Chemical and Biological Thermodynamics: Principles to Applications

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Lecture - 51
Thermodynamic Characterization of Partially Folded States of Proteins

In the previous lecture we talked about qualitatively characterization of the partially folded states of protein. We discussed the use circular dichroism spectroscopy, which can qualitatively establish the relative change in the secondary structure and tertiary structure of a protein compare to the native state. By taking the examples of hexafluoroisopropanol with alpha lactalbumin, what we observed was that at certain concentration of hexafluoroisopropanol the CD signal suggest that the secondary structure is rather strengthened compared to the native structure, but the tertiary structure is lost. And therefore, these features suggest that the protein under these conditions is in the molten globule state.

There are other qualitative methods which can be applied in the studies of the molten globule state or partially folded states. A look at literature suggests that floriation spectroscopy has been extensively used also in the characterization of the partially folded states of protein floriation spectroscopy details is a matter of a different lecture; however, the floriation spectroscopy of proteins has given tremendous information not only about the conformation changes in proteins, but also about the protein ligand binding. Several proteins have tryptophans and there therefore, the floriation due to the tryptophan, that is selectively exciting at 293 nanometer or a suitable wavelength and looking at the emission spectra, gives information about the conformational changes and even the protein ligand binding.

Therefore the changes in the floriations emission intensity and the changes in the floriations emission wavelength where the maxima appears gives lot of information as we will discuss a bit later, how it gives.

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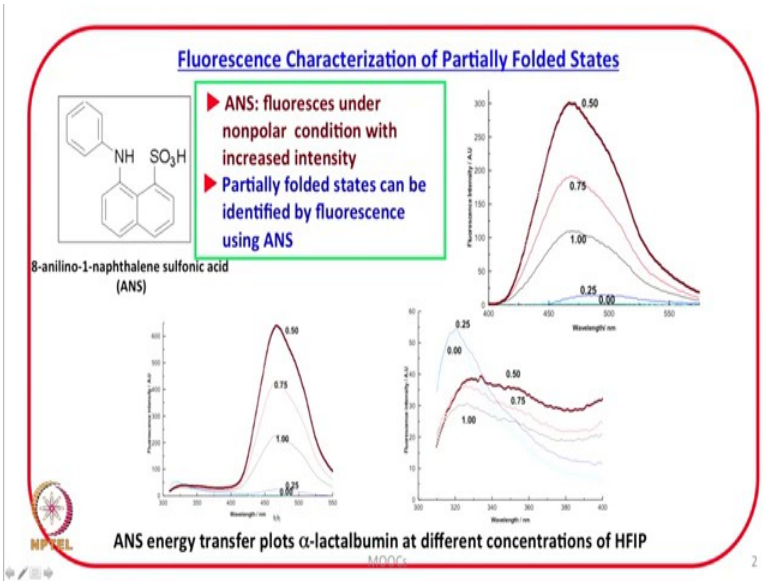

Now let us take a look at the slide. This molecule 8 anilino 1 naphthalene sulfonic acid has been extensively used in literature in the characterization of the partially folded states. How and why? What if we look at the molecular structure of ANS? It has a good hydrophobic component. And it also have a has a sulfonate group. So therefore, this molecule can bind to hydrophobic clusters through hydrophobic interaction over here. And also it has ability to undergo ionic interaction. And therefore, this dye or this molecule has been extensively used in this characterization of the partially folded states.

When the protein is in an in between state that is the partially folded states such as the molten globule states, where it is not completely unfolded. So therefore, the m g sate will have several hydrophobic clusters available. And the molecules like ANS can go and bind to those hydrophobic clusters. And hence this will lead to change in the fluorescence properties of the ANS. And that is why ANS has been extensively used in the characterization of the partially folded states of proteins.

Let us take a look at the comments; ANS fluorescence under nonpolar condition with increased intensity, because of the interaction of the nonpolar moiety with the nonpolar acceptances. That is why the partially folded states can be identified by fluorescence using ANS. What happens is when ANS binds to the partially folded states or nonpolar clusters, the emission intensity goes way high becomes way high. The fluorescence emission intensity of ANS for example, in this particular case where the concentration of

the additive is 0 is here and 0.5 molar of hexaflouroisopropanol induces molten globule in protein. So, this emission corresponds to those conditions where 0.5 molar hexafluoro isopropanol has been added to alpha lactalbumin, and since the protein under these conditions is in the molten globule state you see the fluorescence emission of ANS is very high.

Compare to other concentration of hexafluoroisopropanol where the protein is not in the molten globule state, either it is native 0 means 0 hexa fluoro isopropanol means the protein is in the native state here 0.5 molar molten globule state and above, that it is pushing the protein into denatured state. And that is why the comment if we relook at the comments ANS fluorescence under nonpolar condition with increased intensity and partially folded states can be identified by fluorescence.

