Chemical and Biological Thermodynamics: Principles to Applications

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Lecture - 58
Thermal unfolding of protein by non-calorimetric methods
Addressing thermodynamics of the process

So, far we have discussed thermal unfolding of proteins by the differential scanning calorimetry by calorimetric method and the DSC can also be successfully applied to understand thermal unfolding of nucleic acids, to understand thermal unfolding of membrane proteins, any type of proteins as long as the unfolding is cooperative process and it can also be used for understating the transition in lipids, and we have discussed mostly the biological micro calorimetry.

The thermal unfolding of proteins and other bio macro molecules can also be studied by non calorimetric methods. See by thermodynamic signature associated with the unfolding process, we will mean here the transition temperature, enthalpy of unfolding and the heat capacity change upon unfolding. All these parameters transition temperature, enthalpy of unfolding, entropy change associated with unfolding and the heat capacity change associated with unfolding, can be easily determined by differential scanning calorimetry in single run.

However when it comes to this getting these thermodynamic parameters by non calorimetric methods, we will discuss today that we can get the transition temperature, enthalpy change associated with unfolding and entropy change associated with unfolding very very conveniently. And when I talk about non calorimetric methods, these can be spectroscopic methods for example; u v visible spectroscopy has been thoroughly employed in studying the folding unfolding process is of proteins. Fluorescence spectroscopy has been extensively used. Circular dichroism spectroscopy has been extensively used and similarly we can use some other techniques to study the folding unfolding process and getting the thermodynamic parameters associated with the process.

Let us discuss these issues in some details, now let us look at this slide.

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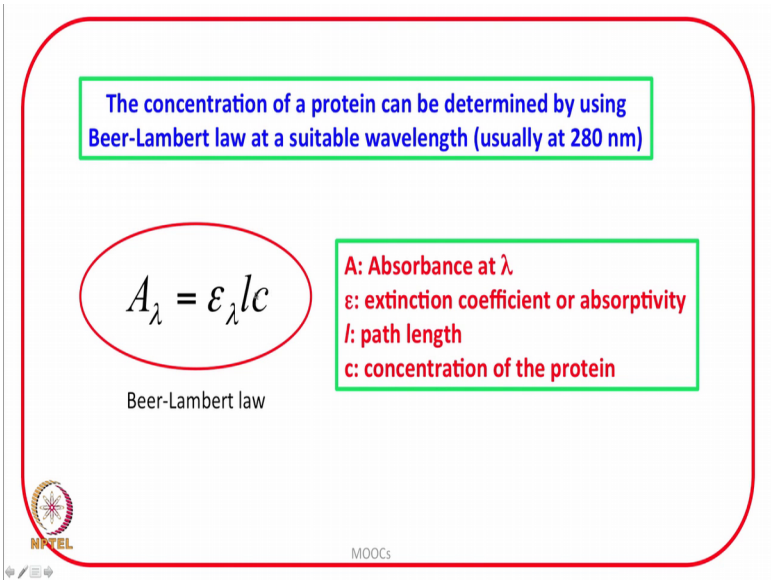

Before we start any experiment, we need to determine the concentration of the protein or concentration of nucleic acid or the concentration of any biological macromolecule that we are interested in. The concentration of a protein which has tryptophan residues is usually determined by the absorbance measurements at 2 9 280 nanometer. The absorbance at a wavelength of 280 nanometer is usually used to determine the concentration of the protein in solution by the application of Beer Lambert law, and the concentration is to be chosen so, that it is within the applicability of the Beer Lambert law; What is the Beer Lambert law let us take a look at this slide.

According to Beer Lambert law A lambda is equal to epsilon lambda into l times C what is the meaning of each term a is absorbance at wavelength lambda e is or epsilon is extinction coefficient at that wavelength this is also called absorptivity, l is the path length which is usually a path length is the length the horizontal length of the qubit that is used and c is the concentration of the protein, and that is what we are interested into begin. So, after we know the concentration of the biological macromolecule very very accurately; because eventually you are going to normalize your measured thermodynamic quantities to per mole and that is why the determination of the concentration is very very important.

