Bio-Physical Chemistry
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Lecture - 15
Protein Stability Curves

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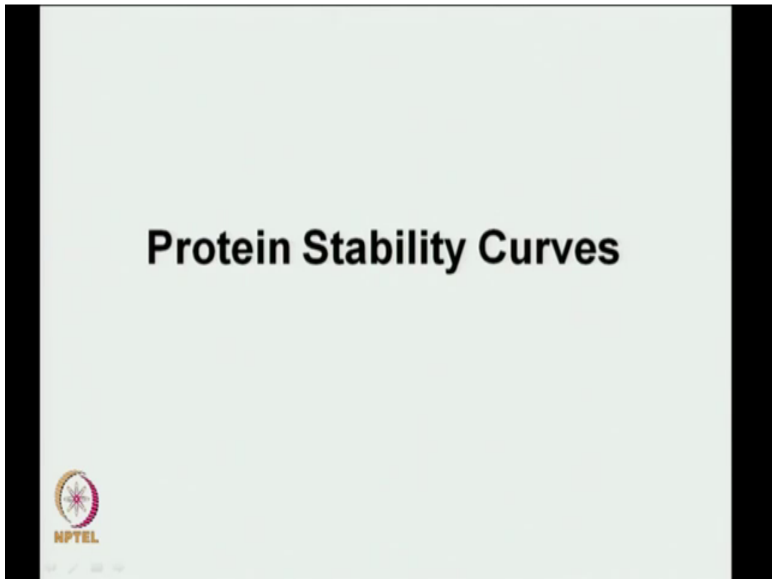

So, we will start on with our discussion with regards to Protein Stability Curves (Refer Time: 00:30). So, I will go through a brief recap of what we were doing last time.

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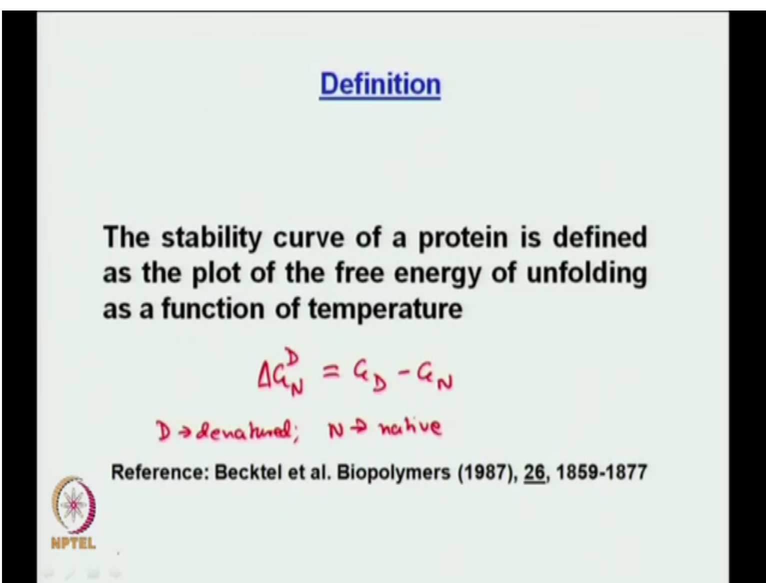

So, we defined the stability curve as essentially they are dependence of free energy on temperature. And whatever we are looking at; whatever looking at is this free energy of denaturation where D stands for denatured, N and for native. And we are looking at the difference in free energies between the denatured the native state.

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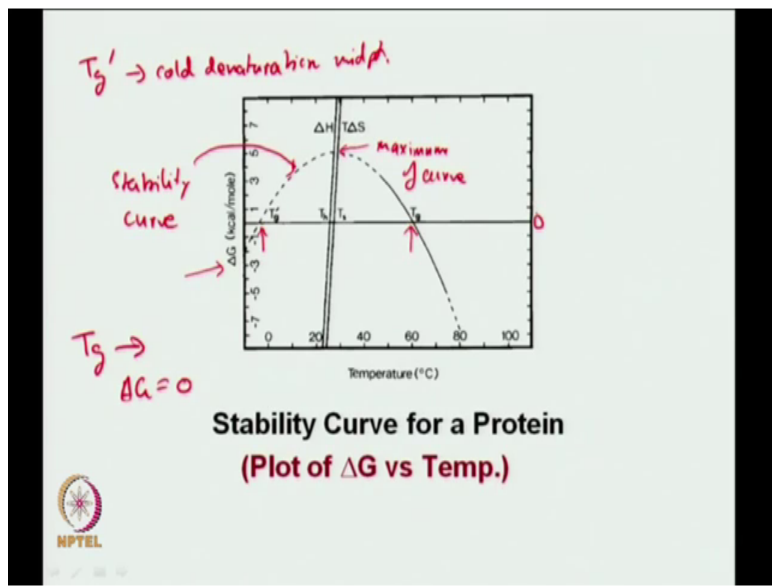

We looked at this stability curve for protein and one of the major points in this case was; if you would compare it with the stability curve or with the free energy curve of a hydrophobic molecule being dissolved in water, then you would see the difference being that T h and T s are very near to each other, in this case for a protein. However, for a hydrophobic molecule like neopentane; this T h and T s were far apart from each other right. Now, there are couple more points in this case.

For example, if you would look at the stability curve which is this plot of again delta G versus temperature. There are two points at which the stability curve cut the 0 line. So, this is the 0 line; so this is the 0 line. So, there are two points; so when delta G is equal to 0, it is essentially; that means, k is equal to 1; k equilibrium is equal to 1. What it means is that, we have equal concentration or equal number of unfolded molecules and folded molecules right.

And that is referred to as the midpoint of any transition. What I refer to what I mean by midpoint we will be more clear in just a few minutes. But the more important thing is that see there are two points; one is we know that when we heat a protein up it has to denature; that means, you destroy all the; you know all the stabilizing interactions and you start opening up. So, one of those points is T g which is the heat induced; that means, a increasing the temperature.

But what possibly we did not realize was that if we would also decrease the temperature; a point would come where the protein will again denature right. So that means, a point will come where? k is equal to 1 again; so that is your T g prime which is the cold denaturation point. I have written midpoint again for a specific reason because wherever we are talking about T g and T g prime, this are; these are essentially midpoints of certain transitions we are looking at and its will become more clear in the next few slides.

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Recall the Thermodynamic Equations

$$\Delta H(T) = \Delta H(T_1) + \int_{T_1}^{T} \Delta C_p dT = \Delta H(T_1) + \Delta C_p (T - T_1)$$

$$\Delta S(T) = \Delta S(T_2) + \int_{T_2}^{T} (\Delta C_p / T) dT = \Delta S(T_2) + \Delta C_p \ln \frac{T}{T_2}$$

$$\Delta G(T) = \Delta H - T\Delta S$$

$$\therefore \Delta G(T) = \Delta H(T_1) - T\Delta S(T_2) + \Delta C_p \left[(T - T_1) - T \ln \frac{T}{T_2} \right]$$

Two reference temperatures: T_1 and T_2

And then we had looked at this thermodynamic relations before. And these are some of the relations we are going to go on using quite often.

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Properties of the Stability Curve

- Curvature is given by

$$\frac{\partial^2 \Delta G}{\partial T^2} = -\Delta C_p / T$$

- Slope of Curve is given by

$$\frac{\partial \Delta G}{\partial T} = -\Delta S$$

So, one more thing was the curvature; it is, you can see the curvature depends upon delta C p and the slope of the curve is given by delta S. And it is needless to say that where the slope of the curve is a maximum where the slope of the curve is the maximum this typically here; what happens is then delta S should be equal to 0. That means, the slope of the curve corresponds to that temperature where it is delta S equal to 0; that means, T s.

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Properties of the Stability Curve

Define the temperature for maximum stability as T_s where $\Delta S = 0$

$$\Delta S(T) = \Delta S(T_2) + \Delta C_p \ln \frac{T}{T_2}$$

Taking the reference temperature $T_2 = T_s$,

$$\Delta S(T) = \Delta C_p \ln \frac{T}{T_s}$$

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Properties of the Stability Curve

- The native protein is assumed to be stable in a certain range of temperature; hence ΔG for unfolding will be positive in that temperature range
- The stability curve crosses the zero line at two points. These points are the low temperature and high temperature melting points and are denoted as Tg' and Tg respectively

So, that is what we refer to; so, just by another diagram which you are going to come very soon. So, one of the properties or one of the assumptions was that the native protein is assumed to be stable in a certain range of temperature. Hence, delta G for unfolding will be positive in that temperature range; because in that temperature range; what we are assuming is that the folded state is more stable.

