Bio-Physical Chemistry
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Lecture - 16
Thermodynamics of Protein Unfolding

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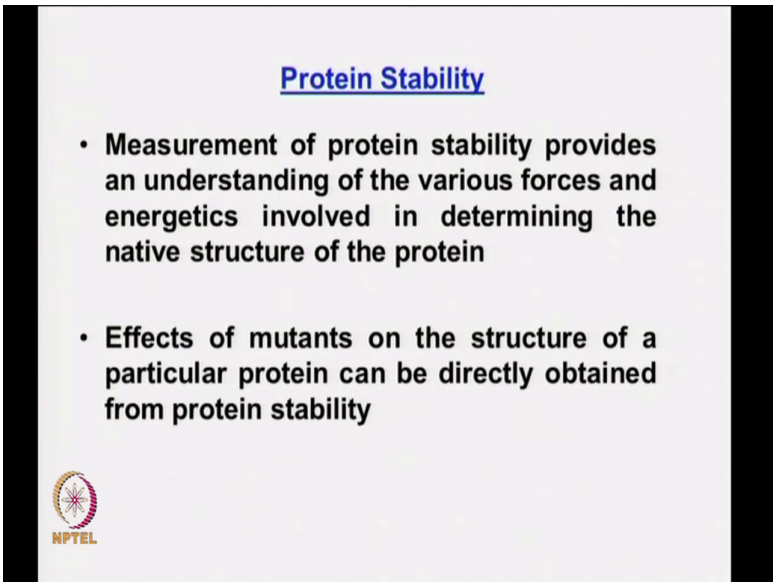

So, we will continue with our discussion on the Thermodynamics of Protein Unfolding. And as we were talking yesterday that protein stability is one of the key issues; that people who are working with proteins especially biophysical researchers or researchers in biophysical or the field of biophysical chemistry they worry about.

And we said was measurement of protein stability gives an idea of the various forces involved and if you would be doing mutations, then that can bring over changes in protein stabilities and how would you look at those.

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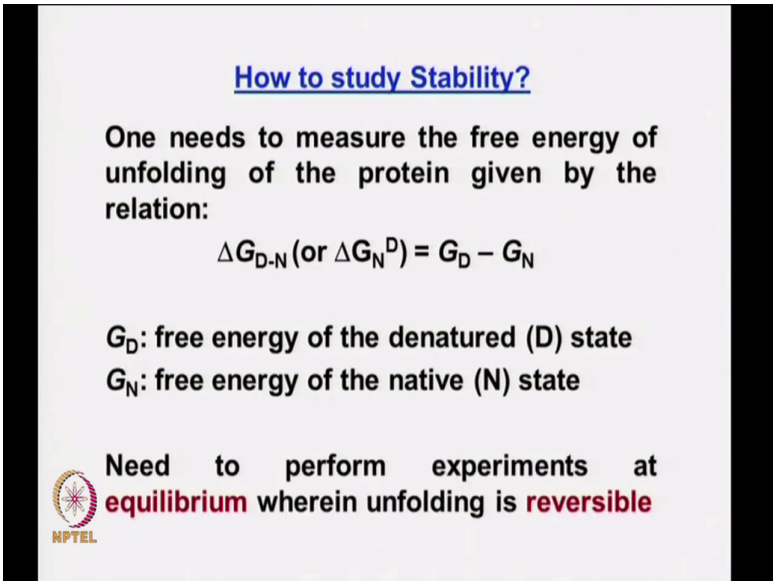

How to study stability? We said that we need to measure the free energy of unfolding right and the free of unfolding is given by this where D is a denatured N is the native right. And we also what we; also made note of was that what we are going to talk about are equilibrium measurements. So that means, we have to be at equilibrium and if its equilibrium that means either we go back or forward; we should be able to come back to the same point without losing any information; essentially it should be reversible.

And why we care about equilibrium or why are we so much focused on equilibrium because you know we have this relationship between del G and K equilibrium right and del G is

directly related to that. And hence if the system is not an equilibrium; there are many cases where we would discuss later that the system is not reversible; that means, the system is not in equilibrium, in that case you cannot actually do this analysis right. So, the bottom line is that you have to have the system in equilibrium ok.

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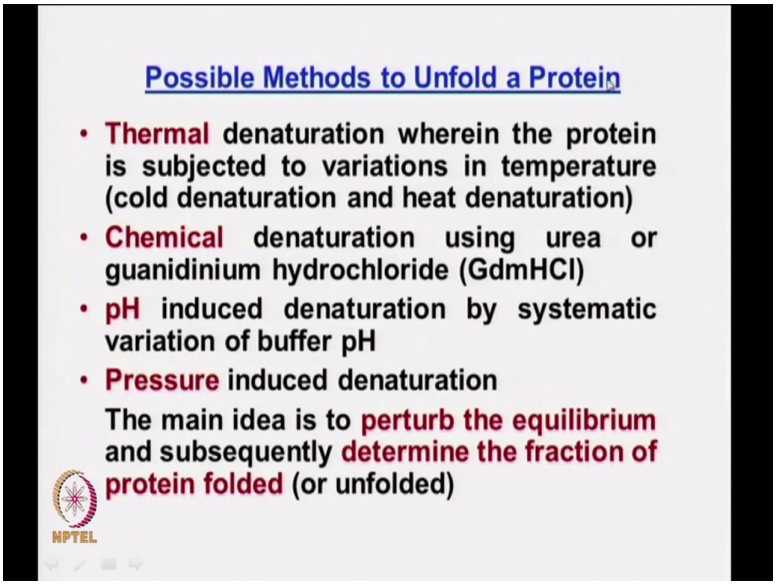

So, what are the possible methods to unfold protein; this we looked at; one was thermal, one was chemical, one was pH and the other one is pressure right. And again the main idea is that you perturb the equilibrium and determine the fraction of protein folded or unfolded. Now, since we are doing what should I say; since we are doing denaturation; that means, you are going to the denatured state, you would rather look at the fraction unfolded or the fraction denatured ok.

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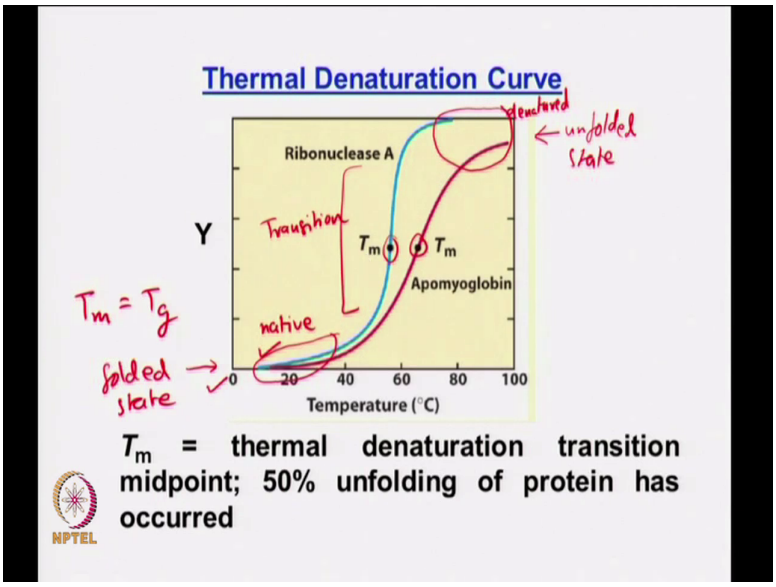

So, this was was actually one of the starting points of our discussion on protein denaturation. So what we are looking at was; we are looking at two different proteins ribonuclease and apomyoglobin; Y is an observable.

Now, what do you mean by an observable? A Y is a signal right; a signal which you observe; that means, you take a protein in a container say in your sample cuvette right and you do a change in temperature; that means, you increase the temperature.

And the effect of this temperature increase or temperature change is registered by some observable; by some signal and that is what Y is. So, what are the; you know few signals that people commonly use? One is electricity; I do not know whether you heard about the

technique circular dichroism. So, circular dichroism will give you an observable signal; so that would be one Y.

There is another technique called fluorescence; so, fluorescence will give an intensity change that will be an observable for us. Now, there is another technique called absorption or UV visible spectroscopy right; so there also you can have a change and that will be a corresponding Y and many like this right. So, that is our observable something we are measuring.

Now, between ribonuclease and apomyoglobin; what we said was, though they have different transition profiles; that means, different ways of going from see if you look at this arrow; if you look at this arrow; so, the blue one is ribonuclease a, the red one; obviously, is apomyoglobin as told out here.

And if you go from the low temperature to the high temperature side; what you essentially do is what essentially you do is; you are spanning across three regions of the transition and what are the three regions? If you look at the low temperature site, you follow the arrow; if you look at the low temperature, where it is written native? It means that you are in the low temperature, the protein is in a stable form and hence you are in the native region right.

And then you increase the temperature and suddenly what happens is; from here say from you follow this arrow from here to here, you have a huge change within a small range of temperature that is actually a transition range right. And then after the change is done then you go from here to onwards. So, that is what? That is your?

Student: (Refer Time: 04:55).