Now, let us go ahead and we talk about the thermal unfolding of a protein and associated absorbance change. When a protein is subjected to heat I other words when we allow thermal unfolding of a protein, when the temperature is increased as we discussed earlier at some point at some temperature there will be cooperative unfolding. The various

chromophores of the protein is with temperature observing different environment, and therefore, the absorbance of the protein also changes. And if we can monitor the absorbance as a function of temperature, then we can determine the thermodynamic parameter associated with the unfolding. It is not only absorbance it can be fluorescence intensity it can be lambda max that is the fluorescence emissions wavelength where the maxima appears or it can be the ellipticity as a selected wavelength because all these properties are dependent upon the environment of the various chromophores of the protein.

Let us take a look at the u v visible based study based thermal unfolding of a protein. This example is thermal unfolding of alpha chymotrypsin as a function of temperature. Now how do you choose the wavelength? Now this study is to be done at a fixed wavelength you fix a wavelength and that at fixed wavelength you change the temperature and monitor the absorbance changes. When you study the thermal unfolding of a protein by u v visible spectroscopic measurements, the wavelength selected is 293 nanometer or it should be a wavelength where the difference in the absorbance of the native state and the denatured state is maximum. You want to choose a wavelength where you can see a maximum change in absorbance and that in most of the protein it turns out be 293 nanometer.

let us see this is how the absorbance at 293 nanometer for alpha chymotrypsin changes when the temperature is increased. When the temperature is increased initially the absorbance appears to remain nearly the same, and then when the unfolding starts you see when the cooperatively unfolding starts there is a sharp change in absorbance and eventually then again its starts the increasing in a slow manner. With the knowledge of pre transition absorbance variation on temperature, post transition absorbance variation on temperature, and the absorbance changes in the transition range the thermodynamic parameters can be obtained can be calculated.

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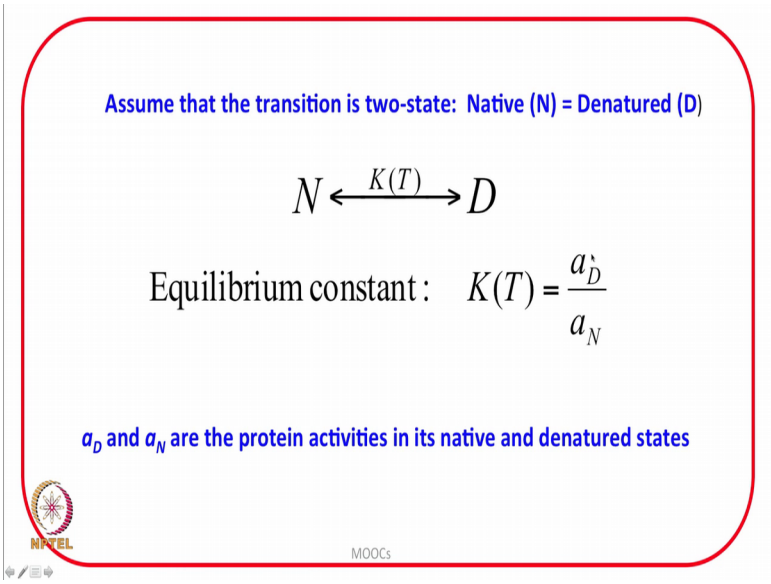

Now, let us discuss about a two state native to denatured unfolding transition. The reaction that we are talking about is N equilibrium with D; that means, you are heating N and it is going towards D and the associated equilibrium constant is KT which is expressed in terms of the activity of denatured form divided by the activity of native form. And if the concentration are very low we are assuming that the activities are same as the concentration. So, the equilibrium constant is defined as if we activities to be same as concentration, then this will be defined as K T is equal to concentration of the denatured form divided by the concentration of native form ok.

