Experimental Biochemistry
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Lecture – 08
Protein Folding and Denaturation- II

We continue our discussion on Protein Folding and Denaturation, we in the last lecture we looked at protein structure.

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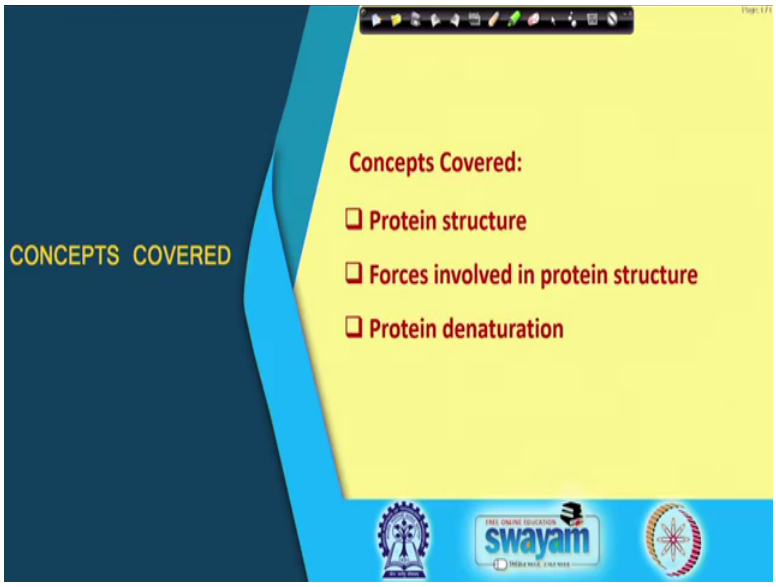

The primary structure, the secondary structure and the tertiary structure which we will look at in much more detail today. The concepts that are going to be covered in this lecture include the protein structure in general, the forces that are involved in protein structure and protein denaturation.

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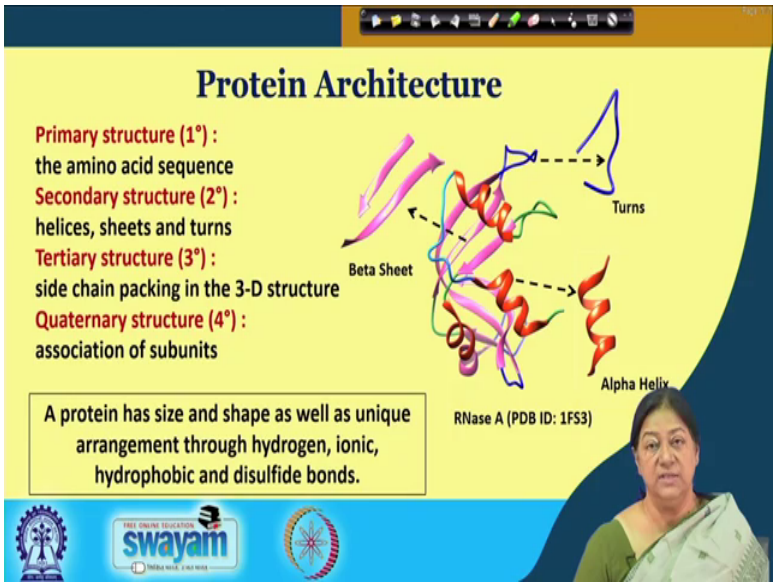

We looked at the primary structure the amino acid sequence last class, the secondary structures that involve the alpha helices the beta sheets and the primary other linkers the turns that would actually link the secondary structural elements together. And when they link up together, they form what is called the tertiary structure; beyond the tertiary structure some proteins from a quaternary structure which involves the association of subunits.

In the tertiary structure that we looked at last time we found out that they were certain parameters to the structure for example, this potion would be an alpha helix, here we would have the strands that form the beta sheet and the and the turns of the linkers that link the secondary structure elements together. And we found out that the important interaction between the secondary structure elements in the alpha helix or in the beta strands that form the beta sheet is hydrogen bonding. But beyond that when we have a folded structure as we see here what are the other interactions or the other forces that are involved that actually lead to the folded structure of the protein, which is called the tertiary structure.

Now, in the tertiary structure we will see that when we have this protein that has a definite size a definite shape and the unique arrangement through hydrogen ionic hydrophobic as well as disulfide bonds. So, it is these interactions the hydrogen bonding, the ionic interactions the hydrophobic interactions as well as the disulfide bonds that

hold the protein together in the non covalent interactions that are predominantly present in the tertiary structure of the protein.

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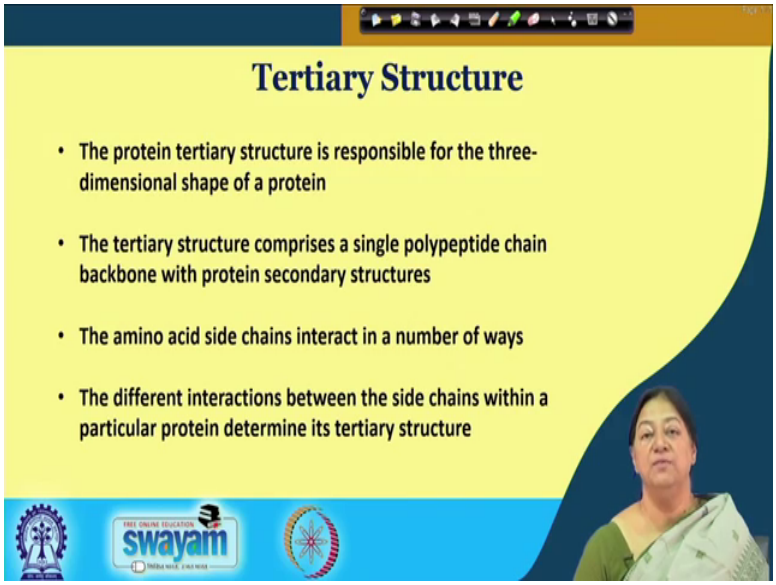

Now, the protein tertiary structure is responsible for its three dimensional shape, it comprises the single polypeptide chain backbone with the protein secondary structure elements in it and the amino acid side chains interact in the number of ways. And it is this interaction the non covalent interaction that gives its stability and what happens is when the side chains interact with one another deformed this folded compact structure.

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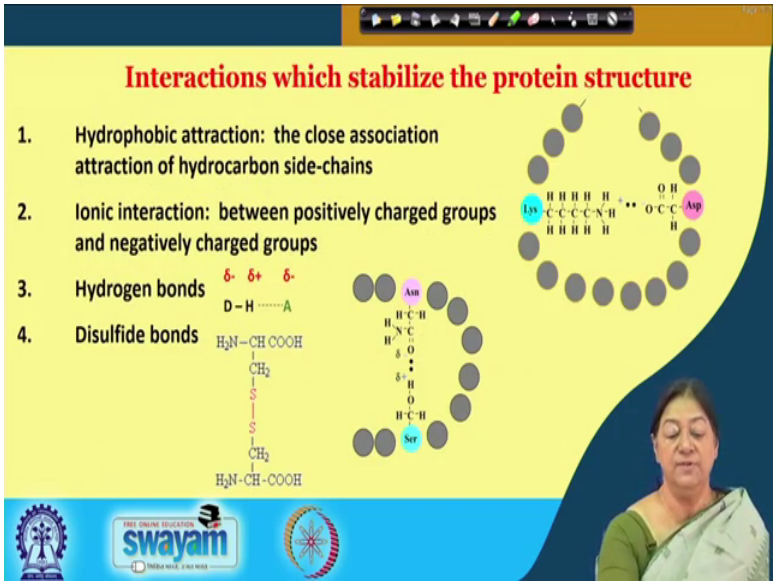

We will look at these forces involves so the interactions which stabilize the protein structure include hydrophobic interaction that is the close association attraction of hydrocarbon containing side chain. Now which are these? We learn these in the first lecture where we looked at the different types of amino acids those amino acids that have in there side chain carbon and hydrogen are those that would form a hydrophobic kind of side chain which would have a characteristic to remain away from the solvent water.