And if you are trying to unfold it; that means, you are doing a process which is non-spontaneous and hence delta G has to be positive. Next is, as we said that the stability curve crosses at two points; one corresponds to the high temperature melting point, the other one corresponding to the low temperature melting point right.

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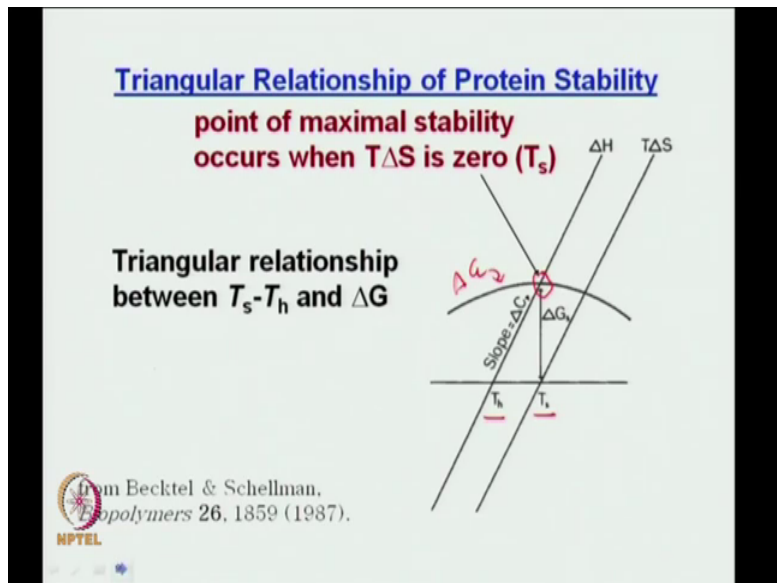

So, this is again the triangular relationship which we were talking about. So, you can see that here the maximum stability corresponds to T s right and the triangular relationship is between T s, T h and delta G. So, there are three right; it is being referred to by a triangle of this form and that is why it is called a triangular relationship.

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Why T_h and T_s are very close to each other for a Protein

$$T_s - T_h = \frac{\Delta G_s}{\Delta C_p}$$

- ΔC_p for unfolding is large and falls in the range of 12 – 18 cal/degree per residue
- Maximum stability of proteins tends to be small, on the order of 50 – 100 cal/residue

Hence T_s and T_h are only within a few degrees

But, I guess the take home point of this one was why T h and T s are very close to each other for a protein? And what we finally, decided on or what we looked at was; that this T s minus T h is given by this is what we derived; is given by the dependence or the ratio of delta G s and delta C p. Now, it is called delta G s because it is delta G at that temperature of what? It is correspond to s right; the T s. Now, what it means is therefore, delta C p for unfolding is large and falls in the range of 12 to 18 cal per; calories per degree per residue.

Now, this is definitely an approximation, but think about it; if you have a number like this; it is 12 to 18 calories per degree per residue right. Now, given a protein you can have 100, 200, 300, 400; you know many residues. See, even without actually doing a formal measurement of delta C p; what you can gauge is all right. At least you can estimate based on this; what is the possible delta C we are going to see right?

Because you are already given the range; it has to be between 12 to 18 calories per degree per residue. And depending upon the number of residues, I can multiply it by that number I can see ok; this is supposedly my delta C p change that is theoretical. Well, theoretical means it is an empirical observation, it is an observation; I should not say empirically, its observation based on experiments. You take this and you compare with what delta C p you get from doing measurements.

Once you do measurements, you get delta C p; you have this value, you can compare you will see they are oftentimes very close to each other; so it is a very good guess. Well, the other point is the maximum stability of the protein ranges between 50 to 100 is 100 calories per residue and this is not too high; this is really not too high. And hence because this ratio of delta G is by delta C p is small which means the T s minus T h; the difference would be small on thus for a protein, they live very close to each other ok.

So, what we will do now essential is; we will this was your recap. So, we will start from here and before looking at thermodynamic unfolding, we will do some more thermodynamics based on what we have learnt.

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So, let us again start from the very basics. So, what I would write here is some more thermodynamics ok. So, what we know from before is; this we know will here what we will do is, we will take the reference temperature, we will take the reference temperature as T g. Remember what is T g? T g is our heat denaturation point right where delta G goes to 0 on heating.

And then what we had is; we had this delta H T was equal to delta H T 1 plus delta C p; T minus T 1 that is what we had from before. So, what then what we said was; I am just rewriting it because it will help us in kind of maintaining the continuity. We will say that T 1 is equal to T g and then what we will also write is; what will you write here? We will write T is equal to what? T h.

Student: T h.

Right T is equal to T h because we know at T h; delta H goes to 0. So, based on that what we can write is delta H; T of h is equal to delta H; T of g plus delta C p; T of h minus T of g right.

And here; this delta H; T of h goes to 0 by definition; delta H; T of g; we will write as delta H g. We will just not write T of g all the time, we will just write as abbreviated as G.

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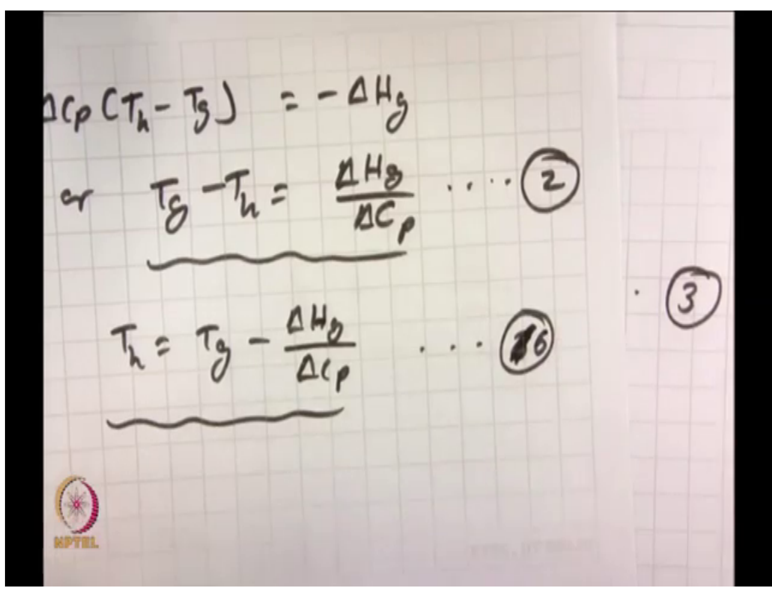

And from here what we got was this relation; delta C p; T h minus T of g is equal to minus delta H g or T g minus T of h is equal to delta H g over delta C p. Now, I forgot what was the number of this equation? I think it was 2, if I remember correctly right ok.

So, we have this; now see what is the beauty of this relation. Or this equation is it relates your T g which is the midpoint of your transition to T h, through the ratio of delta H over delta C p ok. That means, if you are not knowing T h, but you will be knowing T g; I will tell you how to find that, it is easy to find. And if you can measure delta H and delta C p, then you can find T h right.

So, what we can do here? We can rewrite this equation as saying that therefore, T h is equal to T g minus delta H g over delta C p; so this is our equation number 3. So, this is a relation between T h and T g through the ratio of delta H over delta C p.

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Now, correspondingly what we will also do is; we will do the same for entropy now. So, let us look at entropy now; so again delta S of T was equal to delta S; here I think we have

written T 2, then plus delta C p natural log; T over T 2. And since we have said that the reference temperature T 2 is equal to what? T g and we also say the T is equal to what?

Student: T s.

Ts because delta S of T s is equal to 0, then we write delta S of T s is equal to delta S T of g plus delta C p; natural log; T s over T of g. Now, by definition delta S; T s is what? Equal to?

Student: 0.

0.