Denatured state ; that means, you have already denatured, you have already gone over to the denatured site; that means, your system has mostly is consisting of now denatured molecules because you have raised the temperature, unless everything after that is your denatured

correspond to your denatured state. So, one is your pre transition which is the native one; one is the transition where the actual transition occurs the sharp change and the other one is your.

Student: Post transition.

Post transition right; now, the other thing we said was if you look at these two important parameters hm; T m for both the protein. So, what is T m? If you remember, we had discussed that this T was or T g right what we had discussed before T g is the midpoint of some spectroscopic signal; spectroscopically determined transition signal or transition right.

So, this is essentially what midpoint means; that means, it is a midpoint of your transition from the folded to the unfolded state ok; that is what T g is and here T g has been replaced by T m; the same thing. So, that is what it says T m is equal to T g; m either stands for melting or midpoint; does not matter which you refer to.

And what is the characteristic of T m? The characteristic of T m is that this is as it say is the midpoint; that means, your K equilibrium is equal to 1 and if it means K equilibrium is equal to 1; that means, it is K equilibrium is equal to concentration of denatured over concentration of native; that means, both these have equal populations.

Before I move on, there is one more comment I would like to make; the comment is this. See, suppose you are looking at free energy changes right; your free energy change is related to equilibrium; delta G is equal to minus R T ln K equilibrium right, that is what it is.

Now, if your free energy is related to K equilibrium; now what will happen is to get a reliable free energy, we should be in which region or which region of this transition you think? Remember, I can only do equilibrium measurements, I can only do equilibrium measurements or those measurements are meaningful; if I have measurable concentrations of both the denatured state and the native state; isn't it true? See, if my K equilibrium is either on this side or this side; it does not make any sense because my equilibrium is only on either one side or on the other side.

But what I am doing now is; I am trying to do equilibrium measurements an equilibrium measurements are best done; are best done if I have measurable proportions of both of these where would you have that?

Student: Tm, Tm.

Right or you would be having something in the region which is in the transition close to the T m.

Student: Yes.

So, that is why; remember if you are doing any equilibrium measurements, you are better of being in the transition region rather than being in the corresponding baselines which is the pre or the post transition baseline. So, always you focus on the transition region that gives you the best value right. Because here both of those the natured I mean the denatured and the natives states are populated to a significant extent.

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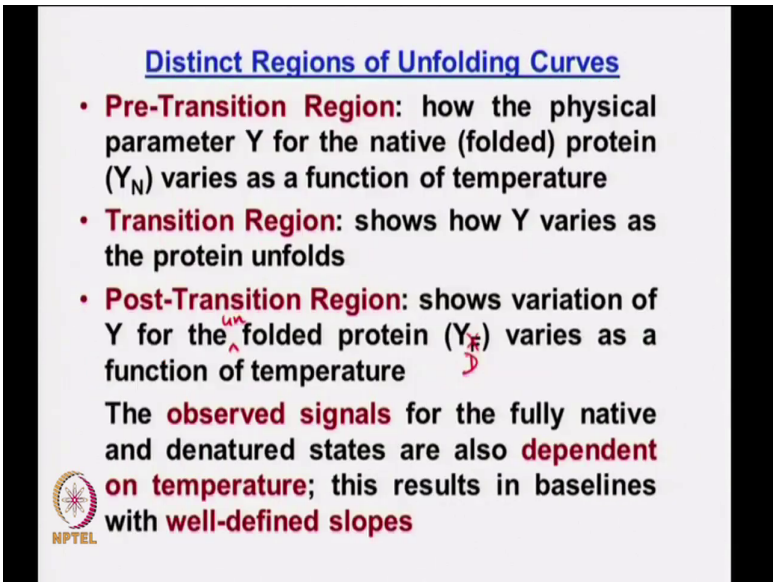

So these are the different regions we talked about yesterday.

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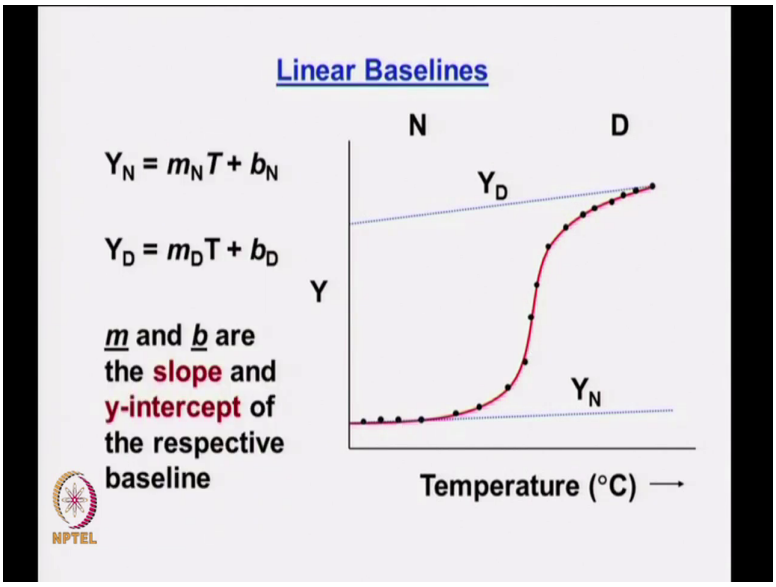

Now, let us look at this; this is also what we refer to, if this is a curve; so what we are doing now is you look at the black points, you look at the black dots out here in your; in this curve, you look at this black dots.

So, these black dots are your experimental points; that means, you have at every; degree at any given temperature, you have measured the signal Y and this is how the transition moves, as you increase the temperature for a particular protein. Now, what is the red line? The red line is a fit, you are trying to fit this curve and extract certain thermodynamic parameters right.

Now, look at these blue lines; let me move further, scroll further. So, what are these blue lines are referring to? You look at these blue line which is referred to as Y; what does it mean? Remember, we talked about pre transition and post transition baseline; see we have to know;

we have to know what is the actual value of Y N; what is Y N? Y N means I will write it down later, Y N means the observable for the fully native state; what is Y D? Y D means the observable for the fully denatured state.

But you see when I am varying temperature; what is happening? This is these slopes, you can see these lines are not parallel to your temperature axis; that means, they have well defined slopes. So, what you do is; you define, you define your Y N and Y D in this form that your Y N is equal to m N times T plus b of N right. So, again what is Y? Y N is the signal we are getting from the pure?

Student: Native.

Native state; this is equal to m N times T plus b N; what is T? Temperature; remember our signal from Y N is depending upon temperature because the slope is not parallel to x axis. So, then I mean the line is not parallel x axis; it comes with a defined slope. So, that slope is defined by m N and then you have; obviously, if you are having a straight line, you will be having an intercept which is b N.

Now, the same thing happens with Y D; exactly the same way. So, this is Y D; this is the blue line, corresponding to Y D and then you have m D times T plus b of D. The slope for the transition for the baseline of the post transition and then b of D is the corresponding intercept, as it is written out here ok; these are two things.

So what you can see is; in Y N and Y D, how many parameters do you have which you need to fit? Because these you do not know, you have to fit somehow; how many parameters do you have? See for Y N you have two parameters; what are the parameters?

Student: m of N.

m of N b of N; for Y D, how any many parameters do you have?

Student: m D and (Refer Time: 11:06).

m of D i m D; b D; that means, you already have four parameters which you have to vary when you are going to fit this experimental black circles to this red line; that means, this is your fit line right.

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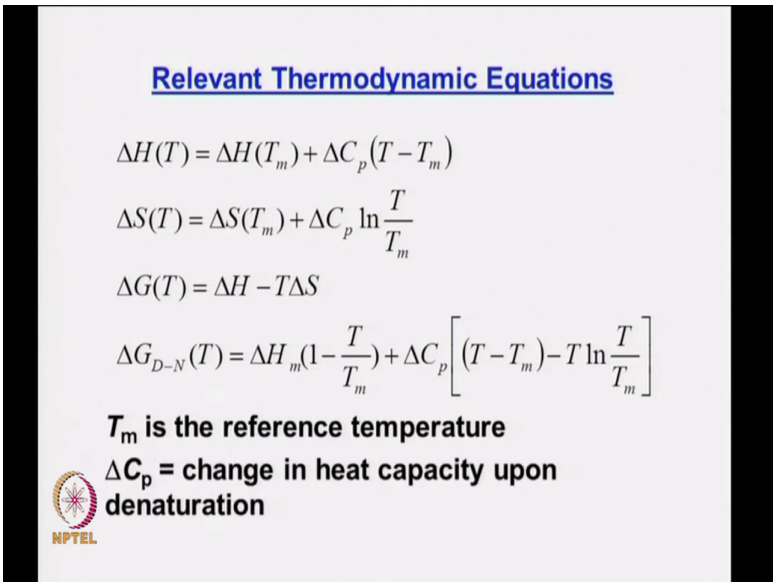

So before, you have going to the relevant thermodynamic equations keeping this in mind; let us look at some basic equations. So, what I am going to do is or what we are doing rather here is this.