So, as we looked at last time a globular protein as opposed to a membrane protein, if we look at a globular protein we would not expect the hydrophobic residues to be exposed to the solvent, they would preferably remain in what is called the code of the protein which is the hydrophobic code where they would be involved in this sort of a hydrophobic in attraction..

We have the ionic interactions; these are between positively and negatively charged groups as has been shown here the lysine which is positively charged and the aspartic acid for this example that is negatively charged. So, we could have then within the other basic amino acids with another amino acids that are acidic in nature. So, these are the types of interactions that would comprise the electrostatic or the ionic attractions between the side chains of the protein, we have to remember that when we considered the secondary structural elements the hydrogen bond was between the backbone carbonyl and the N H and this we are talking about between the side chains.

So, the hydrogen bonds again here we are talking about the interaction between the side chains when we are talking about the compaction of the protein structure or the protein folded structure. So, here we have asparagine which is the amide group and the serine which is an O H C H 2 O H which is the side chain and we see that this doted line represents the hydrogen bond.

In addition to these non covalent interactions the covalent interaction that is present in proteins is the formation of the disulfide bonds which we discussed in an earlier lecture. So, apart from the peptide bonds that are covalent bonds holding the different amino acids together there are also disulfide bonds that can link different faraway parts of the protein together because of the to 16 residues forming this disulphide bond.

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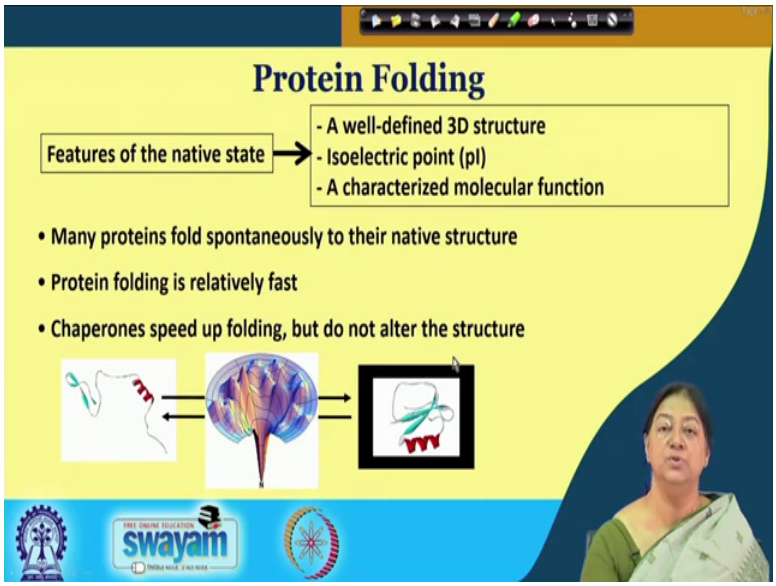

In if we look at the folded structure now, the specific features of this folded structure include a unique 3 dimensional structure which will have a specific isoelectric point depending on the number of the charged amino acid residues that are present and it would have a characterize molecular function. The importance of this means that this these proteins actually folds spontaneously to formed their native structure.

Now, we will look at protein folding a bit more and try to understand the thermodynamics involved in the protein folding, the protein folding process is actually relatively fast and there are some other proteins that are call chaperones that assist proteins to fold, But they do not alter the structure of the protein, but they help in that the assist in the folding..

For example, if we have an open chain like this that has have formed partially formed a helix we would have a large number this is called what is called a thermodynamic or rather the protein folding funnel, where we looking at a very large number of confirmations possible from just the primary amino acid sequence of the protein. So, if you look just look at the amino acid sequence of the protein, we were realize that there are until possibilities in the ways that this can fold, but no we get a single native structure.

Depending on what? Depending on how the protein is going to fold, it is going to have a hydrophobic code; we are going to have polar residues of the surface being a regular globular protein.

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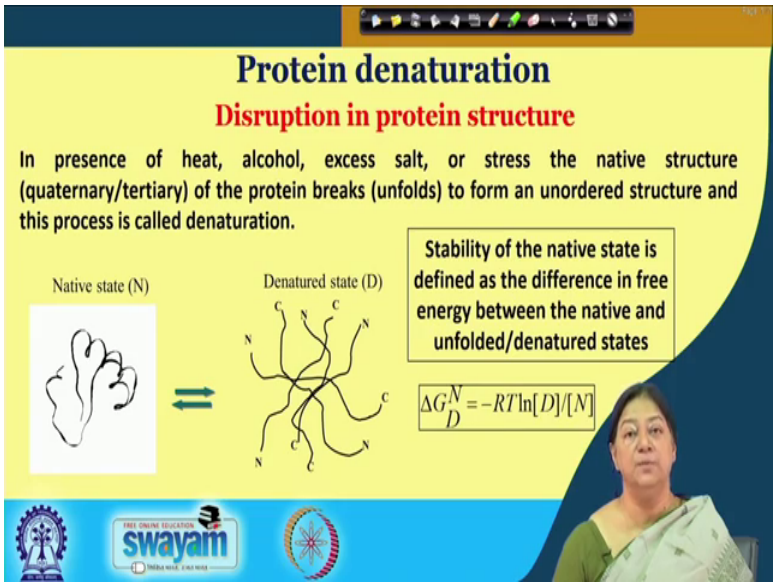

When we look at protein denaturation, now what do we mean? We mean that we are going to disrupt the protein structure. Now in this disruption of the protein structure it means that we are going to disrupt the forces that hold the protein together. So, if we have a native state of a protein and we have a denatured state of the protein this represent by N or D it is also represented by u meaning the unfolded state of the protein.

So, what we are going to do is, we are going to disrupt the structure disrupt the tertiary structure and what do you mean by the desruption it means we are going to bring about conditions that are going to force the protein to unfold. So, we have to go against the hydrogen bonding, the ionic interactions the hydrophobic forces that hold the molecule of the protein chain together.

So, the stability of the native state is defined as the difference in the free energy between the native and the unfolded or the denatured state. Now when we consider these states we will look at a free energy, the free energy is an equilibrium between the denature and the native state. So, there will be conditions where we will have a partial unfolding of the structure and we will try to monitor this unfolding to tell us what the free energy of the folding of the protein is. Now the fact that the protein does fold spontaneously means

that this delta g has a negative value, but what does it mean in terms of the other thermodynamic quantities in protein folding.

