at Chevron, but there would be other cases too that either get there can be a case where you do a stabilization mutation. That means, instead of the Cm going down, it can go up. So, if the Cm

goes up; that means, you come down, then what will happen to the rate? Unfolding rate, it would decrease.

So, you would see just the opposite effect ok. So, try when you would look at a Chevron plot, you would have to try to interpret what it means. It actually tells you a lot of things. So, this is what I have taken from Jackson from Porsche group Biochemistry paper.

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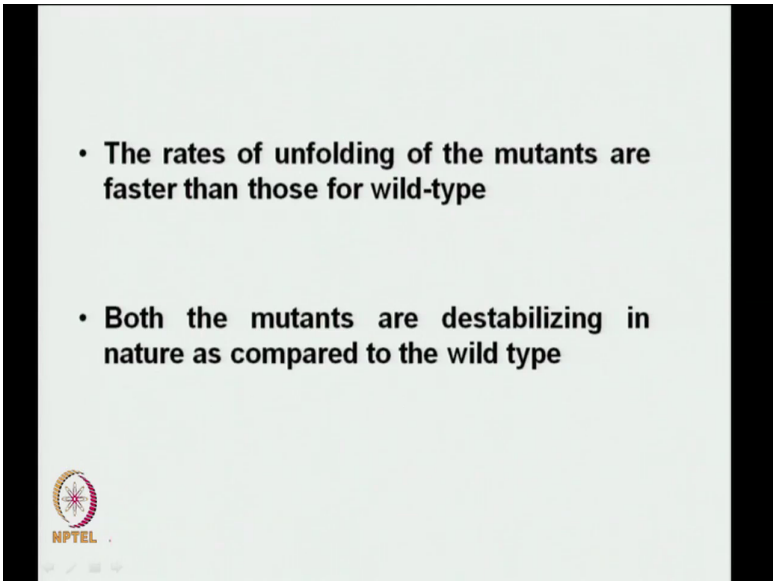

So, then the conclusions based on this the rates of unfolding of the mutants are faster than those for the wild type, 1. Both these mutants are destabilizing in nature as compared to the wild type. How did we realize? We realized by looking at the shift in the Cm values and that is what realized ok.

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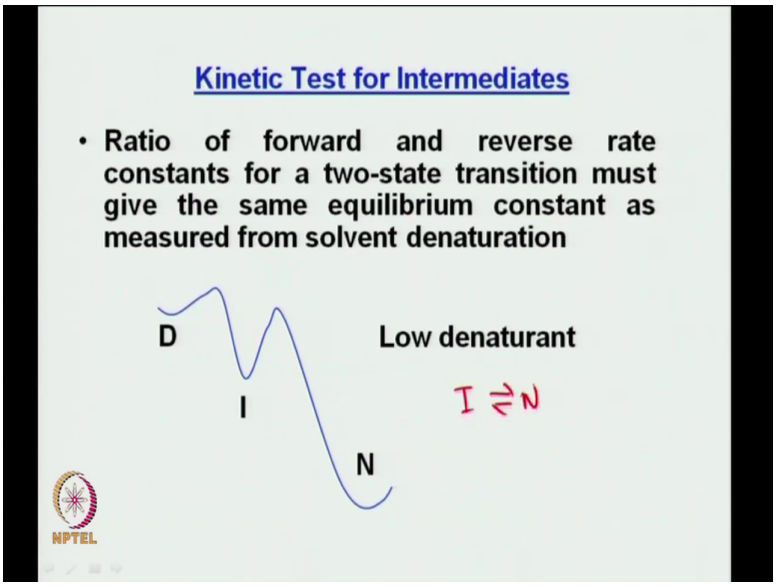

Now, this is the last thing we do today. The kinetic test for intermediates, I will go through it quick. Now, the in some cases what can happen is, in some cases what can happen is for if you look if you are looking at this what should I say if you are looking at this free energy profile right, you are going from the denatured state which is D; D is a denatured state, I the intermediate, then to N.

Now, intermediate has a lower energy than the denatured state. So that means, intermediates more stabilized in the denatured state and remember, the topic of this slide is how would you know whether it is an absolutely two-state transition or whether it is not a two-state transition; but do you have intermediates coming in that means, there are some other intermediates coming in. So, the first thing is remember what was k observed equal to k u plus k f? What is k equilibrium? If you are going from U to F, what is k equilibrium?

Student: (Refer Time: 56:11).

k f by k u right. Now, think about this. When you are looking at your when you are looking at it this kinetics, you are getting k observed which is k u plus k f, you have one equation, but you have two unknowns. You always get k observed right. Now, how would you how do you figure out how do you figure out ku and kf by themselves? You take help of the equilibrium because k equilibrium is equal to what?

Student: (Refer Time: 56:38).