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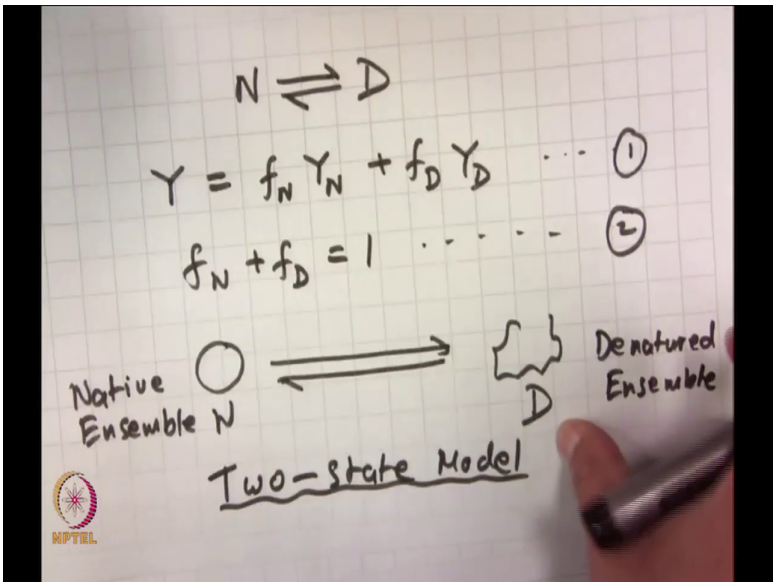

What we are looking at is we are looking at the protein going from the native state to the denatured state ok; that is what we are looking at. Now, my observed signal Y; my observed signal Y, this is my observed signal; at any given temperature right.

See, Y is a function of T right because you see it changes as a function of T that is temperature at any given temperature it should be having contributions from how many species? You can have contribution from the native state, you can have contribution from the?

Student: Denatured state.

Denatured state. So, what I can write is; I can write Y is equal to fraction in the native state, times the signal given by the native state plus fraction in the denatured state; times the signal given by the denatured state clear ok. So, that is say this is 1 for us.

Now, there is one more thing based on the way we have written, N going to D that is a native state of the protein going to the denature state; what I can also write is f of N plus f of D is equal to what? 1 right because these are the only two species present. Now, when we are saying this; now this is this is very important.

When we are saying this; what do we mean? What we mean is; carefully listen to this, what we mean is we have only two states available. What are the two states? The two states are the native state and the denatured state; we are not considering any other state.

I will tell you what I mean by any other states; that means, for example, so to diagrammatically represent it; I have a state which is native ok, it is a compact form; a globular form and I have a state which is denatured right. I am looking at an equilibrium between these two; at any given temperature.

But you can easily raise a point; the point is that what is the surety or what is the guarantee that I would not be having any intermediate states between N and D? I can have, I can have, but this is the simplest way I can represent a transition right.

So, this; this N to D is often referred to as a two state model; this N to D is often referred to as a two state model. Again, please try to understand the relevance of this by two states I mean that I have a native state and I have a denatured state, nothing in between that was number 1.

Number 2 is, if you are considering a native state and a denatured state; please do not think that the native state is only one structure or the denatured states is only one structure. What we mean is that in the native state you have an ensemble of structures; that means, quite a few

structures which belong to the native state; that means, which are; which are characterizing the native state, they are very similar properties.

In the denatured state, we have a quite a few structures; you know quite a few confirmations which are very similar to each other and constitute the denature state; it is not just one structure. It is remember, if you remember what we discussed for protein folding, you lose a lot of entropy right when you come from the unfolded state to the folded state.

So these unfolded state is having a lot of entropy; it will always move around right it is so flexible, hence you it is very hard for you to pin down on one structure. So, the denatured structure will always be having on ensemble of structures.

Now, same for the native; why? Because no protein is rock solid; that means we look at a protein, does not mean that the protein has only one structure; it is, it can have only one structure. Every protein is dynamic, it shows movements and whenever this protein will show movements; there will be certain conformational changes and we will get you will get a host of structures which again corresponds to the native state.

So is it clear what I mean by a two state transition? A two state transition is only having two ensembles a native ensemble and a?

Student: Denature.

Denatured ensemble so; that means, I can write. So, this is my native ensemble and this is my?

Student: Denatured ensemble.

Denatured ensemble ok; so that is one.

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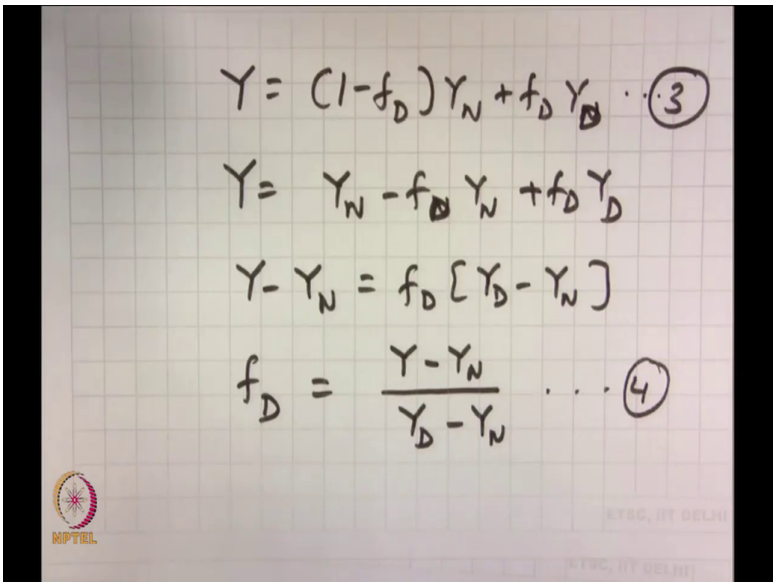

Now, based on this; based on these two equations, what I can do is I can write Y is equal to; now, f of N has, there is a relation between f of N and f of D. So, f of N would be equal to what?

Student: 1 minus.

1 minus.

Student: f of D.

f of D; then I can write Y of N plus f of D; Y of D right; so let this be equation 3 for me; so let us simplify now. So then, I can go and simplifying I said right. So, Y of N minus f; D; Y

of N plus f of D; Y of D or the next line I can write Y minus Y; N is equal to f of D, Y of D minus Y of N or now see what we have done. We have said that f of D is equal to Y minus Y N over Y D minus Y N ; this is equation 4, clear?

Now, what is the significance of this equation; can someone tell me? See, initially we started with f of D; f of N right, but see we are not measuring f of D; f of N directly, what are we measuring though? We are measuring the observable Y.

So, what now you have is; you have expressed f of D which is the fraction of the denatured state in terms of your observable Y. So, if you look at the numerator; what does it give you? The numerator gives you; Y means Y observed, you are doing the measurement; Y N is that, remember the baseline thing Y N is equal to m N T plus b of N ?

And then you have Y D minus Y N ; now to understand it is very simple ; what are you going to do? f of D is the fraction denatured. So, what does Y minus Y N tell you? Y minus Y N tell you, how much you have moved away from the native state; that means, how much you have denatured right; that is the difference. Now, what does Y D minus Y N tell you? Y D; Y N minus Y N; remember, if Y D is belonging to the pure denatured state and Y N to the pure native state, there is a maximum difference you have; you can have between the two observables in terms of Y.

So, hence your f of D is just Y minus Y N over Y D minus Y N; even without actually doing this derivation, you would have been able to write it out. Now, there is equation number 4; there is one more thing we know, we know that we are always maintain equilibrium condition.

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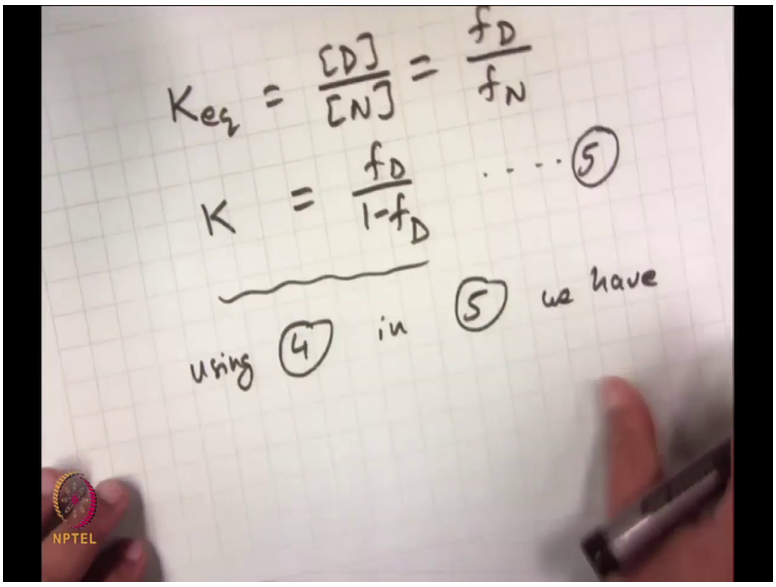

So, what do we have? So, we have K equilibrium; K equilibrium is equal to what should I write? Concentration of D over?

Student: (Refer Time: 19:25).

Concentration of N right; so this can be written as f of D over f of N or I can write K which is equilibrium. Now, I am just writing K is equal to f of D by 1 minus f of D. So, this would be equation number 5 from us.