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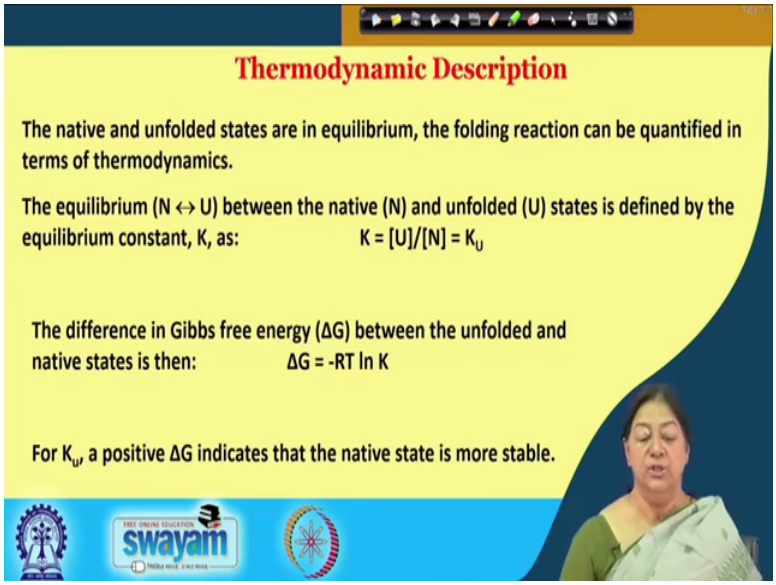

So, if you look at a thermodynamic description we are saying that the native and the unfolded states are in equilibrium and the folding reaction can be quantified in terms of thermodynamics. So, we are looking at an equilibrium that involves the native and the unfolded state, this is the equilibrium constant K that tells you that you have an unfolded state the ratio of the unfolded to the native state. And the delta G between the unfolded and the native state is a quantity that looks like this. Now if we say that K is positive then delta G is negative which indicates that the native state is more stable, now how do we look about that?

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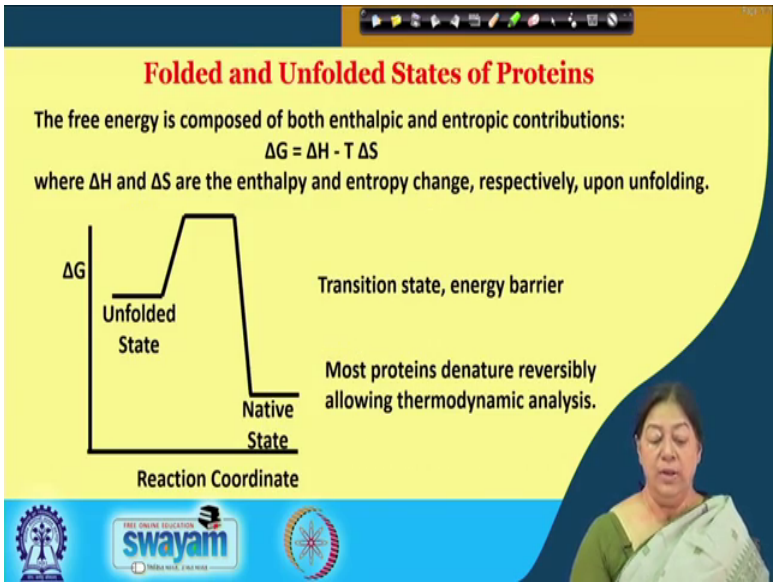

We look about this as the unfolded state and the native state. Now the free energy we know if we look at the go back to our thermodynamics we know that delta G is equal to delta H minus T delta S. What is delta H? Delta H is the enthalpy change and delta S the entropy change and what are we talking about, what kind of an enthalpy change or what kind of an entropy change this is due to the change upon unfolding.

There is a transition state that is the energy barrier that is going to take us from the unfolded state to the native state which is more stable.

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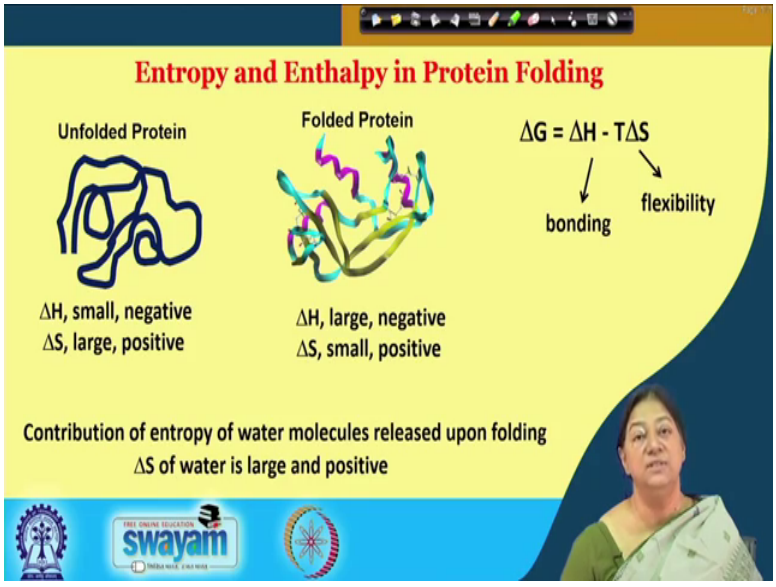

Now, when we look at these different types of analysis possible and try to understand what are protein looks like. So, we have a folded protein, we are looking at the enthalpy contribution and we are looking at the entropy contribution. When we consider an unfolded protein and unfolded protein has a large entropy factor. Now why is this? It is because it is unfolded, it has it does not have any order to it we know that, when we have disorder the entropy is going to be high which means that this delta S is large and positive in contrast delta H is small and negative.

Once we form the different types of interactions the non covalent interactions within the protein structure be at hydrogen bonding, be at the hydrophobic interactions the electronic attractions, the ionic attractions these are all going to contribute in their little ways to the protein structure in general to the stability of the protein structure in general. But the delta S is small and positive because they has been an order brought about in it. So, the delta S brings about is recording the flexibility and delta H the bonding, now what happens is because the delta h is large and negative it over compromises the positive delta S here making the delta G overall negative, which is a relatively a small quantity, but nevertheless spontaneous in nature.

Now the contribution of entropy of the water molecules that are released upon folding renders the delta S of water large and positive, what does this mean? It means that when you have the unfolded protein in water there are the water molecules that are surrounding the amino acid residues when this folds it disrupts the water molecules rendering a delta S that is positive in nature because there is disorder in the water molecules.