So, not only that the ANS energy transfer because if the emission intensity or the wavelength at which the emission is observed overlaps with the absorbance the energy transfer from some amino acid residues to ANS if there is an overlap between the emission wavelength and the absorbance wavelength the energy transfer can gives information also about the conformation of a protein.

For example the wavelength at which the tryptophan amide coincides with the wavelength at which ANS absorbs. So therefore, energy transfer is possible; and since the unfolding of protein also changes the environment of tryptophan that is why the energy transfer can also be used to correlate the changes with the conformational changes in the protein. The comment what I want to make over here based upon this fluorescence observation is, that the ANS fluorescence has been used for a long time in the characterization of partially folded states of proteins. But such characterization is also qualitative in nature. In order to make the characterization quantitative means we should be able to assign the thermodynamic parameters.

Let us see some more examples. Now we will start discussing how the thermodynamics of the process can give information about the molten globule state.

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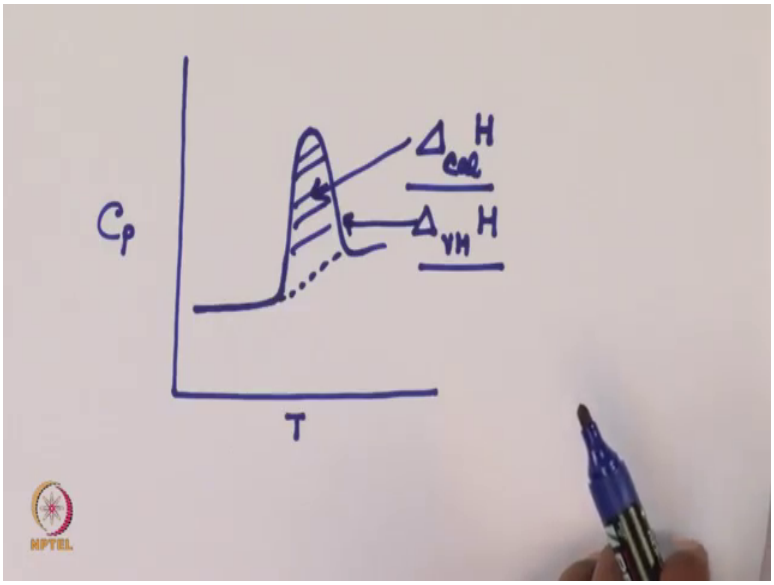

Let us take a look at a typical differential scanning calorimetric output. In a DSC what we have is C p versus the temperature. And we have a differential scanning calorimetric profile, from which we get delta H calorimetry and delta H vant hoff, these 2 are obtained right. Delta H calorimetry comes from the area under the curve and delta vant hoff comes from the shape of the transition.

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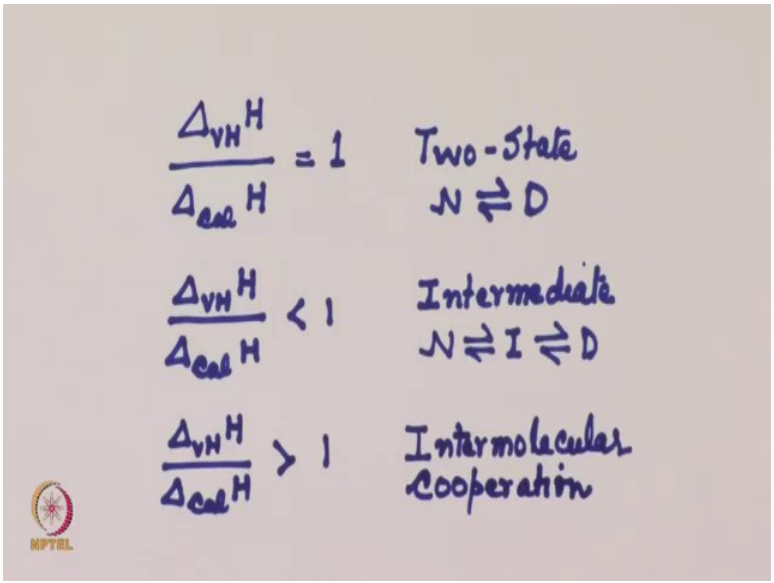

Now, this ratio of vant hoff enthalpy to calorimetric enthalpy gives information about the mechanism of the folding unfolding process. Suppose if this is equal to 1. We have

earlier discussed that if vant hoff enthalpy is equal to calorimetric enthalpy, then the unfolding process is 2 state. 2 state means either native state is significantly populated or denatured state is significantly populated. If vanthoff enthalpy over calorimetric enthalpy is less than 1; that means, there are intermediates, in the unfolding process then the mechanism is no more N going to D then there could be for example, N is equilibrium with I intermediate is in equilibrium with D. It suggest if the vant hoff enthalpy is less than the calorimetric enthalpy the conclusion to be drawn is that there are one or more intermediates appearing in the overall unfolding process.