Let me begin the discussion by taking y T as an experimental observable at a temperature T. Y T where y is the property; it can be absorbance, it can be fluorescence, it can be ellipticity, it can be viscosity, it can be heat capacity or it can be any other property which depends upon temperature in case of proteins.

Now, let us discuss some equations.

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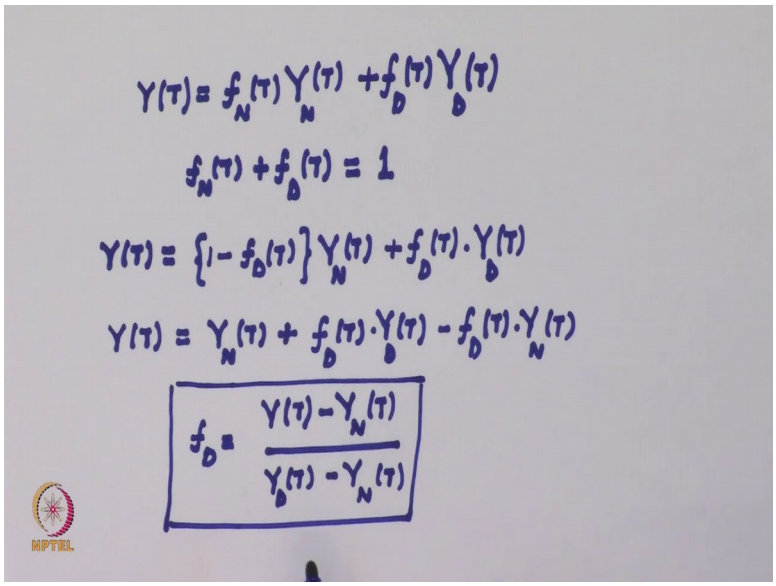

Y the property at any temperature T will be equal to fraction of the protein in native state at that temperature into Y N T the value of that property in the native state at that temperature plus f D at a temperature T into Y D at that temperature. We know that f N at any temperature plus f D at any temperature, all the fractions when you add it has to come out to 1 therefore, this equation becomes Y at any temperature to T is equal to instead of f N, I will write 1 minus f D at temperature T, into Y N at that temperature. Instead of f N I am writing 1 minus f D plus f D at temperature at T into Y D at that temperature. Going a step further Y at temperature T is equal to Y N at that temperature plus f D T into Y D T minus f D at temperature T into Y N at temperature T. Once you solve this what you will get is f D is equal to Y at a temperature T, minus Y N at temperature T divided by takeout f D common over here, it will be Y D at temperature T minus Y N at temperature T. Fraction of the protein in denatured state can be obtained from the observable property at any temperature T, observable property in the native state and that observable property in the denatured, state we can get the fraction if the protein in the denatured state.

Now, let us go to the slides, what we discussed is that we can express the observable property at any temperature and by the suitable rearrangements we can get this expression f D is equal to y T minus Y N into Y N at temperature T, over Y D at temperature T minus Y N and as we discussed earlier this Y can be absorbance, this y can be fluorescence, this y can be ellipticity, y can be any observable property. Now K; K is

equal to concentration of the denatured form of the protein divided by the concentration of the native form of the protein, which is equal to f D because denatured is f D it is equal to f D divided by f N let me discuss over here.