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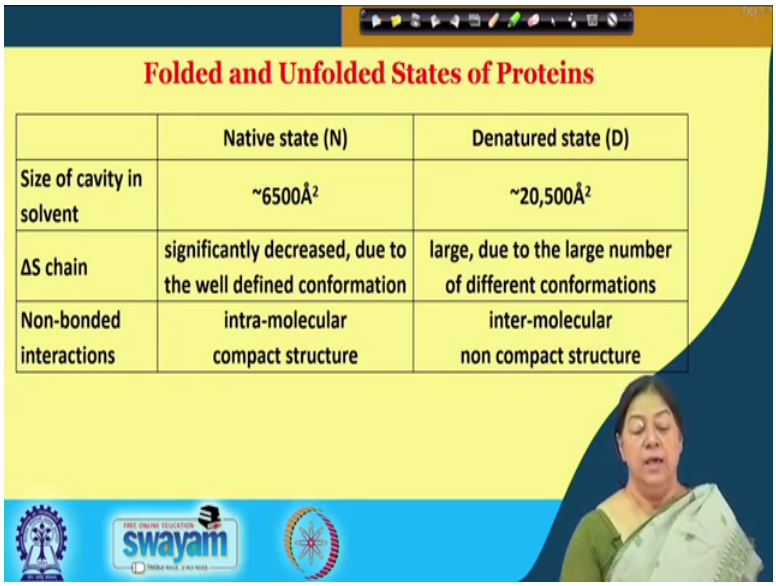

If we look at the native state and the denatured state in this fashion and we look at the size of the cavity in the solvent where it amounts for the amount of the solvent molecules that we can interact in the molecule. We realize that the native state is going to have a less amount of it compared to the denatured state, the delta S is going to be significantly decreased why because it has a well defined confirmation.

On the other hand in the denatured state it is going to be large because they are a large number of different confirmations possible rendering a disorder into the structure, for the non bonded interactions they are intramolecular in nature giving you a compact structure. Here they are intermolecular and there is a non compact structure which means that the chains can now interact amongst themselves also rendering a disorder into the system.

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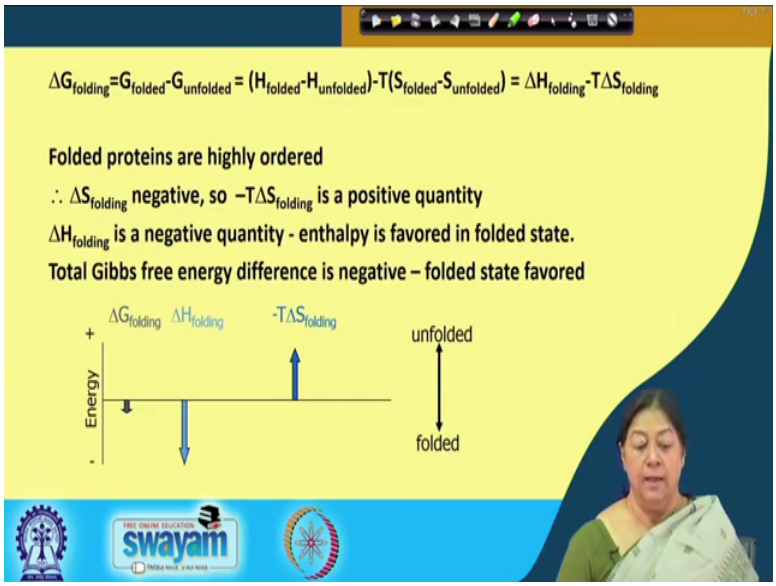

So, when we look at the delta G of folding, it means we are going from the unfolded to the folded state. So, we have an entropic contribution that is given by the S folded minus S unfolded, the enthalpy contribution given by the H folded minus the H unfolded that gives us the overall expression. Now as I mentioned before the folded proteins are highly ordered delta S folding is therefore, negative which men renders minus T delta S folding as a positive quantity. But the enthalpy contribution is favored in the folded state because of the large number of interactions possible making the delta H folding negative.

So, the overall compensation is such that the delta G of folding is a small negative quantity making protein folding a spontaneous process, but the contribution comes from the enthalpy. So, we say it is an enthalpy driven process.

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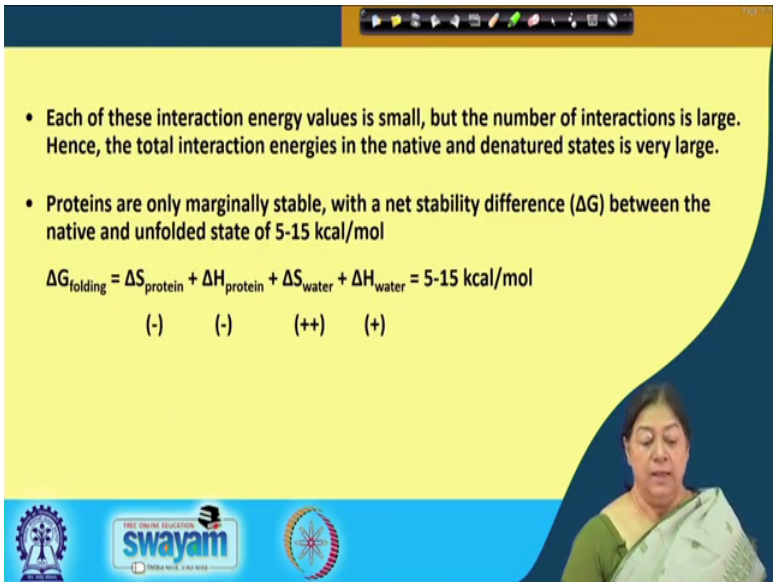

Now, when we look at the contributions the proteins they are actually quite marginally stable and the next stability difference that is the delta G between the native and the unfolded state is of the order of 5 to 15 kilo calories per mol.

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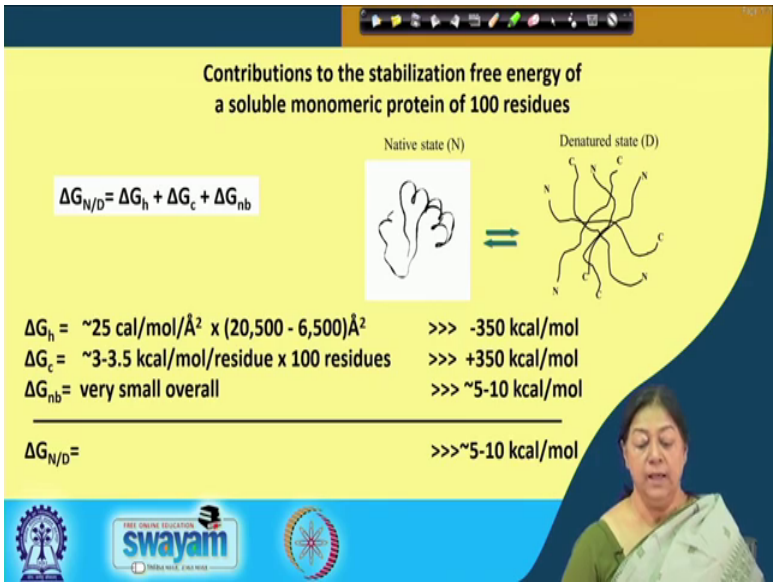

So, if we just look at what the values are going to be approximately for a protein of a 100 residues, if we just look at the delta H contribution from the different types of contribution the non bonding, the covalent the hydrogen bonding. Then we will get value that is the overall delta G which is going to be a very small value because we have the T

delta S that is a positive quantity, we have the delta H that is a negative quantity which renders the delta G a small negative quantity.