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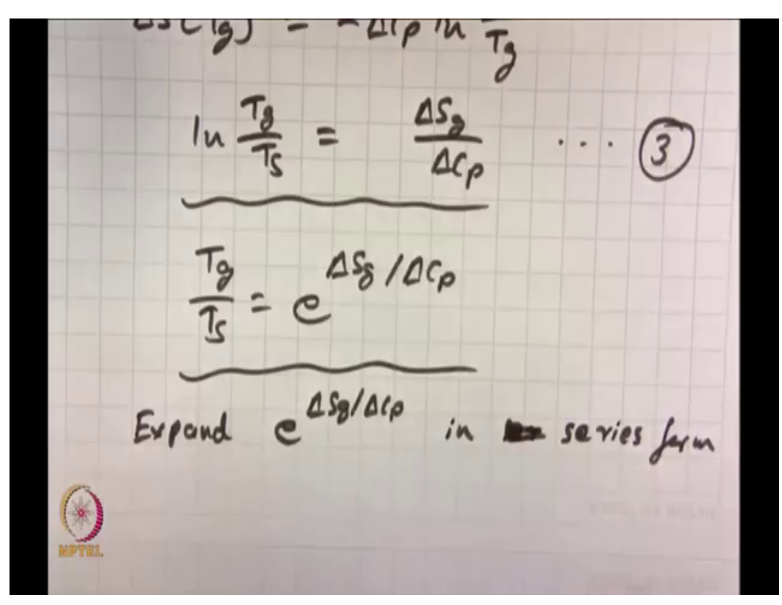

And then we can abbreviate delta S; T g by delta S g right. So, these we are done before right. And we can rewrite this equation as delta S; well T of g, I have wrote it again anyway is equal to minus delta C p natural log; T of S over T of g or natural log T of g of T of S is equal to delta S G over delta C p.

What was the number of this equation? I think it was 3;

Student: (Refer Time: 11:30)

Because I have to maintain the equation sequence. Now, what I will do is; I will do a small change, if you go back to the previous slide or the previous thing we wrote; this 3, what I will change to is; this 3, what I will change to is 6.

So, this one is not 3; this one is 6; because I think last time we did 5; equation number 5. So, we will call start with 6 here this is a kind of a new equation we wrote down and this is a 3 essentially for you. So, from here; now we will go forward; so this we have not done then. I can write T g by T of S is equal to e to the power delta S g by delta C p right; I can write this too.

See after simplified, I have to get a very simple relation between T g and T s; because last time; what we last time is just before this, the question number 6 was a relation between T h and T g; so we need relation between T s and T g. So, what we will do is, we will do an expansion; that means, expand e to the power delta S g, delta C p in series form; in series form.

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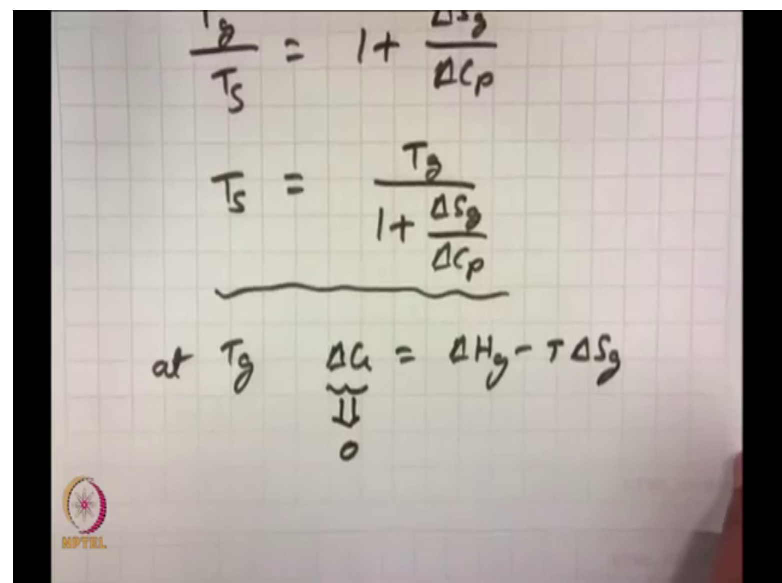

And once we do that; what we will have is T of g over T of S is equal to what is the expansion?

Student: 1 plus (Refer Time: 13:11).

1 plus?

Student: Delta S g over delta C p.

Delta S g over.

Student: Delta C p.

Delta C p and that is what we will stop we will not go to the higher orders because we are doing it for a very small number x which is delta S over delta C p. Then accordingly what we can say is therefore, T s is equal to what? Getting a relation between T s and T g is T g over 1 plus delta S g over delta C p. But that is not the end of it, we can simplify it further; how can we simplify it further? The way we can simplify it is; remember what is delta S g? Delta S g means the change in entropy at the transition point T g; isn't it? Now, at the transition point tg; what is delta g?

Student: (Refer Time: 13:59).

0; so essentially.

Student: (Refer Time: 14:02) delta (Refer Time: 14:03).

At T of g right; delta G at of G; delta G is equal to delta H g minus T; delta S g right. And because at T of g; delta G is equal to 0 because of T of g; delta G is equal to 0, this is our denaturation midpoint or the place where it cuts the axis at delta G is equal to 0.

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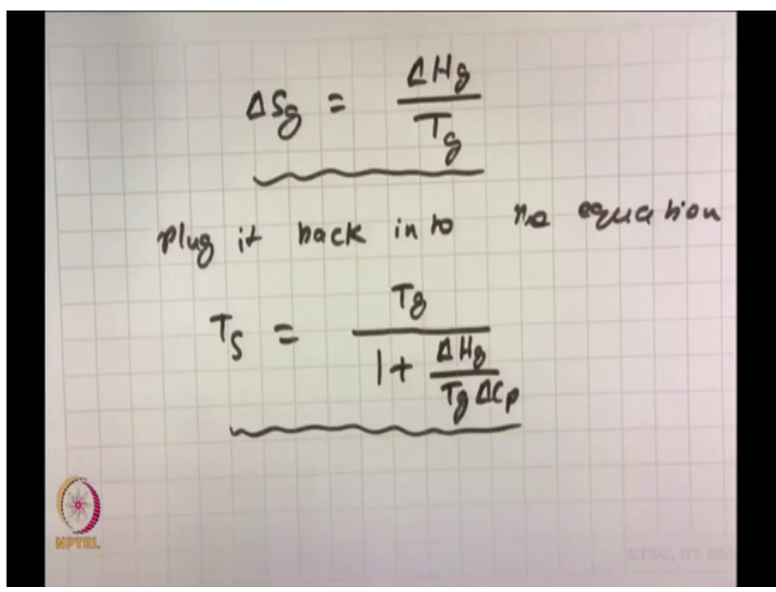

We can write delta S of g is equal to what? Delta S of g is equal to?

Student: (Refer Time: 14:34).

Delta H g by T and here the temperature being what?

Student: T g.

T g very good; so this we know. So what we will do is, we will take this and plug it back into the equation to get T of S is equal to T of g; then 1 plus delta H g by T g times delta C p ok. So, this is another relation we can have.

Now, once we have this what is the further simplification we can do? Remember, what I am trying to do is here I am trying to express T s in forms of in essentially in form of T g and another temperature which is T h; how can I bring that in? What do you think?

Student: (Refer Time: 15:36).

What is delta H over; delta H g over delta C p?

Student: T g minus (Refer Time: 15:44).

Ha.

Student: T g minus T h.

T g minus T h ok.

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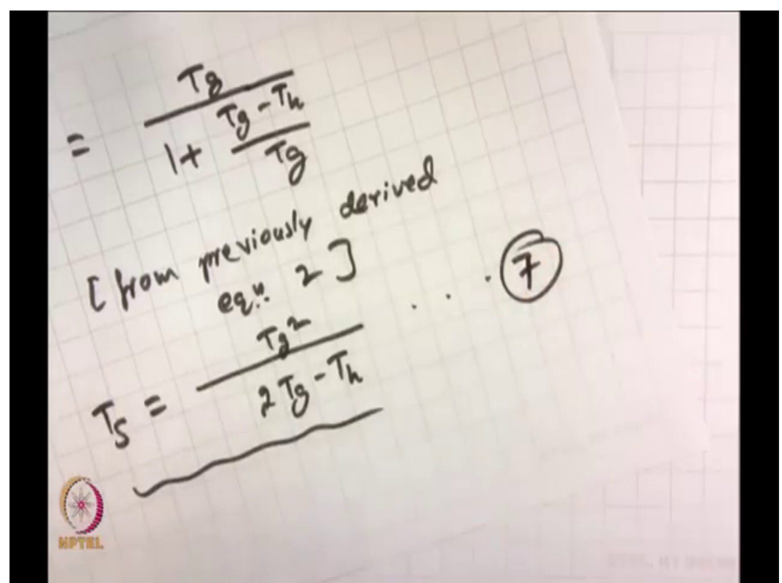

So, what I can then replace it by is; T s is equal to T of g; 1 plus T g minus T of h over T of g because I know what delta H by delta C p is. So, I can write from previously derived; what was the equation number? It was 2, wasn't it? Ha.