The third possibility is vant hoff enthalpy over calorimetric enthalpy is greater than 1, and that suggests intermolecular cooperation, intermolecular cooperation in the unfolding process. What is this intermolecular cooperation? Intermolecular cooperation is that when the protein is unfolding then the 2 unfolded protein can be sought of form a dimer trimer oligomer aggregate. So, extent of aggregation will increase. So therefore, this comparison of calorimetric and vant hoff enthalpy can be used to establish whether the folding unfolding process is 2 states or it involve the formation of intermediate state or the unfolding is involving intermolecular cooperation.

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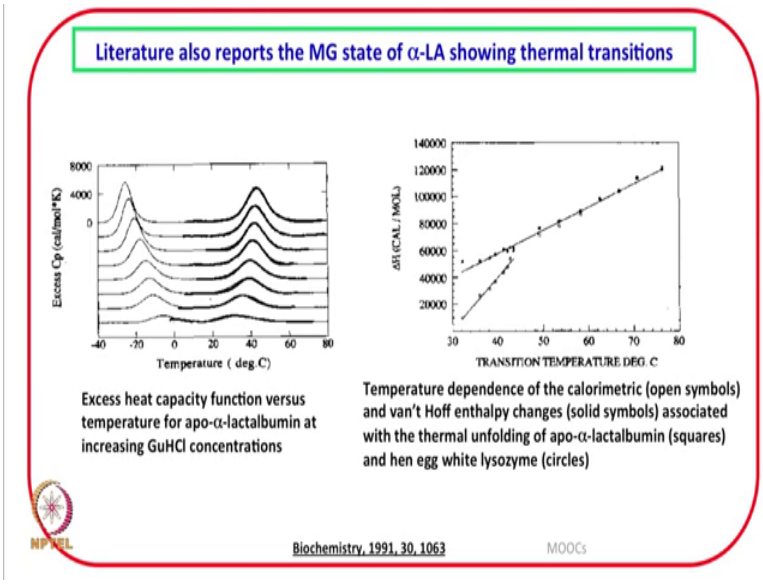

Let us take some examples from literature to discuss this in details. This figure which is adopted from biochemistry is a plot of excess heat capacity against temperature or in

other words it is a DSC thermogram for unfolding of apo alpha lactalbumin at increasing guanidinium hydrochloride concentration.

Apo alpha lactalbumin, what is apo alpha lactalbumin? Apo means you have removed the cofactor you have removed calcium from it. So, once you remove calcium from alpha lactalbumin; obviously, it will lose it is stability and it should go into an intermediate conformation in between native state and denatured state. Let us take a look at the figure. When the concentration of guanidinium hydrochloride is increased in solution, please take a look at how the area under the curve decreases and even there is a left hand side shift towards the transition temperature.

This is called denaturation; so let us not focus on this at present let us focus on heat denaturation. What they observed that when you increase the concentration of guanidinium hydrochloride. The calorimetric enthalpy decreases as well as the transition temperature decreases. They also studied the thermal unfolding of lysozyme along with alpha lactalbumin and made some general conclusions. What they observed that the calorimetric and vanthoff enthalpies for apo alpha lactalbumin in presence of various concentration of guanidinium hydrochloride are not equal.

Whereas the calorimetric enthalpy and vant hoff enthalpy for thermal unfolding of hen egg white lysozymes are equal. I repeat, what they observed is that the calorimetric enthalpy and vant hoff enthalpy for the unfolding of apo alpha lactalbumin in presence of guanidiniumchloride are not same, whereas these 2 enthalpies are same when it comes to hen egg white lysozyme. And the ratio of vant hoff to calorimetric enthalpy suggested the formation of an intermediate state.

So, based upon this nonequivalence you see the calorimetric enthalpy varies like this vant hoff enthalpy varies like this there is nonequivalence, and the nonequivalence the use this information to suggest that guanidinium hydrochloride induces the molten globule state of a protein; however, let us keep in mind that these authors suggested that the molten globule state of a protein shows a cooperative thermal transition which is smaller than the one obtained with the native state as you keep in increasing guanidinium hydrochloride concentration; the height of the peak is decreasing that area under the peak will keep on decreasing, as well as shift towards the left side.