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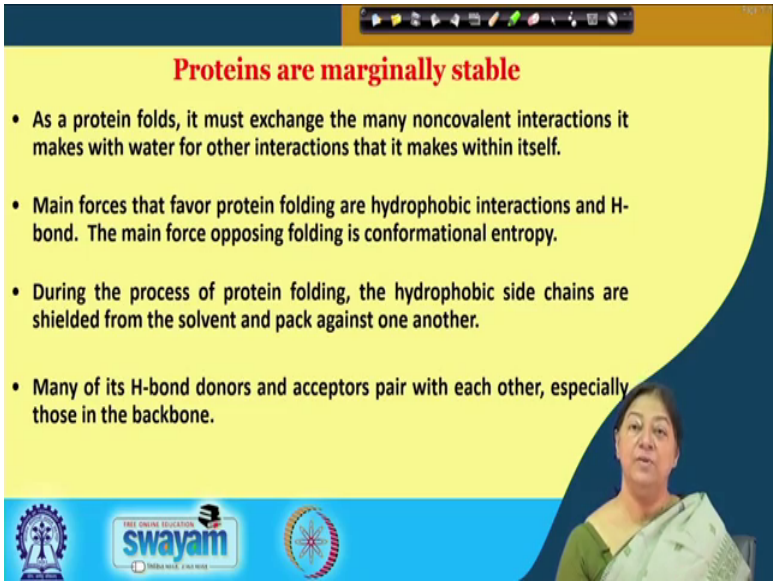

Now, the main force is that actually favor protein folding and therefore, the hydrophobic interactions and the hydrogen bonds and the confirmation entropy something that goes against it because it would tend to be a disorder state. So, during the process of protein folding the hydrophobic side chains themselves are shielded from the solvent and so they try and enter this hydrophobic code within the protein.

The hydrogen bond donors and acceptors where they were talking about the alpha helix, whether they are between the backbone residues backbone residues in the sense of backbone CO and the n H or between the amino acids side chains these hydrogen bond contributions the contributions to the energy the favorable contributions to the energy add up as well.

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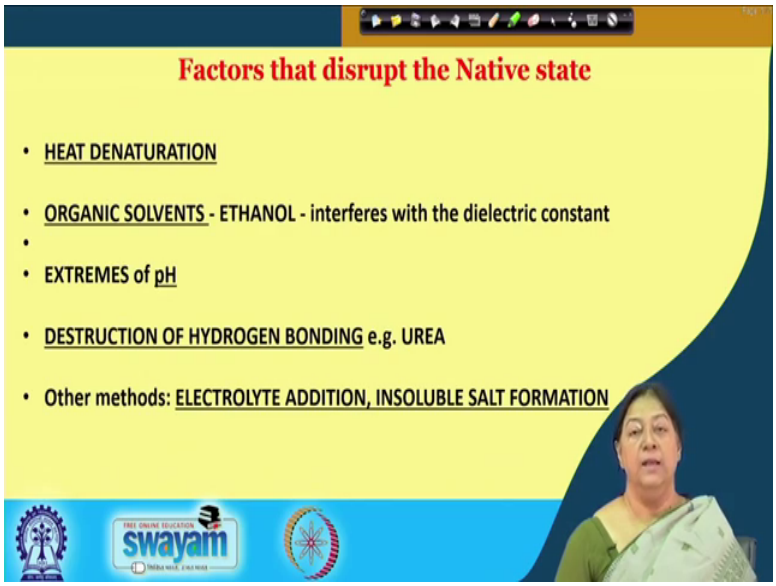

Now, if you want to destruct the native state, we have to look at possibilities variations or what can be used to render the protein to go from a native state to a denature state which means we have to disrupt the native structure. We can use heat, we can use organic solvents extremes of pH, we can destroy the hydrogen bonding by using urea or we can have other methods where we add a lot of electrolyte of form a salt or precipitate of the protein by denaturing it.

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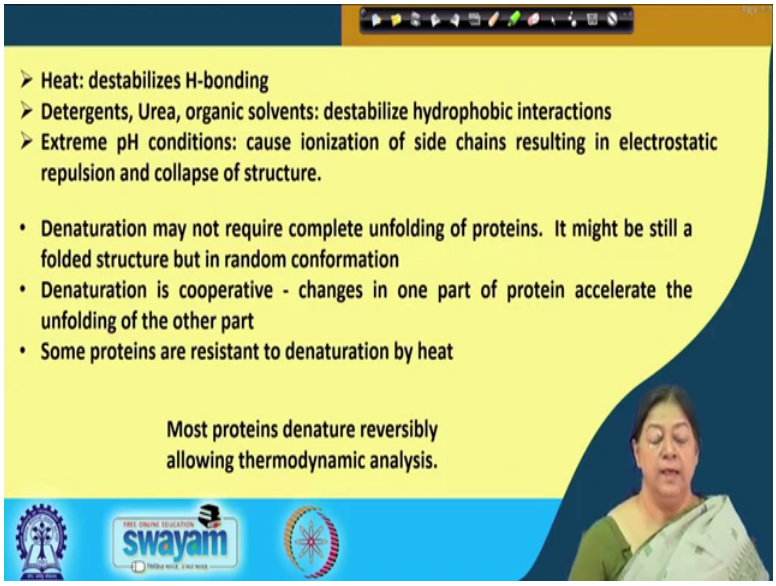

What does heat do? Heat destabilizes the hydrogen bonding detergents, urea, organic solvents destabilize the hydrophobic interactions. Extreme pH conditions we know will cause ionization of the side chains so whether we talking about a very highly acidic pH or very alkaline pH both of these will have a very important effect on the side chain and they will result in electrostatic repulsion and the, they will be collapse of the structure.

Now denaturation may not always require a complete unfolding of the protein, but they it they may be some folded structure present and some in random confirmation. So, there is a degree of unfolding of the protein. So, it is this degree of unfolding that we can measure by different quantities or different measurement procedures.

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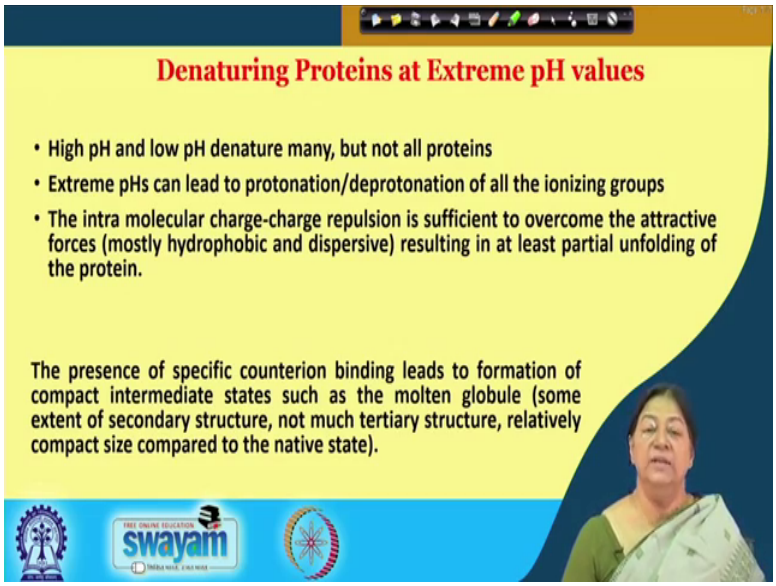

So, if you looking at different pH as I mention high pH and low pH denature most proteins, but not all proteins because some proteins may be stable to a certain extent. They can lead to protonation and deprotonation of the ionize ionizing groups as I mentioned and what happens is there is going to be bound. There is going to be some counter ion binding that leads to compact formation of intermediate states and so on and so forth that are going to result in a disruption from the native state of the protein.