Student: 6; 2; 2.

Yeah, it was yeah 6, 2 is the same. So, from previously derived equation 2 and then we further simplify it. So, if when we further simplify it, what do we get? We get on the numerator; we get T g squared and what do we get on the denominator?

Student: 2 T g (Refer Time: 16:35).

2 T g minus T h.

Student: (Refer Time: 16:39).

So, this we can write as say equation number; let us say we write as equation number 7. This can be written in another form; so you can see we already have a relation between the three temperatures T s, T h and T g. That means, you know either of these two, you get the third one right and there is also very simple relation between T h and T g which is delta H over delta C p.

So, you see all these are you know very much interconnected ok. Once we have this, this can be written in another form I will just give you the form because I have it with me; its essentially just for the simplification.

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So, the way people finally, express this T s is as T of h plus T g minus T of h whole squared over 2 of T g minus T of h. I will just further the simplification from equation 7 and let this be equation 8. So, again it gives you a relation between these three characteristic temperatures T s; where delta S is equal to 0, T h, where delta H is equal to 0 and T g where delta G is equal to 0. You can see; these are huge the impact is huge right, we are talking about three very fundamental thermodynamic parameters ok.

So, that was this; now, see you have one more thing coming in; what we know is that delta G, if we were talking about the free energy; at any given temperature, at any given temperature is equal to delta H minus T delta S at any given temperature. So, I can rewrite it as delta G at any given temperature is equal to delta H of T minus T; delta S at a certain temperature; this is your Gibbs Helmholtz equation.

Now, from here where do we go? From here what we do is; we have a relation, we know what delta H T is, we also know what delta S of T is ok; in terms of T g and T. So that means, what I can write is delta G of T is equal to; what was delta H T for me? It was delta H, if I keep the reference temperatures T g; it would be T of g right; plus delta C p; T minus T g that is your delta H; as a function of temperature right, minus T then what do I have? Delta S; so delta S, I can have it for.

Student: T g.

T g plus delta C p; natural log, T over T of g.

So, what we just did here is; we just replaced the corresponding temperature dependencies of delta H and delta S; you are following up to this one right?

Student: (Refer Time: 19:46).

Now, you can simplify it; how can you simplify it? That means, you take this and just rewrite it so that it becomes a little more clear.

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So, I can have delta G of T is equal to delta H; T g; I will replace by G right. Then, I will write minus T delta S g ok; then I can write plus delta C p, I will combine the other two; I can write T minus T g; then what should I write? Minus natural log; T by T g.

So, what do we done now? What do we done is; we have now expressed delta G in terms of what? Delta H, delta S, G; this temperature you know, but as a function of another temperature T; which you do not know ok. Now, can this portion be simplified? The first two terms; can this be simplified further?

Student: (Refer Time: 20:46).

What happens?

Student: Del G (Refer Time: 20:49); del G (Refer Time: 20:50).

Well, it is del G; yeah ok, it is del G that is fine. So, now what I can do is; what is delta S g equal to at?

Student: (Refer Time: 21:01).

G; T g.

Student: (Refer Time: 21:04).

No, delta S g; what is delta S g equal to?

Student: Delta S g by delta S g by T g.

Ok good. So, therefore delta G of T is equal to delta H g minus T times

Student: Delta H g

Delta H g over T of g plus delta C p; T minus T of g minus ln; T by T of g

Student: (Refer Time: 21:39)

ok.

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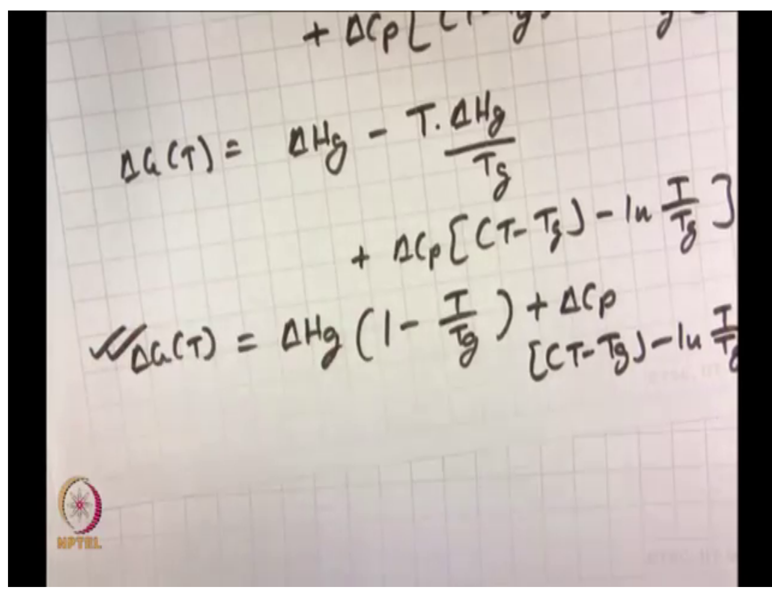

So, I can rewrite this delta G of T as equal to delta H g; 1 minus T over T g; plus delta C p. Then I will just open brackets T minus T g minus natural log T over T of g.

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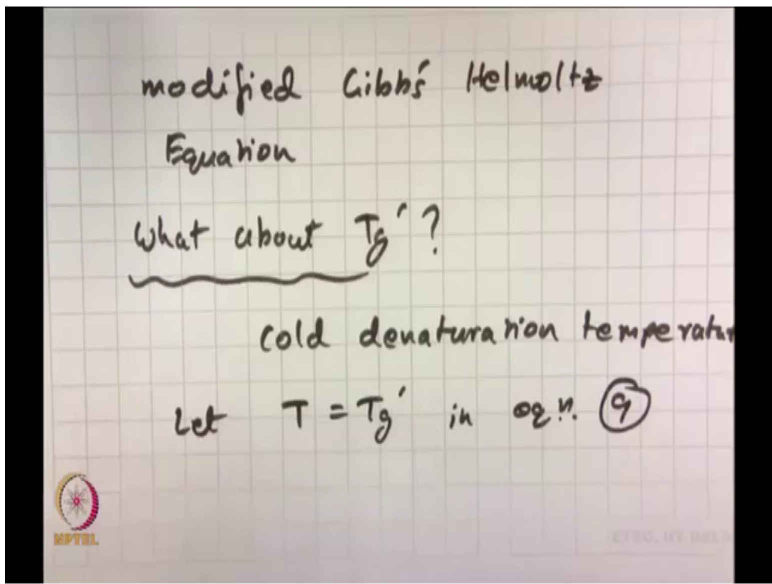

So, this final equation; so this final equation is often referred to is often referred to. So, this final equation is often referred to; I will write the name, is often referred to as a modified Gibbs Helmholtz equation. So, this final equation is often referred to as a modified Gibbs Helmholtz equation.

Now, see the essence of this equation; what you are doing is you are expressing the free energy as a function of temperature right. By what terms? One is; obviously, T of g, but there are two other very characteristic terms; what is? One is delta H G and the other one is delta C p; now why are these so important?

These are so important because both of these can be measured experimentally. And hence if you can measure these experimentally right; then what you can get? You can get the value of

delta G; free energy change at each and every what? Temperature; so that is the beauty of this equation.

See you have removed delta S right; you have removed delta S from the condition was what? That at T of g; delta G should be equal to 0; so therefore, delta H g must be equal to T g times delta S g right. Just to make the; take the point little further; this equation which we just derived is almost all the time or very often used by a biophysical chemists to extract thermodynamic parameters from protein unfolding transitions. And that is why this equation is so important for people doing that kind of research or that kind of work. And that is why; it is not your exact Gibbs Helmholtz equation, it just says; it is a modified Gibbs Helmholtz equation ok.

But see, what we have done here is; we have looked essentially T of g. What about T g prime; what about T g prime? This is our cold denaturation temperature. So, T g prime is a cold denaturation temperature. So, what; in this case, you know what you can do is; what will you do? What will you do is to find T g prime; can you do it from this equation?

To find T g prime; can you do it from this equation? How would you do it? Remember, I am trying to find an expression for T g prime; this equation.

Student: The in place of T g; T g prime (Refer Time: 25:06).

No, I will not do that because I know T g;

Student: (Refer Time: 25:08).

I will not try to replace T g, what will I try to replace?

Student: Replace T.