Now, what is the importance of this step now? Remember, again we are doing equilibrium measurements; equilibrium constant is very important for us and we have to relate the equilibrium constant to your observable. What you have done is; you have equilibrium

constant is related to your concentrations or fraction whatever; that is what you have looked at, but you know there is a relation between what? f D and?

Student: (Refer Time: 25:19).

Y in terms of Y and Y D. So, you actually have a relation between K equilibrium and the corresponding.

Student: (Refer Time: 20:25).

Observables right? So, then let us write that down.

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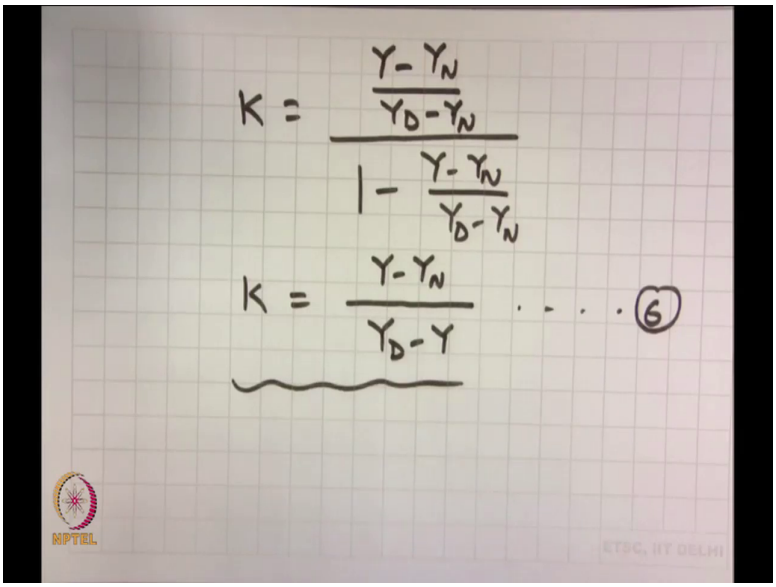

So, what I can write is using equation 4 in 5; we have K is equal to f of D which is given by Y minus Y of N over Y D minus Y of N; full divided by 1 minus Y minus Y; N over Y D minus Y N; this is another expression for K.

And hence, so K would be equal to what would it be? It would be Y minus Y of N and what do you have in the denominator? Y D minus Y and this would be what equation number 6 for us I guess. See again, it makes sense right; what was your equilibrium constant? Equilibrium constant was essentially f of D by f of N; what is Y minus Y N? It is the amount by which you have moved out from the native state right and what is Y D minus Y; Y; so Y D minus Y is the difference between what?

Student: Denatured and.

The denatured and the Y; that means, the amount you have moved away from the?

Student: Denatured state.

Denatured state; so which is essentially f D over f of N ok; make sense ok? Now, what is the last piece of information we need now? There is still one connecting equation, we have to connect; remember we are always going for what? Delta G free energy.

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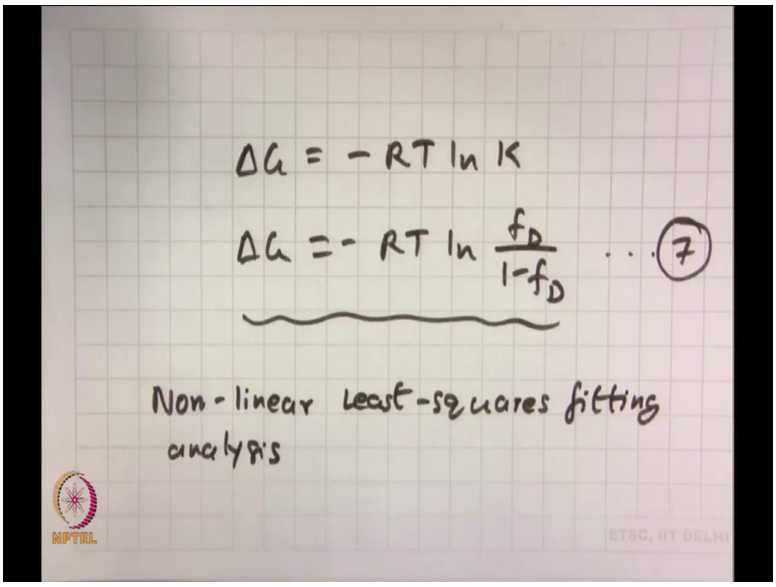

So, the last information is or the last equation is ; it is the last piece of the puzzle the delta G is equal to minus R T ln; K equilibrium or minus R T, natural log f of D by 1 minus f of D and this should be 7. See by doing this, you have actually achieved a huge thing, you have related delta G to what? You know delta G is related to K equilibrium, but not only that; you know K equilibrium is related to f of D; f of D is related to what? Y; Y N and Y D.

So, you have related delta G to what? Your change in observable; having done all these things, see all these things were pretty easy right; you know it. The assumption was that it was a two state assumption that is what it was; otherwise it was just straightforward and everything fell in place. And we finally, managed to connect what? The stability parameter which is delta G; that is what we always go for through the observed signal, you are getting right.

So, now if you carry on with this; after we had these equations, the set of equations; you know you also know this relevant thermodynamic equations right. So, delta H is equal to delta H; T m plus delta C p; T minus T m, where your reference temperature is not T g now, it is T m because I said T m is equal to T g. And you have this was host of relations and finally, you can see; this delta G right, we derived this equation yesterday is related to delta H m, T m, delta C p; it do not have anything else. So, T m is the reference temperature and delta C p is the change in heat capacity upon denaturation.

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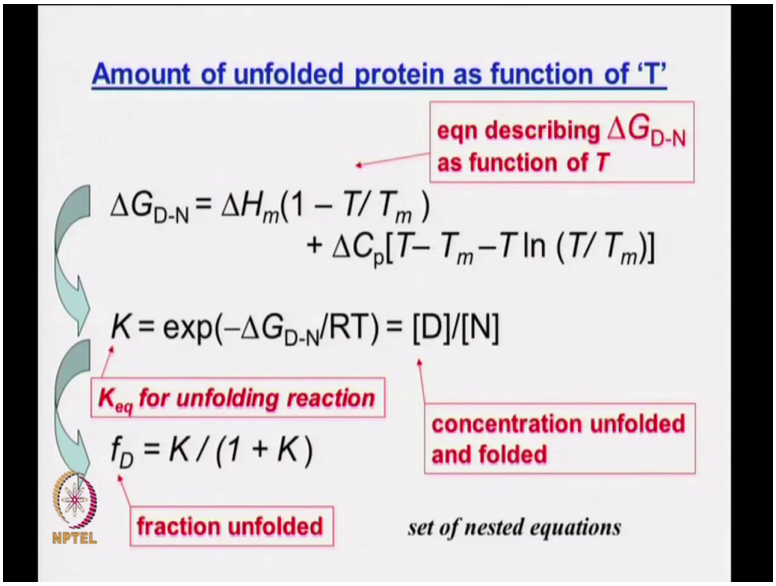

Now, how do you actually fit your curve? Remember that, we had these black circles or black dots and there was a red line passing through the curve. So, that red line was the fit to the curve; it is called a non-linear least squares fitting routine or that is fit rather ; so, this is what you do.

This is an equation describing delta G unfolding; that means, you are going from the native to the denatured state, as a function of T. You have K which is given by this relation, you have f D; which is given by this I have derived that ok. Now, here if you see at the bottom; see at the bottom; so these all three equations are referred to as a set of nested equations. You know what happens in nest? In nest means we have everything together right we have this mesh. So, these are nested equation means all these things, all these equations are related to each other in some sense or the other ok; by through observable parameters.

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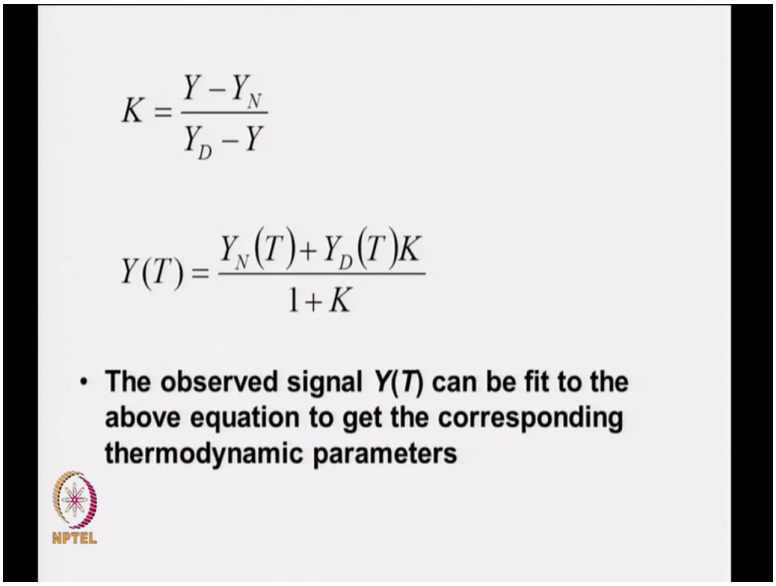

Next, what is K? K is equal to Y minus Y N by Y D minus Y; we just derived and then remember, we are doing this as a function of temperature; hence I can express Y in terms of this Y N; Y D and K simply, I; I am just rearranging this equation to express this like this no problem right.