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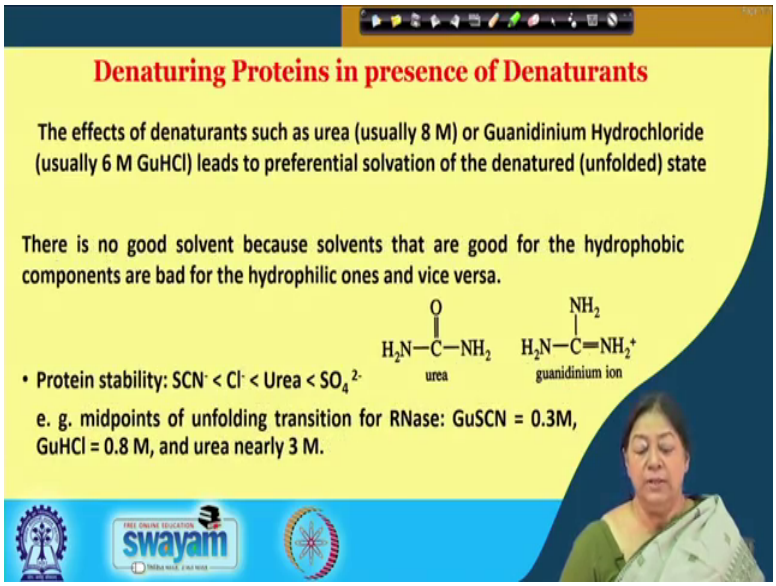

If we look at the presence of denaturants, these are urea and guanidinium hydrochloride which is very commonly used they denature the protein, they unfold the protein. And the sense that they destroy the interactions the hydrogen bonding interactions the salvation of the protein, the hydrophobic components all of this is disrupt disruptive or disrupted due to the urea and the GuSCN.

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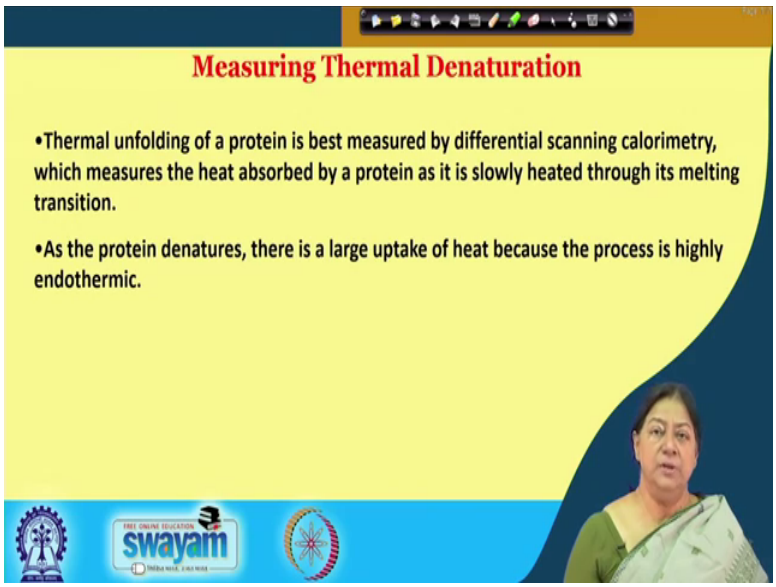

Measuring thermal denaturation we have to have a way to look at how the native structure is getting destroyed. So, if I have a specific characteristic that belongs to the

native structure and that say you have an absorbance, we have studied about UV absorbance we studied about fluorescence spectroscopy. So, the protein native structure is going to have a definite UV spectra, it is going to have a definite fluorescence intensity profile emission spectrum if you look at it.

Now if the protein is denatured what does it mean it means that there is a disruption in the structure of the protein it means that the aromatic amino acid residues could be exposed. As they are exposed the absorbance will increase and we will see more the plot, the amino acid residues we will see more of the light and they will be able to absorb more light.

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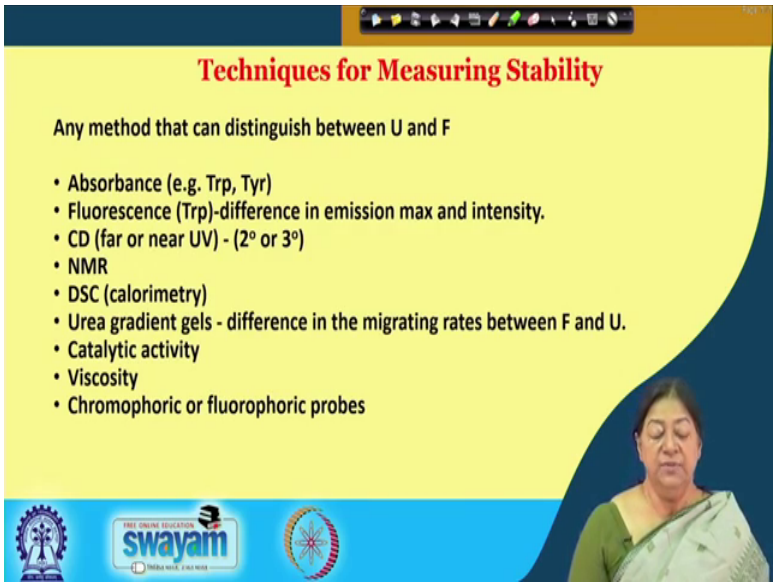

The different techniques therefore, to measure the stability as I say for example, absorbance fluorescence circular dichroism that can measure the extent of the secondary structure. Circular dichroism tells us how much alpha helix we have, how much beta sheet we have. So, if we destroy the structure then the alpha helix will be destroyed. So, the signal for alpha helix will decrease, if we go back from an unfolded to a folded structure, where we know that the protein has the formation of the alpha helix then the signal for the alpha helix will increase. We have can have NMR, we can have calorimetry, we can have catalytic activity, viscosity, chromophoric or flourophoric probes that will tell us whether the protein is has the structure destroyed or not.

In the catalytic activity if there is a specific enzyme we will study about enzyme, we have to have a specific folded structure for the catalytic function. Now if that is disrupted then; obviously, we know that the structure is destroyed.

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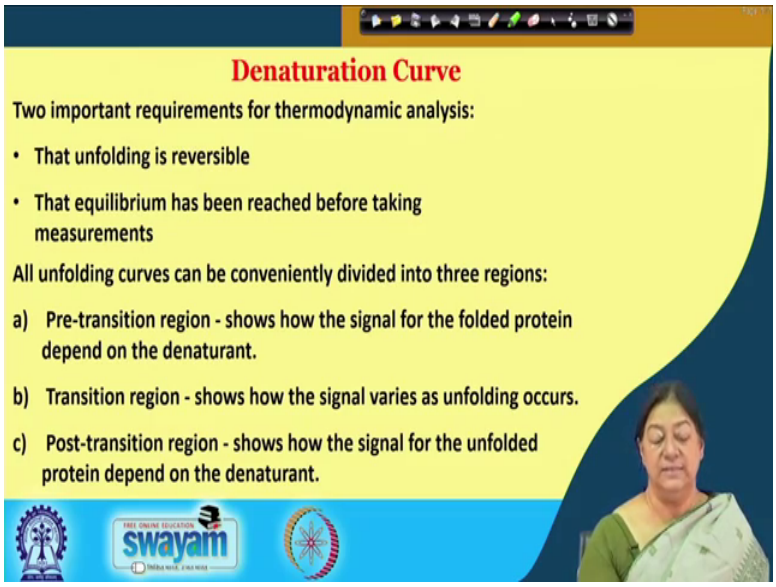

So, when we look at how to analyze this we look at what is called a denaturation curve, the unfolding curves have a pre transition region that shows the signal for the folded protein. How then we increase the denaturant, what do we mean by denaturant? It could be temperature, it could be pH, it could be addition of urea, it could be some other property that is going to or some other component or additive that is going to disrupt the native structure of the protein.