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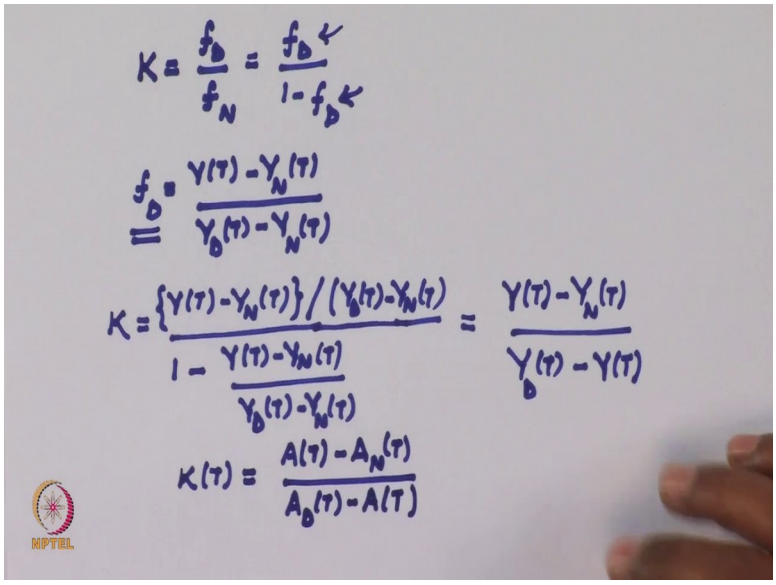

So, let me discuss will be equal to fraction of the protein in denatured form or fraction of the protein in native form which is equal to f D over 1 minus f D, and we have just discussed that f D is equal to Y T minus Y N T over Y D T minus Y N T and this needs to be substituted over here and over here.

Once you substitute this over here let us see what do we get. K is equal to f D which is y at T minus Y N at T over 1 minus Y at T minus Y N at T divided by Y D at T minus Y N at T so; obviously, this also has divided by Y D at T minus Y N at T. Once you solve for this what you are going to get, Y at T minus Y N at T and divided by Y D at T minus Y at T this is what we are going to get. And if I express in terms of absorbance then K at any temperature will be equal to absorbance at temperature T, minus absorbance for the native form at temperature T divided by the absorbance for the denatured form at temperature T minus absorbance at any temperature T. Now let us go back to the slide and that is what is shown over here, that we can get equilibrium constant at any temperature from the spectral measurements, and once I have the temperature dependence of this equilibrium, constant I can easily get the enthalpy.

At any state of at any stage of the transition the concentration of native form will be equal to C0 is the initial concentration of protein into fraction of the native protein, which is 1 minus f D and the concentration of denatured state is equal to C0 into f D; f d is the fraction of the protein which is in the denatured state.

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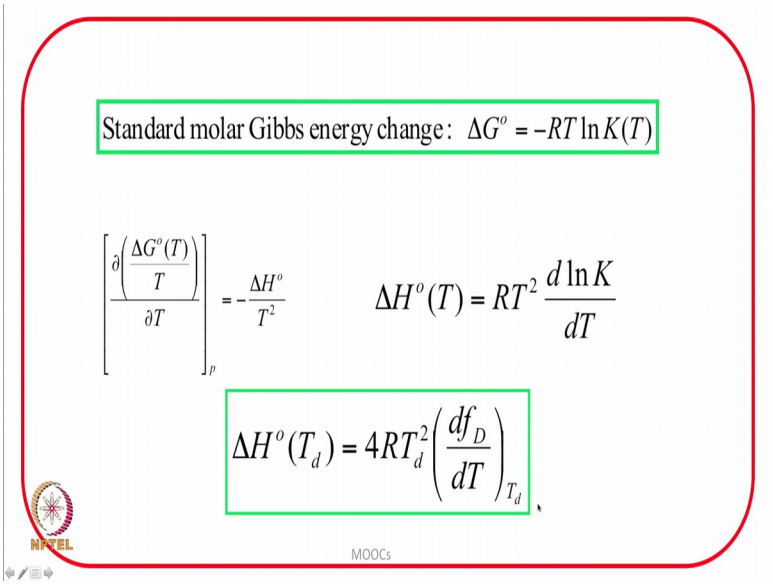

Standard molar Gibbs energy can easily be now calculated from the value of K. So, at any temperature I can now get the value of standard reaction Gibbs energy change and then let us look at this equations Vanthoff equation, we have discussed many times let me show it over here.