I will replace T; the moment I replace T by T g prime, what do I get? I get delta G; T g prime is equal to 0 because that is the other point and the moment I get that 0, I have the other equation coming in. So, what I do is in this equation; what I do is, let T in the equation we just derived let that be equation. So, what should the equation number be 9?

Student: (Refer Time: 25:37).

Ok, let this be equation number 9, let this be equation number 9. So, in this equation let T equal to T g prime in equation 9.

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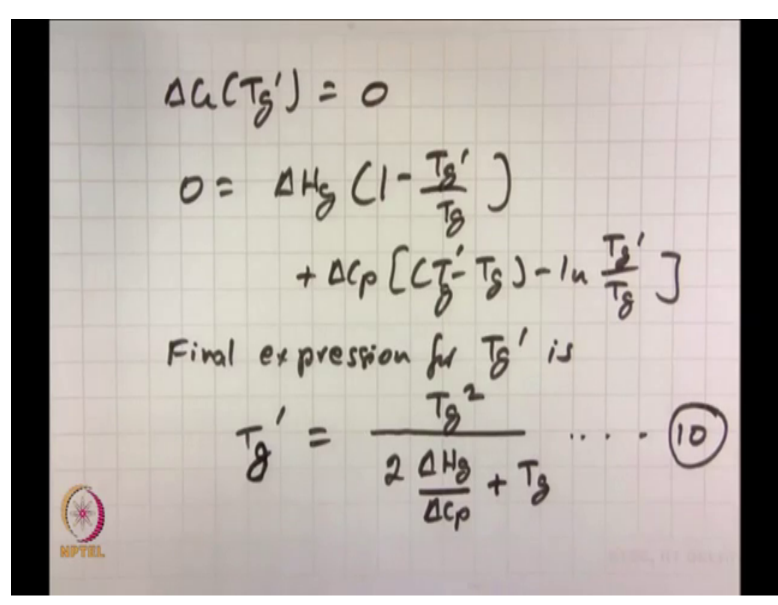

So, the moment we have T is equal to T g prime; so what we can write is delta G, T g prime is equal to 0; is not it? And hence 0 is equal to delta H g; 1 minus T g prime over T of g plus delta C p; T minus T of g, at T is T g prime minus natural log T g prime over T of g ok.

And this equation has to be simplified further to get T g prime, but the problem is that you have a certain factor or a certain term which is what? Which will actually hamper your simplification; it is the natural log of T g prime over T g. See everything else is very straightforward.

So, this is a homework for you; the homework for you is to prove or to get the final expression for T g prime, but; so the final expression for T g prime is; so final expression, I will give you the final expression for T g prime is; give you the expression T g prime is equal to; so T g prime is equal to T g squared by 2, delta H g; delta C p plus T of g. Now, this is the final expression for T g prime.

I will give you a hint; how to do it, I will just give you a small hint. So, you just have to go through the algebra, but first try to realize the inherent meaning of this equation. See, cold denaturation temperatures are not very easy to find; the reason being it often happens that T prime is well below 0 degrees Celsius and hence either you have to change the solvent condition or do something else to go to that cold denaturation point.

But if you have a relation between T g prime and T g; at least you would be knowing because you it is easy to find out T g; you would be knowing where T g prime would come in because you would know what? You would know T g and you would know delta H g and delta C p right.

Now, tell me how can T g prime be increased based on this equation only? That means, my T g prime; I am telling means already very low. How can T g prime be increase; that means, it comes more in the measurable range; that means, kind of close to room temperature or even 5 degrees, 10 degrees; how can we do that?

You look at the numerator; what does the numerator have?

Student: (Refer Time: 29:09)

The numerator has this delta H g over delta C p right? What happens, if delta H g is small and delta C p is big; what do you get?

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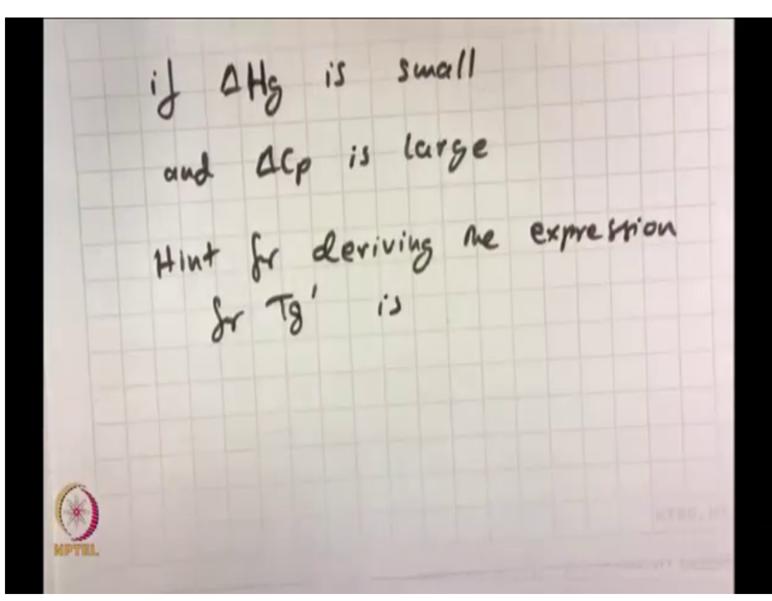

So, the question is; if delta H g; that means, if based on equation 10, delta H g is small, based on the equation 10; delta H g is small and delta C p is large ok.

Student: (Refer Time: 29:41).

Ha.

Student: (Refer Time: 29:42).

Yeah, so well we are not saying it is very small, but I am saying that the ratio is small; you are absolutely right. So, if we look at this; if this ratio is small, what will happen? Then the new; the denominator will approximate to T g say and then you have T square; that means, T g prime would approximate to what? T g.

In other words, what are you doing? You cannot have T g prime equal to T g; essentially there is on top of each other, then there is no T g or T g prime separately. But what you are saying is that; if my ratio is small, then what can happen? T g prime can tend towards T g; that means, tend to go higher up and you can bring it within the room temperature within the observable range; not far below 0, is it clear?

So that means, if my delta H; is my change in enthalpy is low, if my change in C p; that means, delta C p is high; then I can have a condition, I can have a condition which can give rise to T g prime which comes within the experimental conditions like above 0 and I can measure it ok.

So, this is why this equation has extra importance; it tells you what T g prime, the cold denaturation temperature depends upon and knowing T g; you can actually guess what T g prime is based on this equation. So, we have you know kind of seeing two different midpoints or two different points; one is the T g and one is the T g prime. What we will mostly focus on in this course is T g because it is easy for us to handle. In a T g prime as; I said is always its window is always not that easy to deal with and so we will not do that.

But, I will give you just a small hint at how people try to bring T g prime higher up; that means, what they do is; if you add say a denaturant like urea, chemical denaturant like urea. If

you add a chemical denaturant like urea; what it does is, without going into the details; if you are going to denature anything, what will happen?

Delta C p is going to change; huge, but the delta H will be small. If the delta H is small, delta C p is huge; what you are doing is you are not only; remember you are already adding denaturant; that means, you are denaturing the protein. So, if there was no denaturant; chemical denaturant the protein T g would be having a higher value; that means, it will T g means where it unfolds; where concentration unfold is equal to concentration of folded right; just hold on I will come to this..

If without urea; T g was say 50 degree Celsius; now the moment you add urea or chemical denaturant; that means, you already destabilizing the protein to start with ok. So, what will happen to T g? It will come lower and remember because this numerator ratio is small; so T g will also start; T g prime will also start going towards T g.

And hence in that case that is one of the ways by using a chemical denaturant that people can bring T g prime within the experimental window. A very good example is I will possibly take that example later; a very good example is apomyoglobin, you know what apomyoglobin is? Apomyoglobin is a myoglobin where you take out the prosthetic group.

So, apomyoglobin is very subjected to co; I am very I mean its prone to cold denaturation, And the way you can bring it within the easily within the experiemental windows say 20 degrees, 15 degrees is by adding more and more denaturant and it is a proven fact. I will show the data before I have data in my own lab which I can show you later, but you know it can be done; it is essentially all thermodynamics for you.

Anyway ah, so hint which was which I was trying to give you is this. So, the hint is; so; so this would be a homework problem. So, hint for deriving the expression for T g prime is; you use an expression of natural log.