And what we have to do is we have to monitor this disruption. So, we have a transition zone that will actually shows the signal variation with the unfolding and we have a post transition that will tell us that the protein has denatured.

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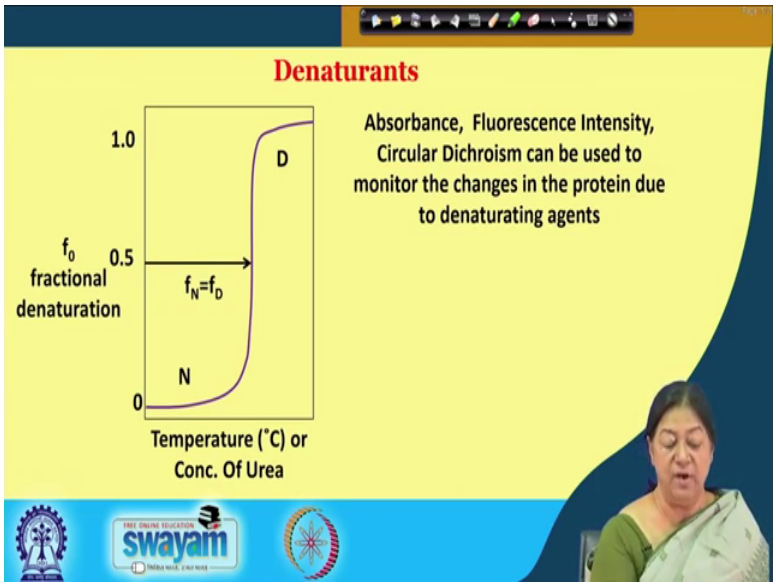

So, we have a fractional denaturation. So, whether with temperature, whether it is a concentration of urea, what is going to happen? We are not in the initial stages there is not going to be any fraction that is unfolded, but gradually that is going to be a state where we are going to get a to a completely denatured structure. So, what does this mean? This is the transition curve and this will tell us we have 50 percent folded or 50 percent unfolded structure. And how can we monitor this? We can monitor by observance, fluorescence intensity, circular dichroism depending upon what kind of system we want to study.

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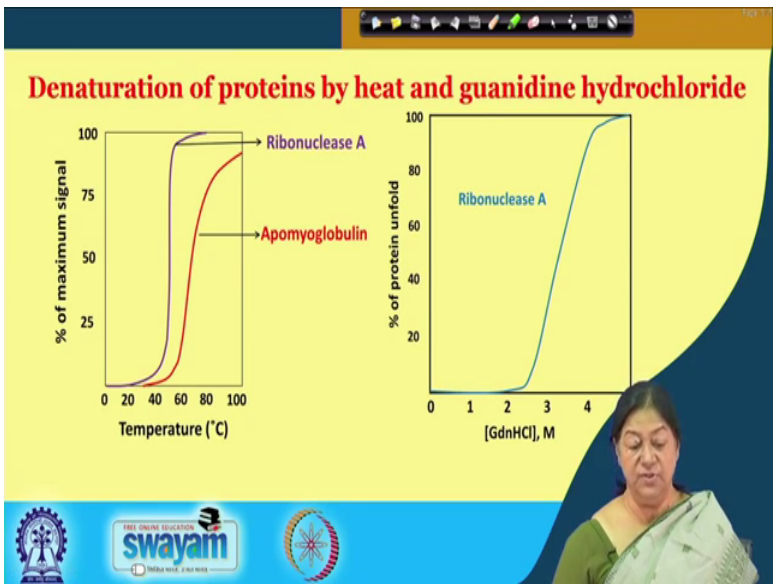

So, if this if we look at the denaturation of proteins by heat by GuHCl here you can see there are two different proteins. Ribonuclease A and apomyoglobulin, what can we say from this we can say that apomyoglobin is more stable. Why? Because it needs more temperature for the disruption to occur. So, straight away from this graph itself the denaturation curve can tell us or give us information about the stability of the protein, here we are looking at the percent of protein unfolded with the guanidine hydrochloride. So, what are we saying? We are saying that at this molarity the ribonuclease A remains in the folded confirmation and gradually unfolds to the unfolded confirmation at a higher concentration of GoHCl.

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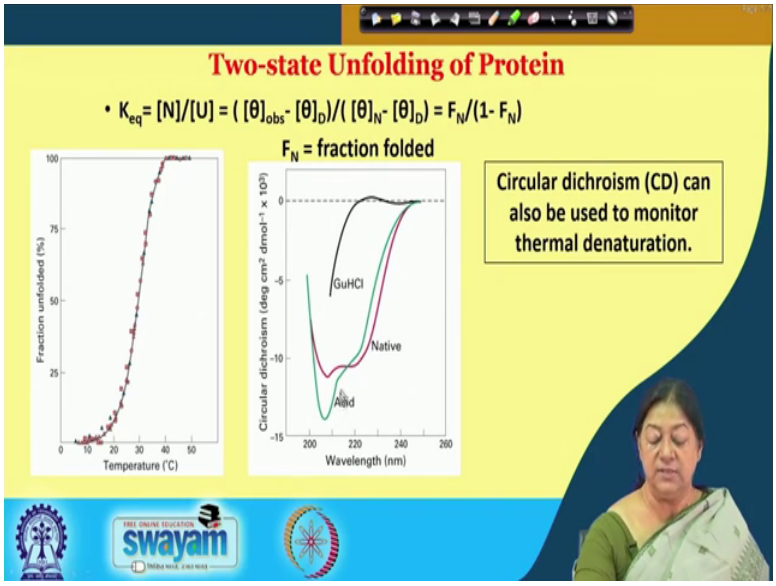

So, this is from this is like a circular dichroism signal, again when we have a disruption this can be used to monitor it. So, we have a methodology so whether we looking at secondary structure changes, whether we are looking at overall changes related to the viscosity of the structure.

The viscosity of the protein the CBE or we are looking at the UV absorbance or the fluorescence intensity; there will be some such measure that will tell us that the protein is getting unfolded.