And the only thing I have done is; I have put in a temperature T, why? Because I know; I am doing a temperature dependent denaturation unfolding a transition. So, see you have Y of T is equal to Y N; T plus Y D; T times K over 1 plus K. You know what the expression for Y N of T is right? What was it? It was m N T plus b N; there were two parameters right.

Now, look at Y of D; what do you have? m D T plus b N; again you have two parameters, right now. Then you have K, what does K depend upon? K depends upon the free energy change right. Now, the free energy change; look at this now, the free energy change is how many parameters now? You have delta H right, you have delta C p.

So essentially, all these parameters have to be fitted together; that means, when you have the transition curve that those circles; if you remember, let us see if I can get that quickly yeah. So, if you remember all these experimental points ok; you are trying to fit these experimental points which this fit is a red line. So, you need all these equations together to fit your experimentally observed transition.

So, the basic equation you are using; let me go through this, the basic equation you are using is this; the last one. The basic equation using is the last one; you know what Y N is, you know what Y D is; we have expressed it, you know what K is and you fit your observed transition to this equation; this is called a non-linear least squares fitting analysis ok; a non-linear least squares fitting analysis that is what it is called ok.

I will write it down. So, what you are doing is; you are doing is a non linear least squares fitting analysis and hence you will be getting typically all your parameters; T m, delta G. Then you will be getting m N; b N; so essentially what you will get is; you will get Y N and Y D which are the spectroscopic signals of your pure native and denatured.

Now, let me make this point before we move on; see it is very hard to get this Y N and Y D. What do you mean by that? When you are at the room temperature; where the protein is more stable, your K equilibrium is almost towards the native state. But even though it is towards

the native state, remember you always have; no matter how much small a fraction of a denatured molecules are there; so, essentially it is not your pure native state.

Similarly, when you go to the other side; you have essentially denatured state, but you still have it mixed with a little amount of; no matter how small it is a native state right, some native confirmations. So, essentially what you do is; it is very hard for you to measure the actual Y N and Y D and that is why this non-linear fitting routine gives you the absolute signal of Y N and Y D.

Because why do we need the absolute signal because the absolute transition is defined by what? The difference between Y D and Y N because those are the maxima; your final transition, your final observable cannot be more than Y D. Your the observable Y N or whatever; you are start starting from you cannot be lower than Y N because you have to start from the pure native and then you go to the pure denatured that is what you are trying for; that is what you are looking for. Is it clear?

Now, this is one way of fitting your thermal transition. So, that is what it says the observed single Y T can be fit to the above equation, to get the corresponding thermodynamic parameters ok; all these equations are interrelated.

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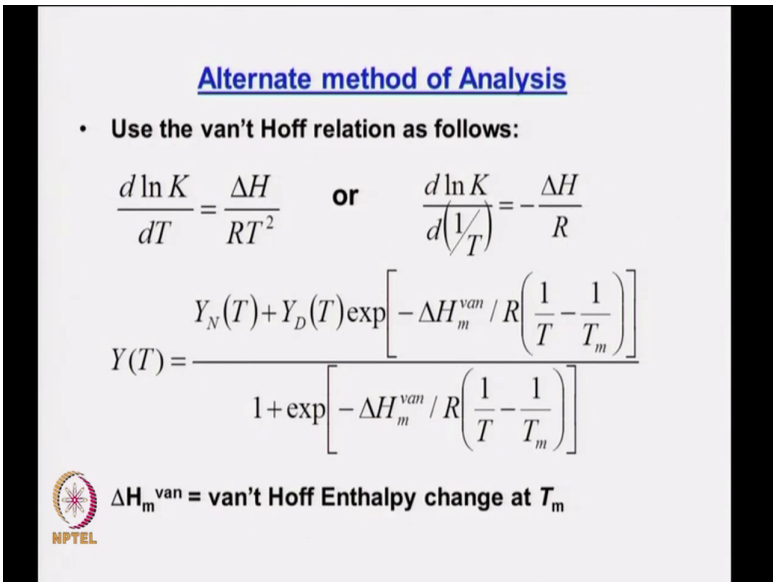

Now, what is an alternate method of analysis? The alternate method of analysis this; you know the Van't Hoff's equation? You have done in thermodynamics; this is a relation between your equilibrium constant delta H right. So, this can also be written as you know; there is a two different derivative forms d ln K or d of 1 by T is equal to minus del H by R ok; where del H is the change in enthalpy; obviously, and K is the equilibrium constant.

. So, now what I can do is; remember, I had this equation of Y in terms of Y N, Y D and K. So, instead of putting the other equation of K; what I do is, I put in this K and what I know from this equation is K will give me this expression. So, I am just putting there and I do an analysis on that, I do a fitting on that based on Y of T ok.

See, what do I have here? What I have here is; I still have Y N, I still have of Y D, I have delta H m, I have T m, but I do not have delta C p here; though I have delta ok. So, this; this

delta with a van on top means on subscript superscript means Van't Hoff s enthalpy change at the midpoint of transition T m.

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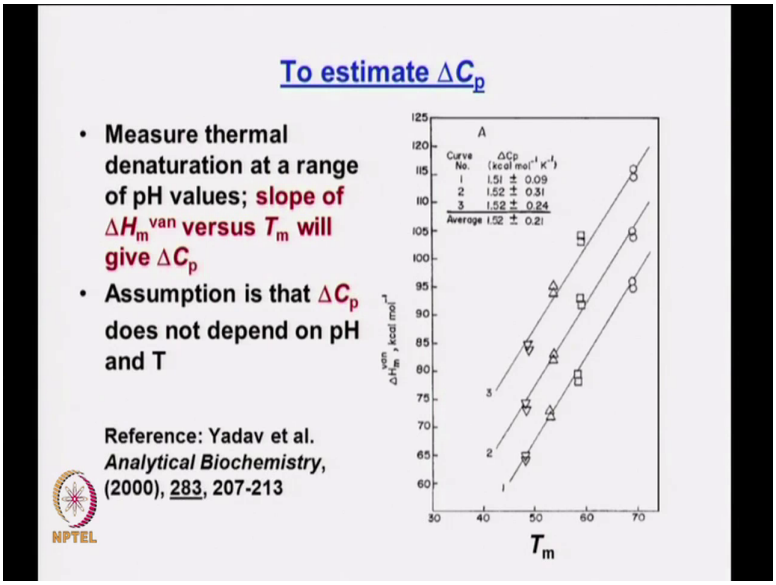

Now, the question is how do you estimate delta C p? If you rigorously, very academically, rigorously if you want to do a delta C p calculation; this is what you should do ; measure thermal denaturation at a range of pH values ; slope of delta versus T m will give you delta C p ok; how?.

So, this is the experiment that was done. What you do is; see what will happen. When you change pH; you know your protein stability is depended upon pH right and T m is what? The midpoint of your denaturation transition, so the T m will also depend upon pH right because you are changing the stability of the protein.

. So, what you do is; you do thermal transitions at different pH values say one is pH 4, one is pH 6, one is pH 8, one is pH 9; whatever. Now, what will happen is when you do this thermal denaturation transitions at different pH values, you will be getting for every pH different T m values right because T m is depended upon the protein stability.

Now, then what you can do is; you can plot your delta for your Van't Hoff's analysis, against this I should have written here and maybe I am see yeah. You can; you can plot delta H Van't Hoff versus T of m and remember this is the slope. So, delta of delta H over delta T gives you what? Delta C p; isn't it? Because the slope of the change in enthalpy or rather the derivative; the change in enthalpy with temperature is your delta C p.

So that is what; it says if you look at these three different curves; if you look at these three different curves, this is our certain pH ok. So, this is another; another pH, this is another pH and you can see; what are the delta C p values available? They have even given you it is 1.51 with this corresponding standard deviation. So, they are very close to each other; this, this is an experiment which has been very carefully done and you can see the average values very close to each other. So, your average C p is or delta C p is 1.52 ok.

So, this is one of the best ways; if you really want to be thorough with your research, this is one of the best ways of getting your delta C p. And remember delta C p is the one which defines the change in delta H, which defines the change in delta S and it also defines what? One more thing, what was; what was the relation of delta C p with delta G? Remember, the stability curve; what was delta C p related to?

Student: (Refer Time: 34:10).

The curvature; delta C is related to the curvature of your stability curve, isn't it? It also gives you that relation or that information that is why delta C p is so important. So, anyway see we really want to do an analysis; a thermal transition analysis, you want to get the corresponding parameters and these are different ways you can do it; is it clear?