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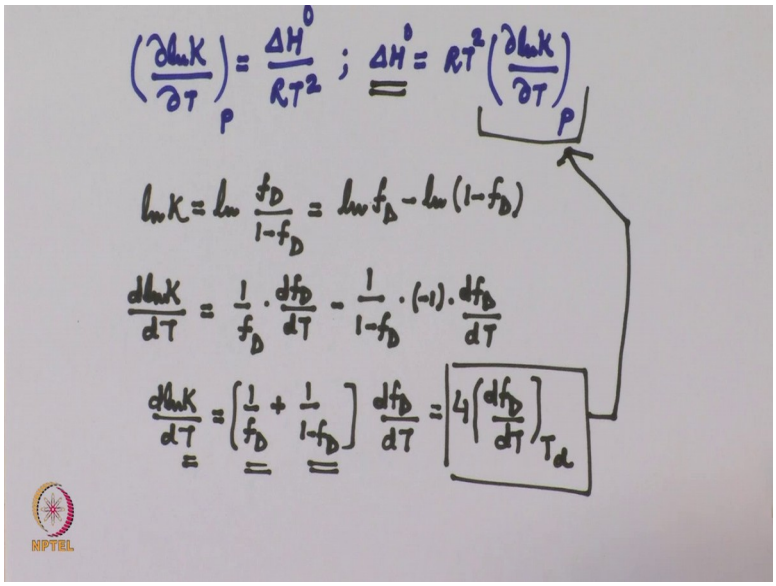

According to Vanthoff equation what we have is d log K by dou T at constant pressure is equal to delta H naught by RT square.

In other words I will write delta H naught is equal R T square into d log K by d T at constant pressure. So, what I will do is now we need to find out this factor. Log K will be equal to log, K we have written as f D over 1 minus f D, and if I write this as log f D minus log 1 minus f D and I take its derivative which will be equal to 1 over f D into d f D by d T minus 1 over 1 minus f D into minus 1 into d f D by d T. So, I have d log K by d T is equal to 1 over f D plus 1 over 1 minus f D, d f D by d T. If I choose the temperature to be transition midpoint, where the fraction of the denatured state is half for a two state transition; if the fraction of denatured state f D is half then 1 minus f D is also half. So, half plus half 1 by 2 this will be 1 by 2; when f D is 1 by 2, 2 will go in the numerator from here also two will go in the numerator. So, will have 4 times d f D by d T and this is actually at transition point. T half T d whatever way want to mention over here and this whatever we derived now can be substituted over here and we can get the value of delta H naught.

Let us go back to the slide now and discuss that from the value of d log K by d T at transition temperature, we can get delta H naught at transition temperature which is equal to 4 into R into T d square into d f D by d T at the transition temperature whatever is the slope at the transition temperature.

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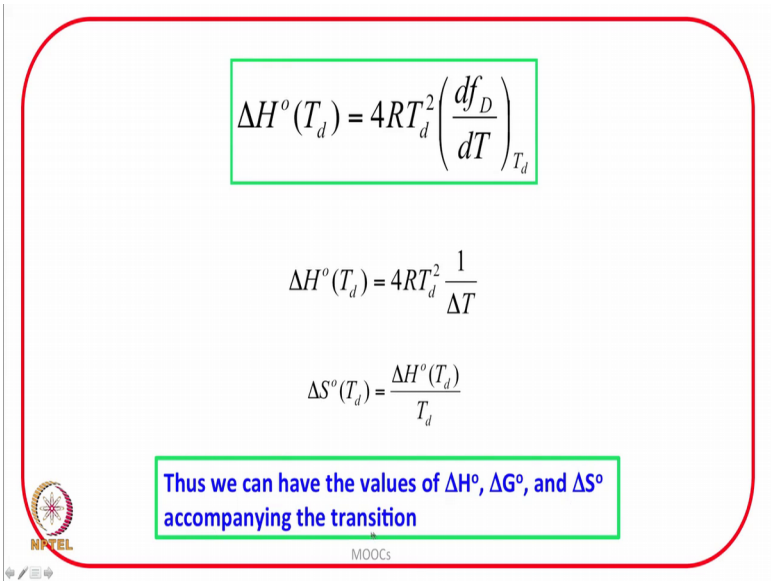