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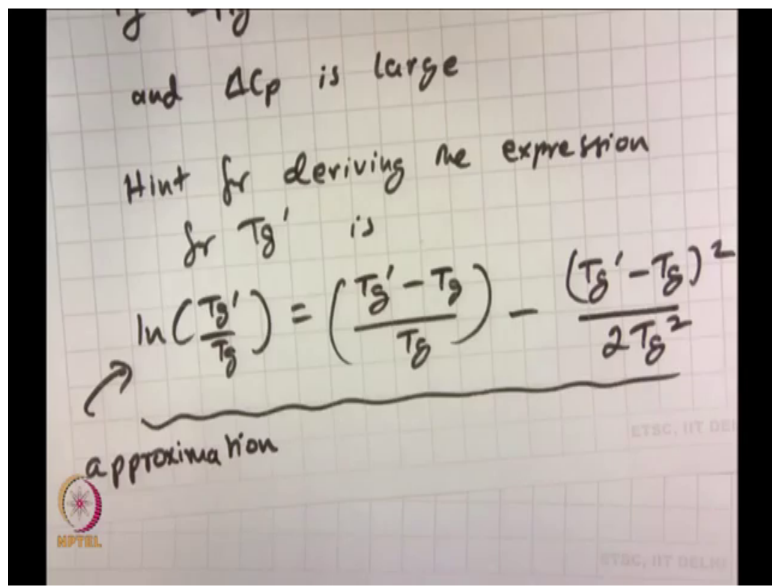

So, of T g prime by T of g; I am giving you the expression for this. So, this is T of g minus well T of g prime minus T of g over T of g minus T g prime minus T of g over 2 T of g. So, this is squared and this is squared. So, this is the approximation we are going to use; this is the approximation, you are going to use to find out essentially what your T prime is.

So, these were just some more thermodynamic manipulations with the corresponding you know T h, T s and T g.

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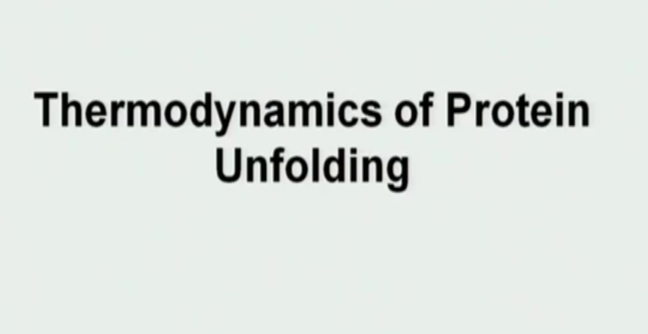

But what where are we finally, going with this? This is what we want to address the most important concept of the topic is thermodynamics of protein unfolding. That means, if a protein is unfolding; if a protein is unfolding, how can we get delta G, delta C p, delta H, T m and all these things.

(Refer Slide Time: 35:34)

Protein Stability

- Measurement of protein stability provides an understanding of the various forces and energetics involved in determining the native structure of the protein
- Effects of mutants on the structure of a particular protein can be directly obtained from protein stability

Now, what do I mean when I say protein unfolding? Let us talk about protein stability little bit, why do we want to measure protein stability? We want to measure protein stability because it provides an understanding of the various forces and energetics involved in determining the native structure of the protein ok.

See, you take one protein; say a myoglobin, you take another protein say human serum albumin and you see that one protein is more stable than the other. The natural thought which comes to your mind is why is it more stable? Where does the stability come from? And the only way possible you can address it; one of the ways you can address it you try to denature it and see how hard how hard this one is to denature, as compared to the others.

And you know that this denaturation midpoint or this transition point where k is equal to 1; T g is dependent upon this delta H g and delta C p. So, immediately once you know T g; you

have an idea of what? The delta H g and delta C p, this relations and these are your thermodynamic parameters and you also know this when a protein is unfolding; the change in delta C p is huge ok.

So, all those things will now hopefully fall in place; where is it really important? One was this, the other one is; suppose you take a protein and see you take a protein which has say 100 amino acids; say about 100 amino acids. You are trying to figure out which set of amino acids might be very important to the stability of the protein. So, immediately what you will you do; if you are trying to figure out an amino acid its contribution to stability.

The best thing for you to do is take that amino acid out and mutate it by or replace it by other amino acid essentially mutation. Now, if that amino acid is very; is a key unit for the stability of the protein, what will happen? The stability of the protein will say decrease right. And because of the stability of the protein decreases possibly the protein because it; becomes unstable it; it will you know melt a denature more easily.

And this corresponding thermodynamics this corresponding change in stability is essentially your change in free energy because free energy is the one which finally, defines the stability right. So, this free energy change with this free energy change; you would be able to address whether by doing a certain mutation that protein is become a more stable or less stable.

It is not always; it is not always that you put a mutation in; nothing will happen. It can happen especially if you do a mutation; for example, you see if you take a protein where do you think; if you do a mutation your protein would be least affected you know, where do you think? If you do a mutation your protein will be least affected where do you think? Will it be the core? Will be the surface?

Student: Surface.

Least affected; not most affected. Say I think it will be the surface right

Student: Ha.

Because see surface you mostly have polar charged residues right. You have many of those all those interacting with water. You can take one polar charge residue out right; you replace with see a little bit non polar one, but you have still many other things out there; because it is a surface and they are helping you solubilize as a protein.

So, in that case it might have, but your expectation immediate expectation would be not that much right or it when be special case, but not that much. However, it will think about the code, the code is essentially what? Hydrophobic. So, there is a very delicate balance between these forces; it is a packed interior. So, in this; there is reason why you know different proteins have different amino acids in the; in that core.

See, if you would be replacing say one of the hydrophobic amino acids say by a relatively polar amino. So, what would happen? It would immediately destabilize because your polar, your core is essentially what? Hydrophobic it tries to stay from water and hence the delta G would immediately change. But these were just two extreme examples and people have done many many mutations and that is essentially how people do to see which amino acid contributes to what extent to a protein structure ok.

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How to study Stability?

One needs to measure the free energy of unfolding of the protein given by the relation:

$$\Delta G_{D-N} (\text{or } \Delta G_N^D) = G_D - G_N$$

G_D: free energy of the denatured (D) state
G_N: free energy of the native (N) state

Need to perform experiments at equilibrium wherein unfolding is reversible

How to study stability? To study stability, one needs to measure the free energy of unfolding of the protein right. We know what free energy unfolding is its delta G; D of N right or D N. So, here what we will see is; this is delta G D N; it is a different way of expressing it. It is the free energy of the denatured state; minus the free energy of the native state, we always going to look at this.

So, need to perform experiements at equilibrium when unfolding is reversible. Now, this is important. When you are talking about delta G is equal to 0; delta G has an immediate relation with equilibrium constant minus r t; natural log k equilibrium.

Now, what it means is; no matter what you do, you have to make sure that the system is always at equilibrium; that is why these are equilibrium measurements your system is always

at equilibrium. If it is equilibrium, what is the definition of equilibrium? Definition of equilibrium is; you go back to your initial first order thermodynamics.

You are making a change in a system right; you change the system by a very small amount and you immediately revert the change, then you are going to come back to the same system. You change by very little right so that it is a essentially your infinite number of steps right. And then again you come back; you will retrace the same path.

When you when you talked about reversibility of unfolding, what does it mean? That if you are unfolding the protein by a certain path, now if you once you have unfolded the protein; now if you want to trace back the path, it should essentially what? Superimpose on the path which was traced to the unfolded portion; so that is what it means by reversibility.

So, you went towards the unfolded state and you are coming back from the unfolded to the folded state essentially following the same path ok; that will ensure reversibility. Actually, that is not very easy especially when you are doing experiments based or based on temperature changes or as a function of temperature, but this we will see later.

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Possible Methods to Unfold a Protein

- Thermal denaturation wherein the protein is subjected to variations in temperature (cold denaturation and heat denaturation)
- Chemical denaturation using urea or guanidinium hydrochloride (GdmHCl)
- pH induced denaturation by systematic variation of buffer pH
- Pressure induced denaturation

The main idea is to perturb the equilibrium and subsequently determine the fraction of protein folded (or unfolded)

Now, so we are talking about unfolding of a protein; how would you unfold a protein? There are many different ways of doing that and people have tried typically all these ways. Thermal denaturation right; thermal denaturation means you are just taking a protein in a certain solvent say buffer and you heating it up into a thermal denaturation good.

Now, you can also do a chemical denaturation; that means, instead you keep it at say room temperature at 25 degrees Celsius, you add denaturants like urea or guanidinium hydrochloride and these two are the most favored denaturants for people when they do denaturant induced unfolding; so you can do that.