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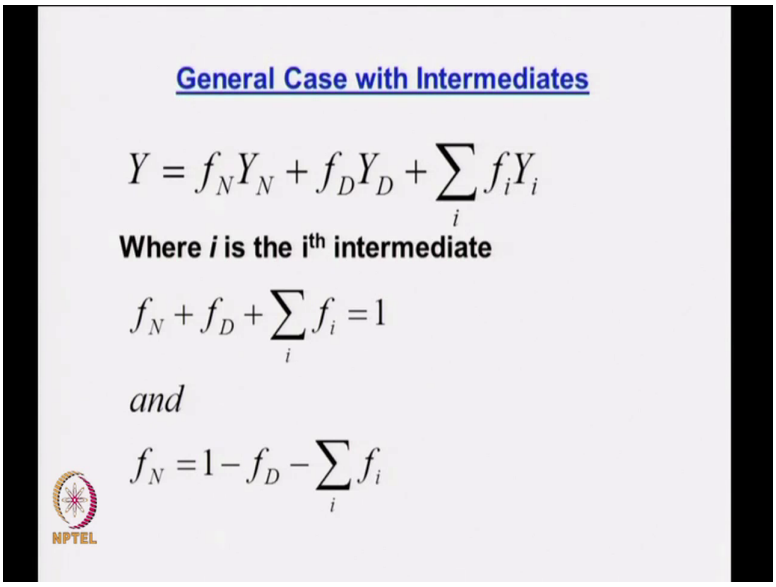

Now, this is where we considered only two states N and D; what happens if you have more than two states?

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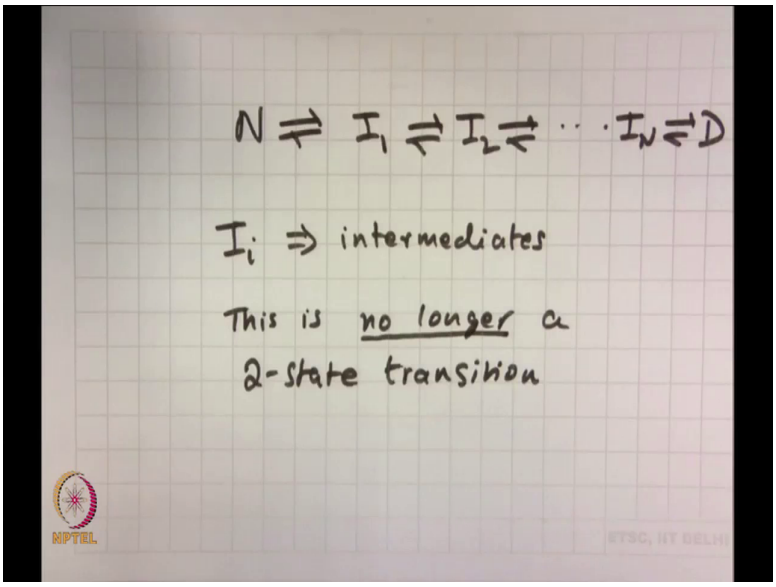

So, what I am talking about now is ok; we have looked at a two step scenario; now, suppose I have a scenario where I can have say N in equilibrium; say an intermediate I 1, in equilibrium with the intimate I 2 and so on and finally, I have say in equilibrium with D.

So, all these I i's are referred to as my intermediates. Then this is no longer a two state transition; this is no longer a two state transition ok, but I can take care of it, at least I can try to take care of it like this. What I say is initially, Y was equal to; when it was two state, if you look at this equation which is on the slide; Y was equal to f ; Y of N plus f; D Y of D right that was; so up till; up till this arrow where the arrow is it was a two state.

But now you are saying you have intermediates. So, what do you do? You take a summation of all the intermediate that can be present and with the corresponding fractions. So, that is the

small change you bring in your basic equation. Then everything follows the same way; i is the i th intermediate and then you know that f N.

So, the summation of all the fractions should be equal to 1 right and f N is given by this; the fraction of the native state is given by this. So, then what follows is; I will go through the same drill; that means, again I will try to relate f of D or f of N; I will try to get K and I will try to get delta G and go through that fitting routine possibly.

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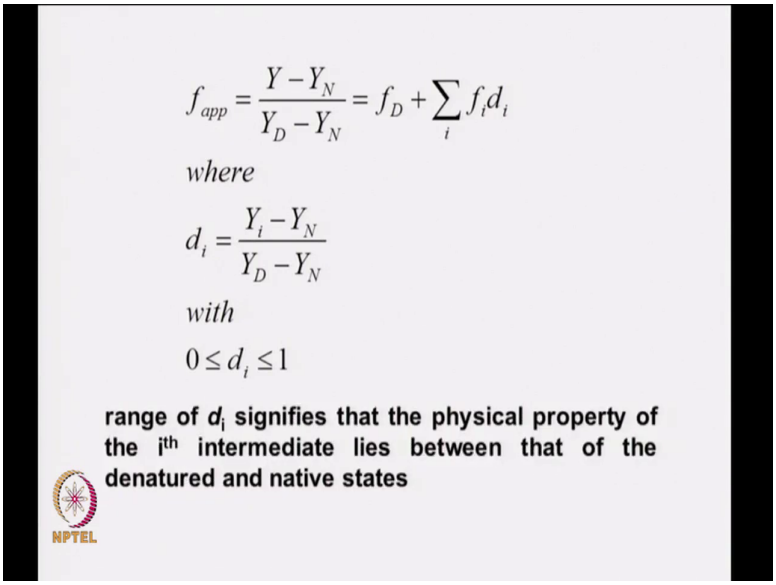

So, what are the next set of equations? Here, what I do is instead of writing f N or f of D; what is see? I right f apparent. Why do I write f apparent? Because I know there is this is not only one denatured state I can have many intermediates now.

Then what I say is if f apparent the expression still remains the same, but what has changed is you can see; it is f D plus sigma f i; d i where di is given by this. So, Y i minus Y N over Y D minus Y N Now, the significance of Y i is; Y i is the corresponding signal of the ith intermediate and Y i minus Y N signifies what how much you have moved out from the native state right and already Y D minus Y N is you know always the absolute value.

The constant is the di is between 0 and 1; what do I mean between 0 and 1? That means, if d i is close to 0, where do I where am I? If d i is very close to 0 of d i is very close to 1, if d i is very close to 0; I am say very close to the native state, if di is very close to 1; I am very close to the?

Student: Denatured state.

Denatured state. So, my assumption is that whatever intimates I have; they have to be between the native state and the denatured state, hence 0 and 1 right ok. So, as it says range of d i signifies that physical property of the i th intermediate that lies between that of denatured and native states essentially that physical property ok.

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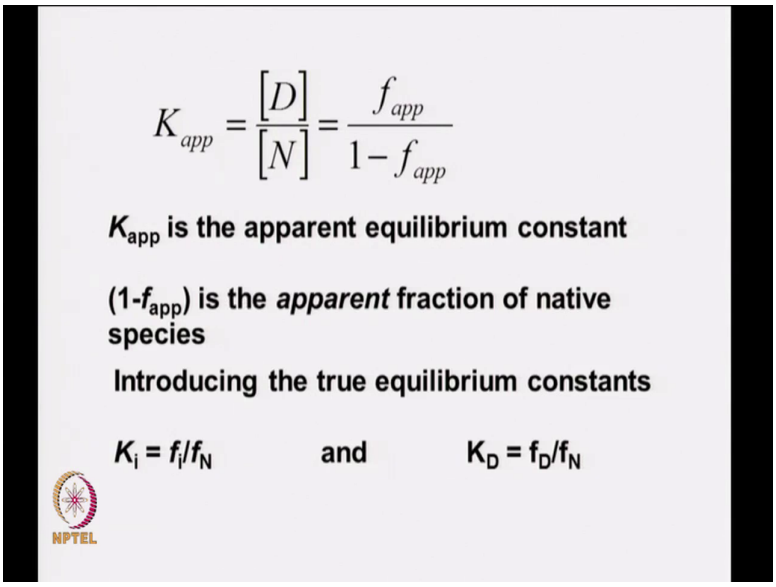

Now, I go for K apparent; again the equilibrium constant. So, this is given by the same relation, we already know what K apparent is; it is a apparent equilibrium constant. And 1 minus f apparent is the apparent fraction of native species; is the fraction of native species right because f apparent constitutes of everything, starting from your denatured state to all the intermediates. So, 1 minus that would essentially give you what? Your fraction of the native state.

Now, what you do is; you introduce true equilibrium constants, what do you mean by true equilibrium constants? If you have an equal equilibrium between the native state and intermediate; I 1 say, then equilibrium constant between this K is equal to concentration of I 1 divided by concentration of N right. So, that is why you see what is K i for me? K i is fraction

of the i'th species which is the intermediate over f of N and K T is definitely what it is defined by ok.