Right. So that means, you have two equations, two unknowns then you can easily figure out what is ku what is kf, that is the beauty of having both kinetics and thermodynamics. Number 2, if you are going to make any mutation, what you would see is I have seen a change in delta G.

Now, if I ask you or if someone asks you, do you know which arm is getting affected more? Is it the folding arm or the unfolding arm? You cannot tell it from thermodynamics. Why? Because you cannot look at the transition state. The only way you look at the transition state is by what? Kinetics. So that means, you have to come back to kinetics and see which one is getting affected? k u or k f and then, you give the answer right.

So, these two things have far reaching consequences and a so frequently used in you know in biophysics of proteins ok. So, but anyway, so what it says is if it is a two-state scenario, my k equilibrium will always be concentration of F over concentration of U that means, the corresponding microscopic rate constants right.

And whatever k phi k and k u, I get from my kinetics; they should match because that k f by k u should be equal to the equilibrium constant I am getting at that given concentration of denaturant. If they match, then I know exactly I am talking about a two-state scenario right.

But then, you see what is the problem here. The problem here is what would be a rate limiting step? In this case, in this case rate limiting step would not be D to N; the rate limiting state would be what I to N exactly. So, you would be having I to N. See if you are ever going to if you are ever going to have these kinetics coming in, the kinetics would not be determined by D, but the kinetics would be determined by the rate of inter conversion between I and N.

So, you are missing out on what? You are missing out on D, though you started with D ok. So, what this means is that if I have a system like this, but then one thing you assure right, the equilibrium between I and N would not be the same as a equilibrium between D and N.

Hence, if you observe a difference between this and if you observe the difference between your k equilibrium here and the k equilibrium you are getting from your kinetics by k f over k or k over k f, then you know for sure there is an intermediate coming in ok.

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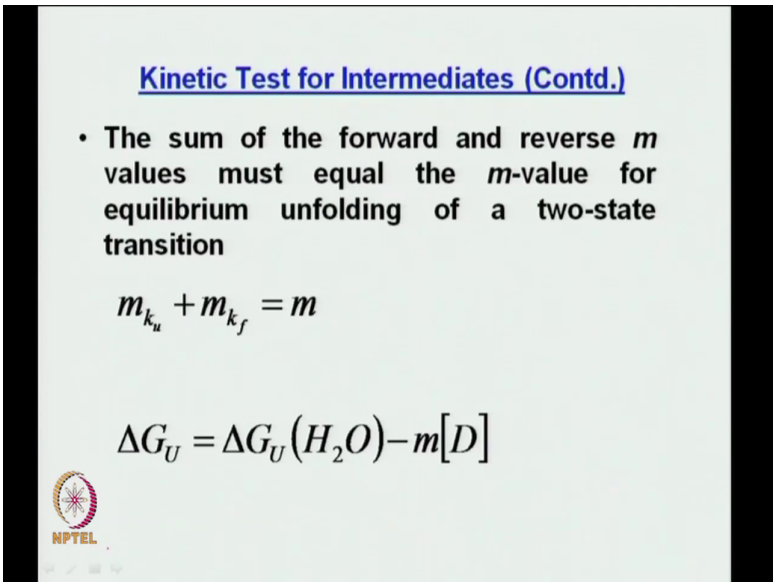

Second thing is the sum of the forward and the reverse m values must be equal ok. What does it mean? Means m k u plus m k f should be equal to m; where, m was defined like this before because m is the total change between U and D, you have to be able to have that and this is also easy in the sense that if you fit k f and k u, I mean that folding and unfolding arm, you can get m k and m k f.

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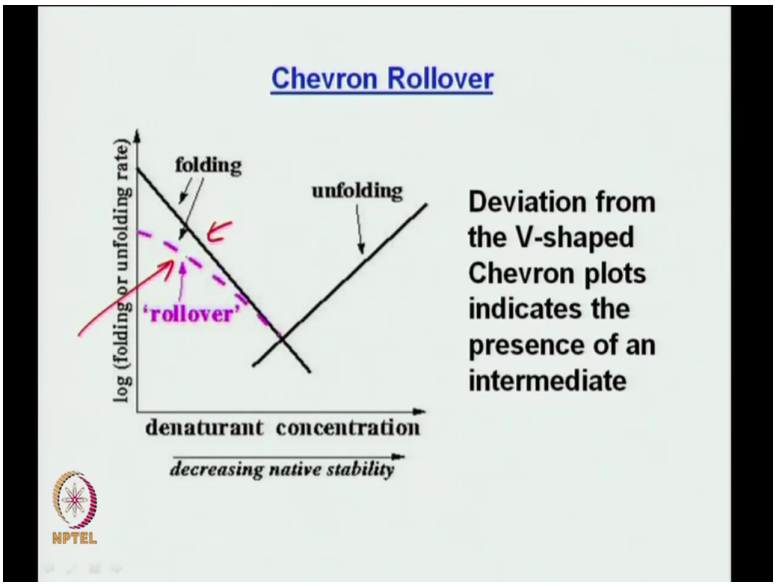

And last, but not the least, this is something known as Chevron Rollover. Remember, when we are talking about Chevron plots, they are almost linear and we said that maybe there is an ideal case. What happens if it is not a two-state scenario; see in that case, what happens? If this is the unfolding arm, this is a folding arm, when you go to the folding arm, instead of following this pattern, instead of following this pattern, it actually goes like this; it shows the curvature.