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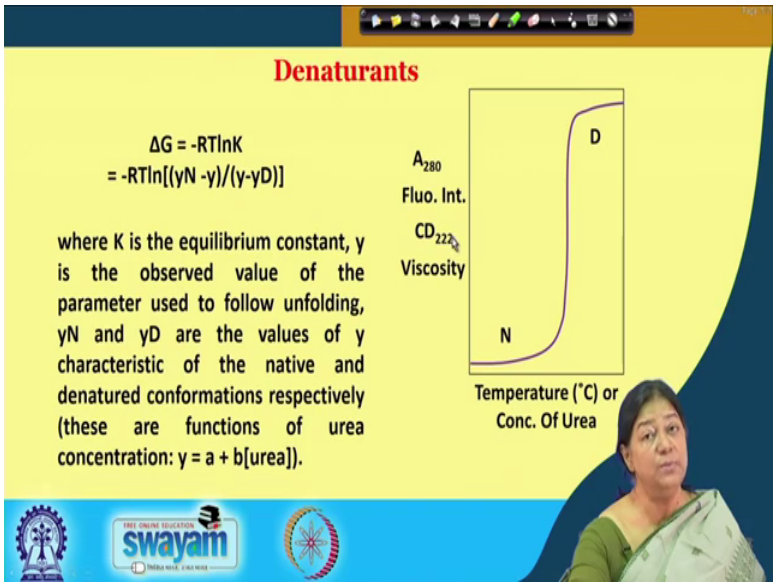

And it is this measure that we will use to determine how we can calculate the delta G of unfolding. So, say we have an equation like this; we want to know what the value of K is. So, whatever signal we are using or whatever parameter we are using to follow it there will be a factor called yN that is the parameter or the signal value for the native confirmation. We will have a yD that is the signal for that completely denatured protein.

The intermediate concentrations of the intermediate y parameter values will correspond to variations of y, if you look at if you look at this. So, these will be variations of y while we either increase the temperature or we add more of the denaturant. So, in that case what we are going to have is, we are going to have an a we are going to have two extremes, one extreme is going to be the signal at the native state one extreme is going to be the single for the denatured state. And all the intermediate states will tell us the degree of unfolding which is a measure of this equilibrium constant at that particular temperature or that particular denaturant concentration.

So, if we say we are looking at the temperature variation and the concentration of urea. So, we have a native state that unfolds to the denatured state, this is usually a transition in such a way that there is a disruption. So, once the disruption begins in the structure of the protein there is a complete collapse of the structure, and how do we measure this? We can measure this by the absorbance at 280 nano meters.

We can measure this by the fluorescence intensity, we can measure this by the circular dichroism signal at 222 nano meters that corresponds to a disruption of the alpha helix or we could even measure the viscosity change in the protein. What will happen in the viscosity change? If we have discrete molecules where we have a folded structure we will have a specific viscosity, as we increase the concentration of the denaturant what is going to happen? Is the protein is going to unfold, now as it unfolds it is going to get tangled up we because we remember we study that this is a long protein polypeptide chain.

So, what is going to happen it is going to get a entangled with other protein chains the one hydrophobic part of one protein may interact with another hydrophobic part of another protein so that it can stay away from the solve the polar solvent and so on and so forth. So, there will be a larger network and this network will increase the viscosity of this protein solution with the addition of urea or with the addition of a denaturant and the detergent, that will disrupt the protein structure, but our idea is to monitor this disruption. So, we have a signal for the native structure, we have a signal for the denatured state.

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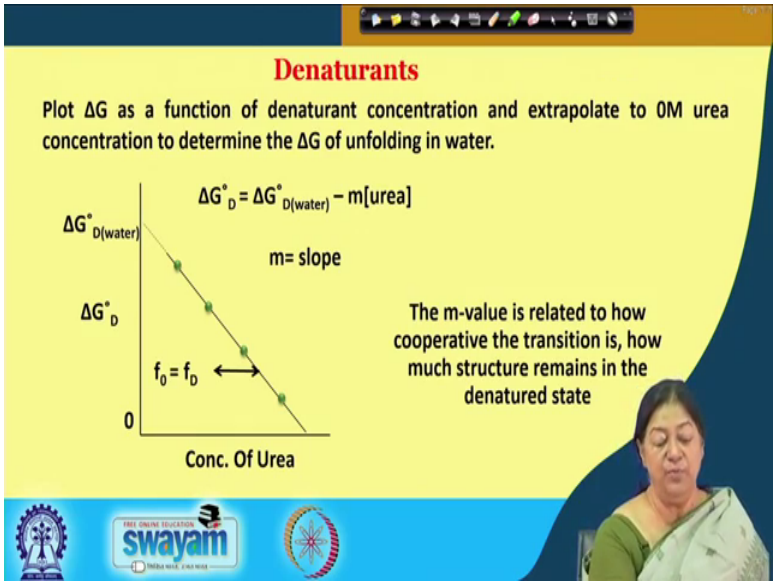

And we are looking for a specific value here. So, now, if we can calculate the delta G so we can calculate the delta G for different values of y of rather different values for the concentration of urea and then we will look at or we plot the values of the delta G versus the concentration of urea has been shown in this case and we get a specific slope the m

which is the m value here. It depends or it tells you how cooperative, what do you mean by cooperative? Like how much structure remains in the denatured state and how suddenly it change goes from the folded to the unfolded, and what can we measure from here?

We can extrapolate this to a zero concentration of urea and determine what the delta G 0 of folding is.

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The standard references would be all of this would be present in standard biochemistry books and biochemistry experimental biochemistry books.

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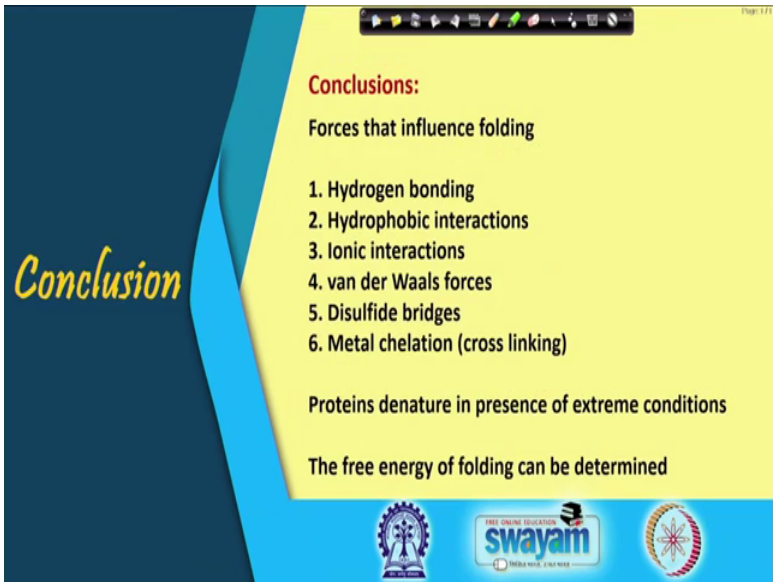

In addition what we did learn is we have to understand the forces that influence folding because if we were to denature a protein then we have to disrupt or we have to destroy these forces that hold the protein together. The forces that influence folding are the hydrogen bonding, the hydrophobic interactions, the ionic interactions the van der Waals forces, the disulphide bridges and if there is any metal cross linker metal chelation.

The proteins will denatured in presence of extreme conditions like heat concentration of urea, extremes of pH addition of organic solvents and some such external additive or that is going to disrupt the inherent structure of the protein. And we showed or we can see how we can actually determine the free energy of folding of the protein. This will also be demonstrated to you in terms of how you can look at the UV absorbance changes or the fluorescence changes or viscosity changes to determine the free energy of folding in a protein.

Thank you.