Anything else you can suggest? What should the other one be? This you should be able to tell me.

Student: pH (Refer Time: 42:51).

pH, yes the other one is pH and we know why pH changes would bring about denaturation; this we are talked about in terms of the charge densities and on all these things. Anything else?

Student: Cell concentration.

Cell concentration ok; there is one more thing.

Student: Mutation.

Ha.

Student: Mutation.

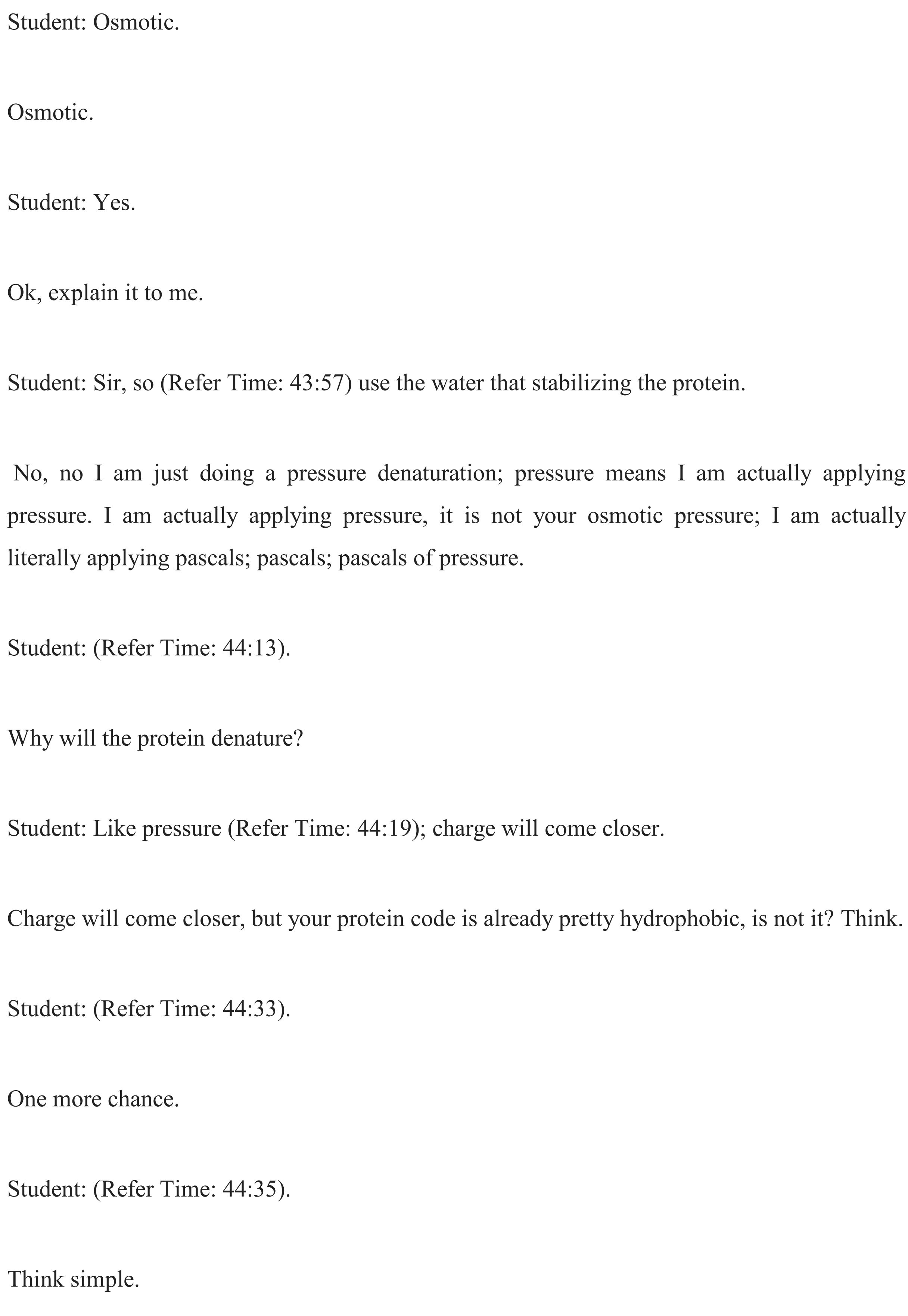
No, mutation; mutation is not your way of denaturation, mutation is you do a mutation and you see how it is affect with the protein stability right. The other one possibly ah; we look at is pressure, your pressure will also actually denature your protein ok; that means, do not be under too much pressure all the time. Anyway, so pressure does induce protein denaturation and can you quickly tell me how you think about a pressure denaturing a protein?

Student: Osmotic denaturation.

Ha.

Student: Osmotic denaturation.

What denaturation?



Student: Osmotic.

Osmotic.

Student: Yes.

Ok, explain it to me.

Student: Sir, so (Refer Time: 43:57) use the water that stabilizing the protein.

No, no I am just doing a pressure denaturation; pressure means I am actually applying pressure. I am actually applying pressure, it is not your osmotic pressure; I am actually literally applying pascals; pascals; pascals of pressure.

Student: (Refer Time: 44:13).

Why will the protein denature?

Student: Like pressure (Refer Time: 44:19); charge will come closer.

Charge will come closer, but your protein code is already pretty hydrophobic, is not it? Think.

Student: (Refer Time: 44:33).

One more chance.

Student: (Refer Time: 44:35).

Think simple.

What do you have in water? What do you; what, what do you have when you take a protein in buffer? Apart from protein, what do you have? You have solvent right. Now, if you would be putting pressure in; remember a hydrophobic core is devoid of solvent

Student: Yes

Right. If you would be putting pressure in; if you would be pressure, your water concentration is huge; now what will happen?

Student: (Refer Time: 45:02).

The water can actually go inside the hydrophobic core because you putting pressure remember? When the water goes inside the hydrophobic core, what will happen? The protein will denature; that is one of the easiest ways of thinking about protein denaturation and that is true.

If we; if you think about it, what do you will think? See, if you would not be thinking about the water part, what do you will think is if I would compress the protein right; I can compress to a certain extent without changing anything, but if I do a forward compression; what will happen is my London dispersion forces; repulsive forces will take over and protein will for apart essentially as you said. But that is not the only thing remember when you have a protein in water you have so many water molecules; what is the concentration of water essentially?

Student: 55 molar.

55 molar; that is huge excellent that is huge. So, that will go inside because you are compressing the whole system right; you are not only compressing protein molecules and they will denature that is also an interesting way of doing it. But I will tell you what; what the problem is the problem is.

If you are going to do pressure denaturation this is high pressure situation; the experimental, the experimental what should I say the experimental setup is not so easy to deal with; that is why you need very you; not modern I should say dedicated setups which can handle huge amounts of pressure ok.

So, in fold; in unfolding what is the main idea? The main idea is that you perturb the equilibrium and once you perturb the equilibrium; then you determine the fraction of the protein that is unfolded ok.

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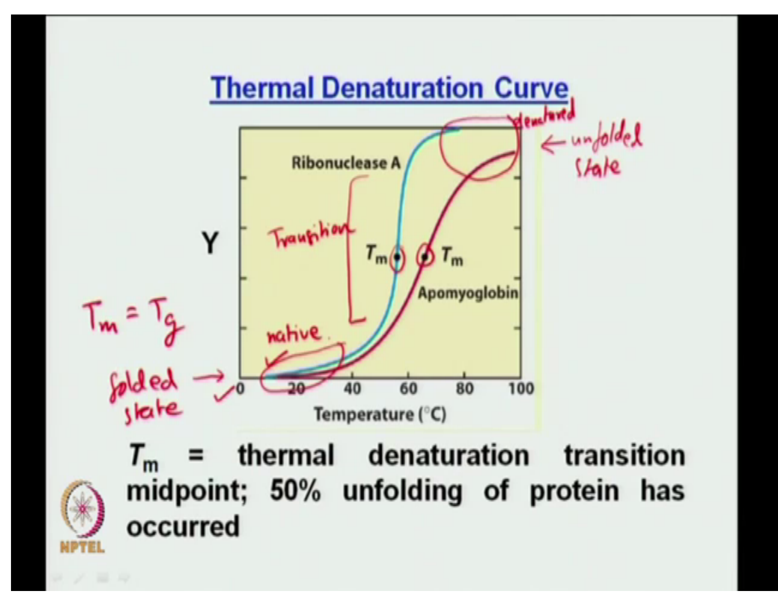

So, this is a typical thermal denaturation curve right. So, look at what we are seeing is; what we are seeing is what we are seeing is we looking at two proteins, one is ribonuclease A and the other one is a myoglobin. Now, what is T m? T m is essentially equal to T of g; T m is

essentially equal to T of g. So, where does m come from? m comes from either melting or the midpoint of the change.