So, another form of writing is delta H naught at T d is a 4 R T d square over 1 over delta T what is. This delta T this delta T let me explain over here that if we are considering two state transition and let us say we are talking about fraction of denatured or native state whatever you want to put and T, and let us say the change is happening over like this and then if I take this as let us say fraction of denatured state, if fraction denatured state will be 0 over here and fraction denatured state will be 1 over here, here. So therefore, if I choose this region only this region and take this as delta T the corresponding delta f D is equal 1 over here.

Therefore delta f D over delta T will be here equal to 1 by delta T and this is what is being put here in this equation, 4 R T d square instead of d f D by d T, I have put 1 over delta T. So, therefore, once we have the enthalpy of transition at transition midpoint, we have earlier discussed that we can entropy we can get the entropy change associated with the transition at transition midpoint. Thus we can have the values of delta H naught delta G naught delta S naught accompanying the transition, and as I discussed that we can use u v visible spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy or any other technique where the observable is changing with temperature we can get the thermodynamic parameters associated with the transition with the protein from native state to denatured state.

For example let us take a look at the slide, this slide shows thermal unfolding of human alpha lactalbumin by fluorescence spectroscopy. So, when one studies the thermal unfolding by fluorescence spectroscopy, what we do is you usually choose a certain wavelength at which you will see the intensity changes or you will take the whole emission spectra by selectively exciting tryptophan residues over a range of temperature; that means, the observables are parameters are 2. One is how the intensity at that selected wavelength emission intensity changes with temperature, second how the value of lambda maxes. Lambda max is the value of wavelength at which the intensity is maximum. So, the changes in the value of intensity or changes in the value of lambda max both can give information about the thermal unfolding of the protein how let us take a look at this figure.

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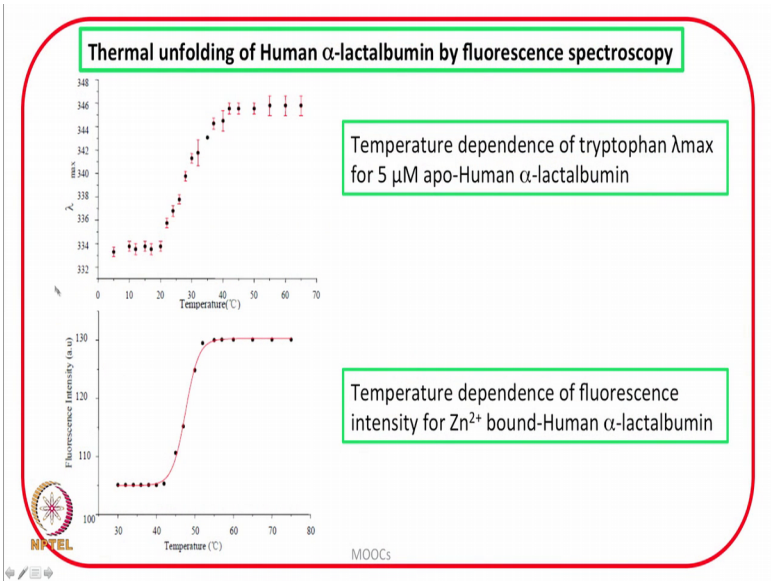

The upper figure shows how the wavelength lambda max changes the temperature is in increased. As the temperature is increased the wavelength increases; that means, there is a red shift and the sigmoidal kind of curve is obtained and here in the lower panel what is shown over here is the how the fluorescence intensity changes with increase in temperature. So, in this uppercase the observable is lambda max and in the lower case the observable is intensity. So, we can use this in place of Y and get the fraction denatured and then address the thermodynamics of the unfolding process in the manner that we just discussed.