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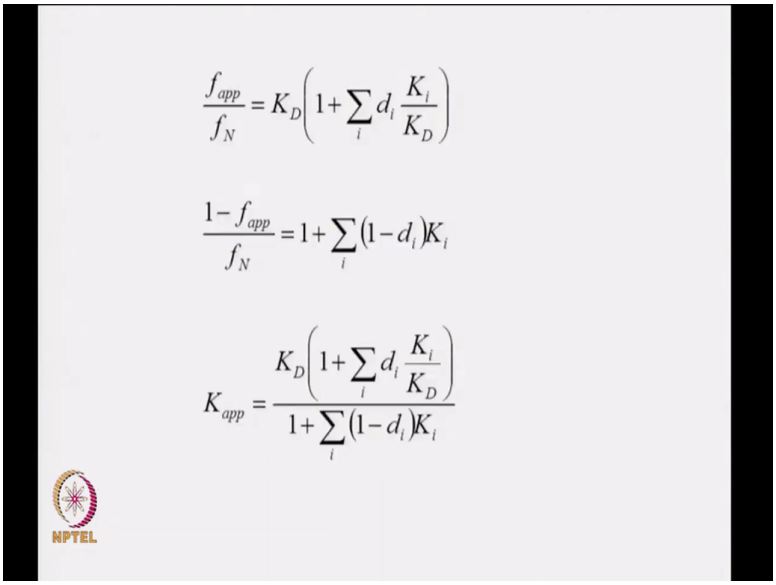

Now, you can go through this; you will get a host of relations like this right. So, that is not the point; let me try to make the last point. So, look at what your K apparent is now; your K apparent, your K apparent you can do this derivations; your K apparent is given by this relation what is it have? Your K apparent has K D right, it also has K i and it has the d i; the one corresponded corresponding to that ith intermediate, the physical observable ok.

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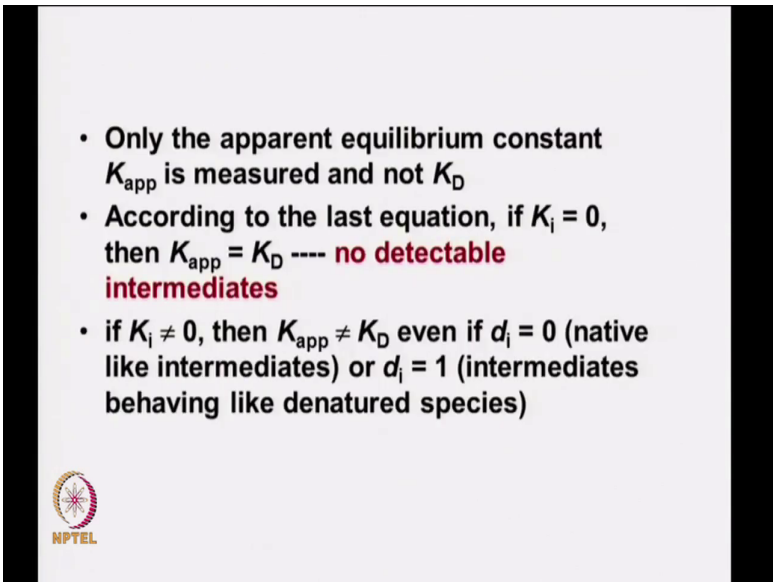

You do not need to write down so quick; it will be on the slides, maybe you want to derive it, but I am not so keen on that now; anyway. So, the point is only the apparent equilibrium constant K app is measured and not K D because as we just saw; K app not only has K D, but it also has information on K i right.

So, this is why it is called K app because your apparent because the equilibrium constant is not only K D, it also has contributions from your respective K i's ok. So, you just cannot do it like that; that is why it is called the apparent equilibrium constant. See, how much it has become more complicated when you just shifted from a two state to multi state model.

Now, what you can do is; if K i is equal to 0; what will happen? If K i is equal to 0; what will happen? Then your K apparent is equal to?

Student: K D (Refer Time: 41:09).

K D; you fall down to what you have just derived. So, this is your most general equation, from this you can go anywhere ok; that is the idea. Now, if K i is not equal to 0 and K apparent; then obviously, is not equal to K D, even if d i is equal to 0. Now, this you have to understand; if you look at it, see K i is not equal to 0 right.

Even if d i is equal to 0; what do you have? You still have K i remaining; that means even if d i is equal to 0, your K apparent is can still K apparent it still depends upon K i.

So; that means even if d i is equal to 1 which is native like intermediate; that means, very close to the native state or di is equal to I mean di is equal to 0 or di is equal to 1, where intermediates behaves like a denatured species still your K is K apparent because your K i is not equal to 0.

So, that is you know another way of looking at it; especially when your transition is not a two state, but involves many intermediates. You can try; you can try to drive this by yourself, it is very easy; it is just a lot of algebra you have to go through, but you know it is the same thing.

But now the question is we have talked about a two state model and we have talked about a multi state model; how would you differentiate between these? That is the obvious question right. How would I know looking at a transition ; how would I know whether this one is essentially a two state model or this one is not consistent with the two state model; that is an obvious question right, there should be reason why we are going through this because we have to try to answer that.

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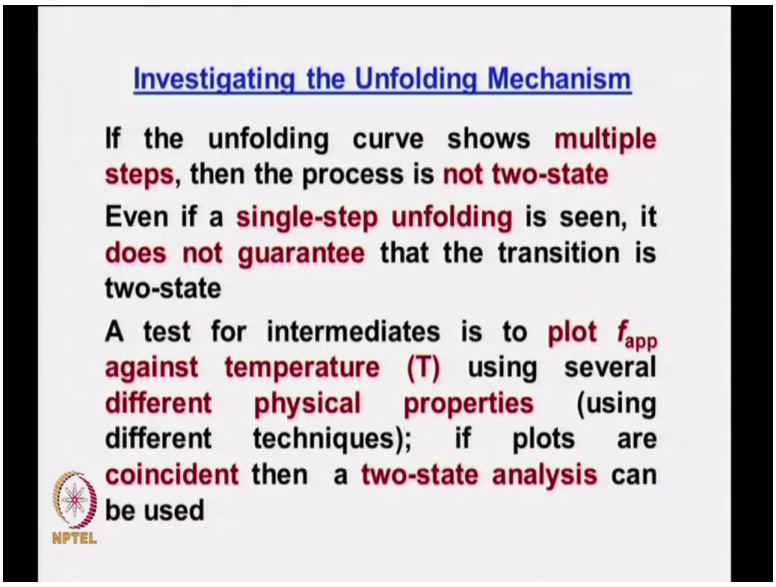

So, this is what brings us to the next topic which is called investigating the unfolding mechanism. How do we investigate that? Is it a two state? Is it a multi state ? If the unfolding curve shows multiple steps, then the process is not two state; now what do I mean by multiple steps?

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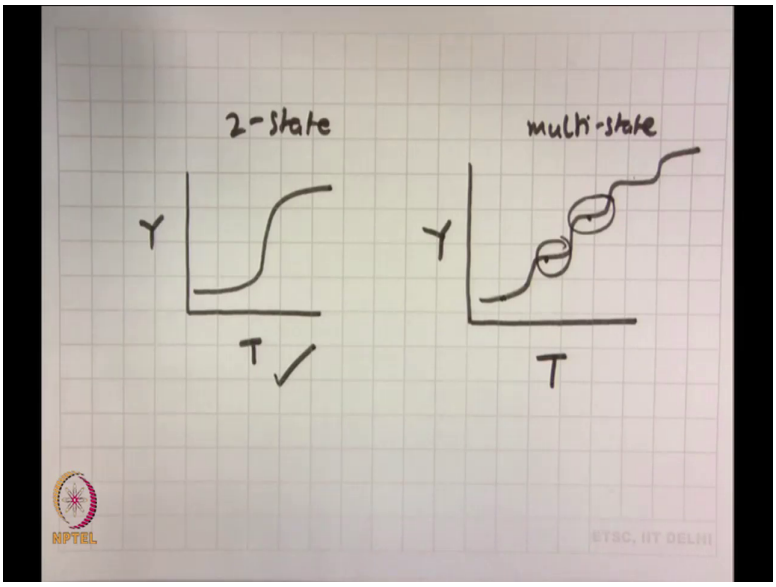

Multiple steps ; what I mean by multiple steps is this ; that means, remember for a two state, say if something is strictly two state; you see a transition which goes like this say right, this is a two state transitions, this is Y and this is T ok.

Now, if you have more states; if it is a multi state transition, then it might happen; it might happen that you have transitions which look like this and so on. Then, if you have transitions like this; you can always see what happens. From here to here is one state, from here to here is another state, from here to is another state; that means, you are all looking at different intermediates and finally, you go to your denatured state.

So, if you have; so suppose you are doing this measurement where you are measuring Y and if your Y is behaving like the one you have for the multistate; that means, is the multi state model; it is not a two sided model. However, if it is being as Y as the two state model; that

means, you do not have any other transitions, you can say there is a possibility of this being a two state model, but you cannot say that will be a two state model.

Why? Why? Because in a two state model; look at this in a two state model what will happen is or look at the multi state. In a multi state, if the corresponding equilibrium intermediates; the concentrations are really low, then you would never be able to figure out these transitions because the presence of those species are really low.

What essentially we look at is either this one and the last one. So, then apparently it will look like a two state model, but is actually not a two state model. That is why when we see a transition like this, when we see a transition like this; we do not say that it is definitely a two state model, but we rather would prefer saying is; it is consistent with the two state transition, but we do not know for sure ok; we do not know for sure because of the complexity of the process right.