So, this is called Chevron rollover and whenever you have a Chevron rollover that means, its rolling over from its linear path and whenever you have a Chevron rollover that is a signature for you to say that there is an intermediate in the pathway ok. So, deviation from the V - shaped Chevron plots indicates the presence of an intermediate; clear? So, again this is the importance of a Chevron plot; not only with respect to the kinetics, but also with respect to the test of the presence of intermediates right.

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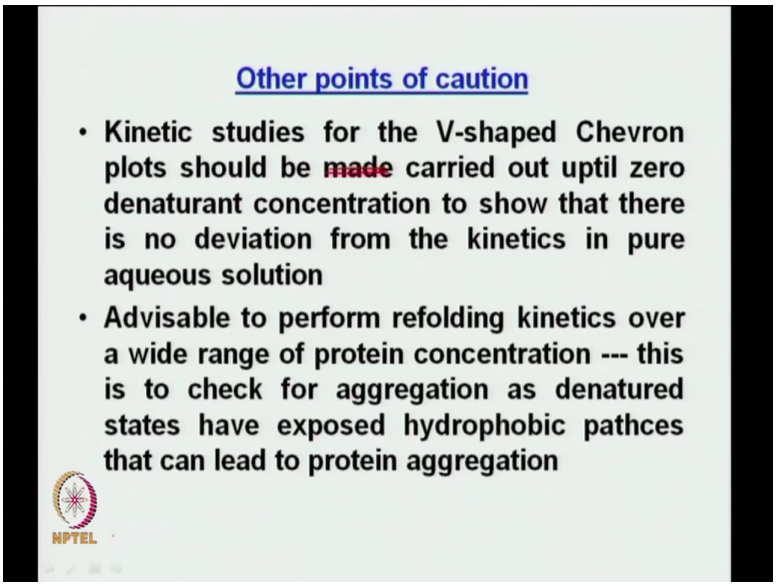

And just other points of caution before I end. Kinetic studies for the V - shaped if V - shaped Chevron plots should be made or should be made or carried out or should be carried out up till zero denatured concentration to show that there is no deviation from the kinetics in the pure aqueous solution ok.

There is one thing we will have to do. So that means, that remember the Chevron rollover you had, the Chevron rollover you had was a very see low denatured concentration. So, you have to go back to the denatured load; you have to go back to as low a denaturant concentration as possible to see that they merge with each other or you are not seeing any intermediates.

And the next is it is always advisable for those of you who would be taking these things up kinetics and protein folding, if you are ever sticking to this field. If I have not already driven you away from that field, then it is advisable to perform refolding kinetics over a wide range of

protein concentrations ok. Now, this we have never talked about; but remember when you are, but this we have talked about, when you unfold a protein, what happens is you expose a lot of hydrophobic patches.

Now, hydrophobic patches do not like water. So, instead what they would do is they would try to look for other hydrophobic patches. So, if you have too much of a high concentration of protein, then it would be easy for hydrophobic patches to find out another hydrophobic patch and then, they would come together aggregate and give rise to aggregates and if you are the moment you have aggregates, then it becomes a problem because aggregates are very hard to work with right and then, they would show you many deviations from your Chevron plot.

So, you might think that this is actual intermediate for me, but it might not be an intermediate; it instead it might be an aggregate. So, the best way for you to check aggregate is see aggregation is essentially demoralisation, agglomeration whatever; it would depend upon a concentration of protein.

The higher the concentration, the higher would the data of aggregates. So, what you do is you vary over a certain range of protein concentrations and see within what range your ku or kf is rather in this case, it is a refolding which is kf is reproducible.

That is the range you take. The moment of aggravation you will see, you will be having differences coming in. So, if you have ever for our for people who are doing experiments, proteins are always tending to aggregate depending upon what type of protein you have. These cautions you have to maintain ok. So, this is typically, whatever I had to talk about kinetics done; thermodynamics was done kinetics is done now.

So, next class what you would start with start with is would actually start to look at instrumentation, rapid mixing methods, fluorescence, CD and all these things that is the last part of our course ok.