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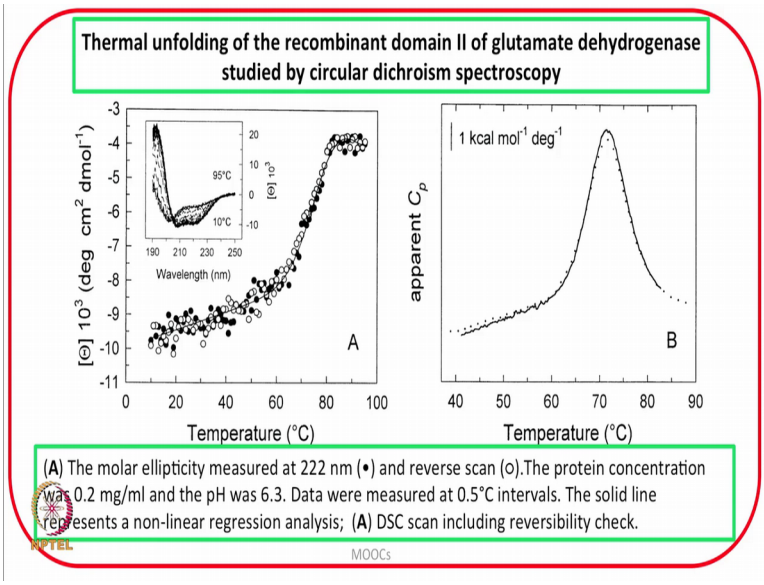

Similarly, one can use the circular dichroism spectroscopy, when we use the circular dichroism spectroscopy we usually talk about the near u v c d region and the far u v c d region. That is we either talk about the tertiary structural changes or the secondary structural changes. Electricity in either in the tertiary structural changes range or the far u v c d can be used determine the thermodynamics of the process how? Usually the electricity change at 222 nanometer, which is accompanying or which is signature for the alfaholics structure in the protein. So, theta electricity at 222 nanometer you see also shows a sigmoidal kind of change when the temperature is increased. In this particular figure the black dot and white dot correspond to thermal heating and checking of the reversibility of the unfolding process; obviously, in this particular case since the black circles and the white circles are sought of overlapping therefore, demonstrating that there is a good reversibility of the unfolding in case of the recombinant domain 2 of the glutamate dehydrogenase.

And when you compare with it the differential scanning calorimetry, where also the reversibility is also be check by heating and cooling or heating and reheating, you can compare the thermal transition temperatures obtained by both the techniques it is almost nearly the same. So, this example also demonstrates that the circular dichroism spectroscopy can also be easily used to derive the thermodynamic parameters associated with the thermal unfolding of the process.

So, even though we can get the thermodynamic parameters associated with the thermal unfolding of the biological macromolecules by known calorimetric methods, but these are indirect technique which gives the energetics of the process. What I mean by this indirect is that the enthalpy change evaluated calculated or determined, the enthalpy change determined by the non calorimetric method is the Vanthoff enthalpy, it is not the true enthalpy because calorimetry gives you the true enthalpy without considering any model whereas, the vanthoff enthalpy is model dependent, it depends in the type of model you choose and that is why the calorimetric methods are the most accurate methods when it comes to determination of enthalpy of interaction.

So, therefore, although calorimetric methods will directly give you the energetics of the process, the similar information can also be obtained by spectroscopic techniques; although the calorimetric method give you the most reliable values and most accurate values. So, in this lecture we connected the basic definition of equilibrium constant, with the observable properties whether these are spectroscopic properties or non spectroscopic properties, and we showed that how the temperature dependence of the equilibrium constant can be connected with determination of the enthalpy of binding, entropy of binding and free energy change of binding or enthalpy of unfolding entropy of unfolding or free energy of unfolding.

So, therefore, it is possible to address the thermodynamics of a process by choosing an appropriate methodology by choosing an experimental technique, which is most appropriate to give you the changes in a more elaborate way or the changes which are large enough to address the thermodynamics of the process in the most efficient manner.

Thank you very much.