So, that was one; is this clear? What do you mean by a two state and multi state; if you have a transitions like this ok? Let us go back to the slides and let us look at the next slide. Now, even if a single step unfolding is seen, even if a single step unfolding is seen; it does not guarantee that the transition is two state. Even if a single step unfolding is in; it does not guarantee the transition is two state; with that is what we just discussed.

You might be having those intermediates which have very low concentrations or very transient existences; they do not contribute that much to a folded, that much to a signal you will not be able to detect. But that does not mean that they are not there; they are there, hence it is not strictly a two state.

What is a test for the intermediates? A test for the intermediates is to plot f apparent, against temperature T using several different several different physical properties; that means, using different techniques. What does it mean? Remember, the d i; the d i was the physical property of every intermediate; so think about this.

Suppose, you are doing a c d; suppose you are doing a fluorescence and suppose you are doing some other technique ; that physical property d i would under all circumstance, not be the same for these different techniques. Why? Because these techniques are looking at different things, they are probing different things. In fluorescence, you are probing the fluorescence of A fluorophore; now we will talk about this later in details much more.

You looking; you are looking at a fluorophore which is actually very local; that means, it is situated somewhere in the protein; it is one point say. When you are talking about c d and just take my word of mouth right now; I will explain this to you later. When you are talking about a c d; say suppose you are doing a secondary structure analysis, then you are looking at the whole protein unfolding; that means, you are looking at the whole protein.

In fluorescence, you are not looking at the whole protein; you are looking at a specific portion of the protein. So, immediately then you have a difference in d i ok. Then, if we take another technical, you will be having something else coming out right. So, that is why di's are definable different in different techniques. So, what it says is; now what it says is if the plots are coincident then a two state analysis can be used or it is consisted with the two state analysis.

Now do you understand this? See, we just discussed that if you are going to use different techniques, then your d i is not necessarily the same. If your di is not necessarily the same, remember your K equilibrium or K apparent was depend upon d i; K i and K D. So, f apparent was also depended upon that.

So, if your d i is not the same and if you would be using this different techniques; when you would plot f apparent versus temperature for the different di's, your plots would be different because the d i is not the same right. If the plots are different, then you immediately know that you have not a two state, you have a multi state transition.

However, if plots are almost superimposable with each other or almost identical with each other or very similar to each other; then what you can say safely is that yeah whatever d i I am

looking at; I am looking at the same thing. And essentially I have only two states the native state and the denatured state ok. Consistent with see I am not saying it is a two state; I am just telling it is a consistent with the two state model right.

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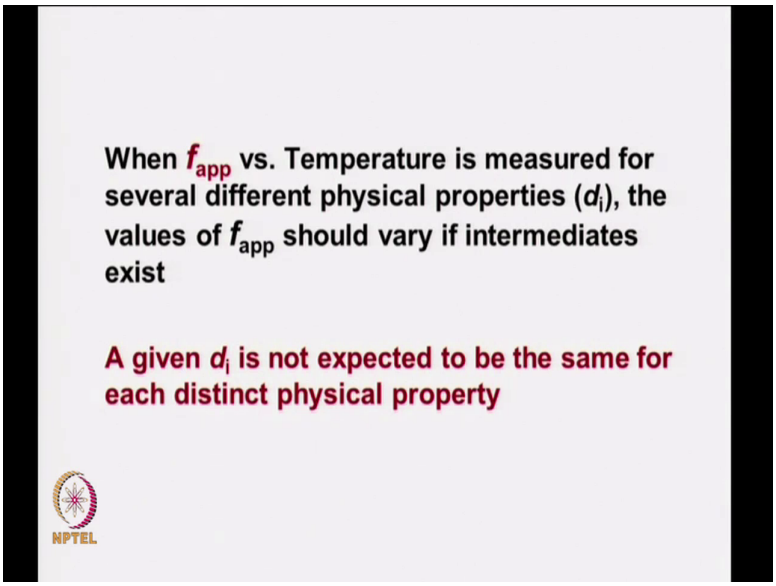

So, as its written out here when f apparent versus temperature is measured for several different physical properties, the values of f app should vary if intermediates exist; that is what we just discussed because d i is a different for the different techniques. Now, a given d i is not expected to be the same for each distinct physical property that is what we said. In one case you are doing c d, in one case you are doing fluorescence different properties; not necessarily the same.

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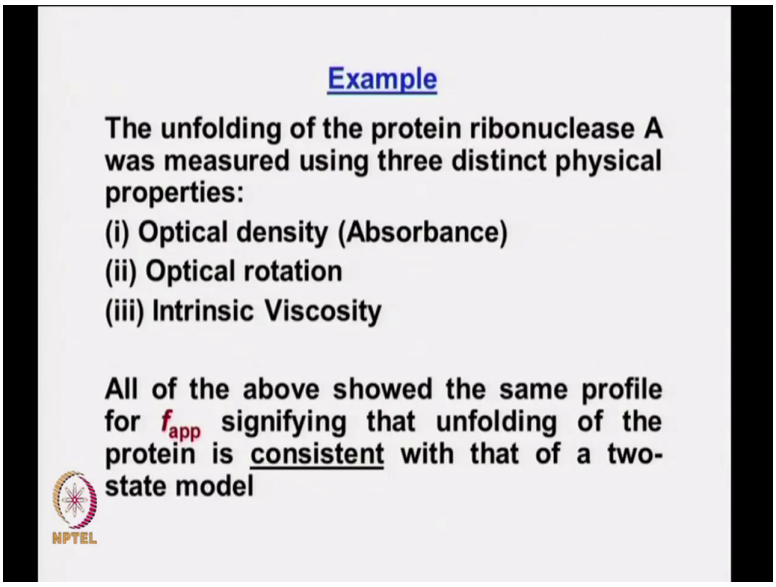

So, example is look at this unfolding of protein ribonuclease A; what did they do? They looked at the unfolding of protein ribonuclease A using three different techniques. What are the techniques? One is optical density which is UV visible spectroscopy absorbance, the other one is optical rotation; you heard about this, the other one is intrinsic viscosity.

So, they looked at viscosity which is intrinsic to the protein and I do not have the plot, but what they saw was all of the above showed the same profile for f app; that means, f apparent. What does it signify? That the unfolding of the protein. Remember, again I am specifying this word consistent with the two state model; consistent with a two state model. I am not saying it is the two state model; it is very hard for me to say that ok, but it is definitely consistent with two state model right.

So, this is how given a thermal transition or given any transition per say; you would possibly try to analyze it, keeping all these different things in mind; is it clear? So, we started from this non-linear fitting routine based on a two state model. Then we moved on invoked intermediates, put an intermediates and saw how the K apparent and f apparent would change. And then what is the significance of this d i? How does it differentiate between a two state and a multi state and this is how it does it for us.

So, in other words the bottom line again is this; I am using too many bottom lines, but really this is the bottom line that if you would try to figure out whether a transition is two state or not; what would you do? You would actually use different measurements; that is it. You would use different probes and see whether the unfolding transition, you get are very similar to each other or not. And that would be consistent with a two state model if they are similar; that is what the take home message is ok.

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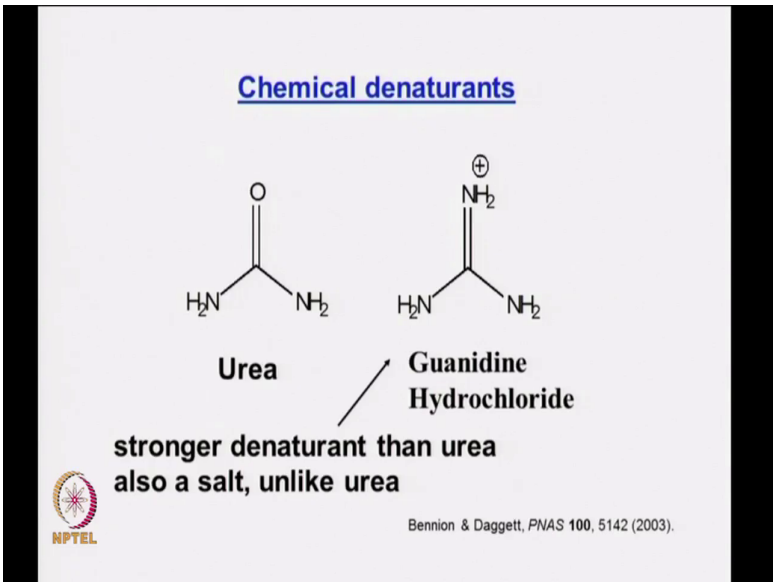

So, that was about you know thermal denaturation. What I will do tomorrow is; we will do chemical denaturation, we will look at chemical denaturation what it is all about. And I; I will see whether I will I can finish off with chemical denaturation, if I can then I will move on to pressure or pH. If not because chemical denaturation is also very kind of interesting; it is based on the same principle, all the basic equations remain the same.

But remember in the case of temperature you vary what? Temperature right. So, the delta G varies with T, but in this case; you are doing it in the same temperature and you are varying what? Concentration of denaturancy, its urea or this guanidine hydrochloride; that means, it is essentially isothermal experiment isn't it? That means, your temperature is the same. So, you; it is no longer delta G of T ; it is delta G as a function of certain something which is known as urea concentration, that is what we are going to try to find ok; good.