Now, what change are we talking about? So, what is Y? I will tell you what Y is later. But Y essentially is an observable; that means, you are changing the temperature right by some way or the other and then you are you are seeing a change in the signal Y an observable right.

So, what this one is telling you is here; this observable corresponds to the folded state of the protein. Because at low temperature, what will happen? The protein is essentially stable right, when you got to very high; when you go to height temperature. So, this high temperature corresponds to?

Student: (Refer Time: 47:45).

Unfolded state, see what have you done? What have we done is we have started from here, we have started from here essentially here right; there the protein was in the most stable form that is the native form of the protein ok. So, this is I can write the native form of the protein; we go on increasing temperature.

Initially, what you see is the increase is not that much, but after you cross a certain temperature say in this case 40; what do you see that? Change is huge right. Then after you reach about say 60 for ribonuclease A, or 80 for myoglobin; here you can again see, this is your denatured form. That means, you have managed to unfold the protein based on the temperature variation you have done.

So, what is this observable track? This observable is that observable which changes when you change the temperature of the protein and because it changes the temperature what is changing? The protein is starting from the native state at the lower temperatures and it is going to the unfolded state and you are monitoring that change using the observable Y.

So, this is called a thermal induced transition or heat induced denaturation or a thermal transition. So, thermal transition means a transition which is affected by change in temperature and that transition is from what? The native a stable state of a protein to the unfolded state of the protein because you are supplying huge amounts of energy and you are breaking the stabilizing forces within the protein itself ok.

So, this essentially if you think about it, I can say for I can say this part is your transition because that is where the maximum change is occurring. Now, we can understand the relevance of T m; what is T m? Remember, I told something before it was T g prime what was it was a cold denaturation midpoint; why did I say it was a midpoint?

Because your T g and T g prime they refer to the midpoint of your transition. And hence this is your midpoint; this is your midpoint; this is your midpoint. So, that is why from now onwards, we will replace T g by T m both having the same meaning. There in that case G; why did it we take T g? Because we took T g because delta G was 0; now we replace it by the thermal melting midpoint and we say T m.

There is one more important thing to observe in this figure. You look at ribonuclease A and we look at the other protein which is apomyoglobin; which one has a higher T m?

Student: Apomyoglobin.

Aapomyoglobin has a higher T m, what does it mean?

Student: (Refer Time: 50:58).

Well, what it means very simply is, that the temperature at which you have k equilibrium is equal to 1 for apomyoglobin; that means, equal concentration of folded unfolded is higher than that of?

Student: Ribonuclease.

Ribonuclease. So, possibly because of this maybe ribonuclease is.

Student: Less (Refer Time: 51:21).

Less stable than apomyoglobin is possibly more stable than?

Student: Ribonuclease.

Ribonuclease because T m by definition is that point where the concentration of folded and the concentration of unfolded are equal to each other right k equilibrium is equal to 1, delta G is equal to 0. Now, there is one more issue; have you heard of the word cooperativity? Have you heard of the word cooperativity when you are doing your biochemistry courses? Tell me based on this or based on your definition of cooperativity which one of these proteins is more cooperative in its behavior?

Student: Ribonuclease A.

Ribonuclease A right, what does it mean by cooperativity? What it means by cooperativity; you start, you open one; everything else gets open. And this cooperativity is typically determined by the suddenness of the change in transition right. So, this is the other important point which you can interpret about on this figure or based on this figure.

And then we are going to look at thermodynamics anyway, but these are some very you know when you look at a figure. That is why people say that figure is what thousand words when you look at a figure, you can make a lot more interpretations because it is just the figure its experimental data it tells you so many things.

And it says where T m is a thermal denaturation mid point which you all already know.

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Distinct Regions of Unfolding Curves

- **Pre-Transition Region**: how the physical parameter Y for the native (folded) protein (Y_N) varies as a function of temperature
- **Transition Region**: shows how Y varies as the protein unfolds
- **Post-Transition Region**: shows variation of Y for the folded protein (Y_N) varies as a function of temperature

The observed signals for the fully native and denatured states are also dependent on temperature; this results in baselines with well-defined slopes

So, there are three regions what we saw; the three reasons are one is the pre transition region, what do you mean by the pre transition region? If this is the transition; if this is the transition, anything before the transition is a pre transition ok.

Now, what is the transition from? The transition is from the folded state to the unfolded state. So, if you are talking about a pre transition; that means, before the transition occurs from the folded to the unfolded. So, pre transition is essentially that belonging to the folded state right ok.

Then; so that is what it says how the physical parameter Y which is the observable Y for the native folded protein varies as a function of temperature. But look at one thing; if you see

this, if you see this area ok, if you see this area; I can say this is typically my pre transition period or the pre transition region.

But do not you see one thing that see even this pre transition what is; where it is mostly folded protein your baseline; that means, your transition is not actually parallel to the x axis, instead what; what does it do? It actually depends upon temperature; that means even your pre transition baseline has a slight temperature dependence.

That means if you are going to model it; if you are going to model it, you have to make sure that when I do a pre transition modeling of the because the transient region also is also composed of the pre transition; that I also better model the pre transition baseline as a function of temperature that is one right.

Next, look at the next slide sorry I mean the next point; next is the transition region. The transition region shows how Y varies as a protein unfolds. Because in the pre transition region your Y was essentially Y N, it was not varying that much it was varying little based on temperature, but now your transition region is where the maximum change is happening right. So, this is the transition region where the maximum change is happening ok.

And then you have the post transition region; obviously, the post transition region shows variation Y for the folded protein where say it should not be the folded protein should be what?

Student: (Refer Time: 55:13).

It should be unfolded protein; let me write as, I think we wrote this as D right and this should be unfolded; sorry for the mistake. So, the post transition region shows once you have done the transition, you have gone to the denature state of the unfolded state. So, that baseline which is called the post transition baseline is belonging to the denatured state.

Even that also; if you remember shows a slight dependence on temperature see; it shows a slight difference on temperature, it is not exactly parallel to the x axis; so that also has to be modeled. So, that is what the last point says; the observed signals for the fully native and denatured states are also dependent on temperature. And this results in baselines with well defined slopes; slopes means slopes depending upon; that means, this signal Y N or Y D depends upon temperature.

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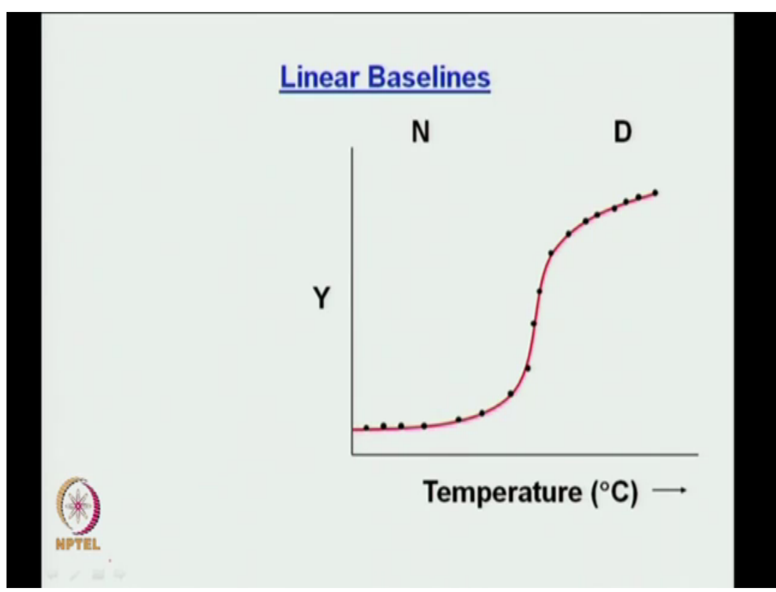

Stop here, I have run out of time. So, tomorrow what we will do is; now we know what a transition is, what a T m is. We will start looking into the thermodynamics of this transition; that means, if you are measuring this transition. How would you fit this transition; that means, you fit this curve to get thermodynamic parameters; because this is finally, the ones you are looking for your A delta C p your delta S these are the ones we are looking for.

So, that is where we are going to start from tomorrow.