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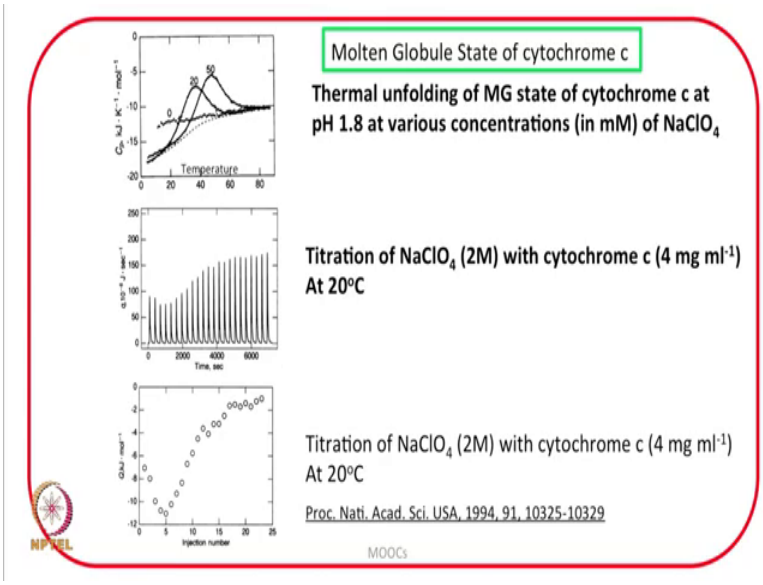

Similarly, molten globule state of cytochrome c has also shown cooperative thermal transitions in the differential scanning calorimetry. As I mentioned in one of the lectures that when you lower the pH of a protein for example, in the case of cytochrome c if you lower the pH to 1.8 that corresponds to 0 line 0 line means there is 0 molar additive in that, there is no thermal transition observed.

However, when you add 20 millimolar sodium per chloride we get a well defined thermal transition, when you increase the concentration of NaClO 4 to 50 millimolar the thermal transition temperature further increases. That means, NaClO 4 is inducing renaturation or is inducing structure in the unfolded protein. And these authors termed these states as molten globule states. And they also performed isothermal titration calorimetry on the interaction of NaClO 4 with cytochrome c and gave the information on enthalpies of interaction. As you can see in a specific manner NaClO 4 is interacting with cytochrome c.

And these kind of experiments can give the enthalpies of interaction along with some other thermodynamic parameters. We will discuss about these bit later, but let us for the time being concentrate on differential scanning calorimetry. What I have discussed is that some authors have shown that the molten globule like state show a differential scanning calorimetric profile well defined differential scanning calorimetric profile, the height of which or the transition temperature is less than that compare to the native state. But there

are some other controversial reports also available in literature. Let us discuss about those immediately after those reports. Another report appeared in literature where the performed differential scanning calorimetry of apo alpha lactalbumin and holo alpha lactalbumin.

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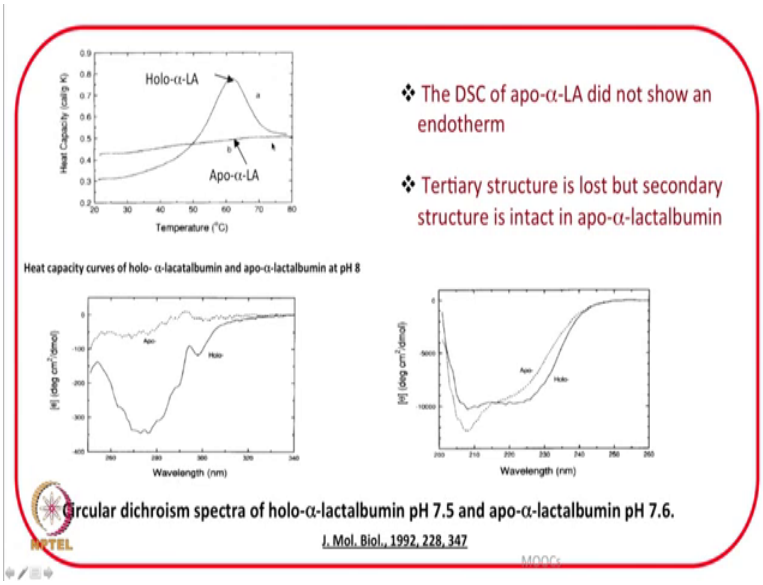

And let us discuss what they observed, let us take a look at this figure. This figures shows the DSC of holo alpha lactalbumin this well defined thermal transition corresponds to holo alpha lactalbumin and the flat line represents the DSC profile of apo alpha lactalbumin. What these authors showed that the DSC of apo alpha lactalbumin did not show an endotherm.

And simultaneously if you look at the CD of apo alpha lactalbumin, if we look at the secondary structure and tertiary structure the far UVCD and near UVCD the secondary structure of apo alpha lactalbumin is almost similar to the secondary structure of the holo alpha lactalbumin, whereas the tertiary structure of alpha apo alpha lactalbumin is almost lost where as it is intact with the holo alpha lactalbumin. That is why the comment is made tertiary structure is lost, but secondary structure is intact. So, here comes the controversy, because in this example the authors showed that the molten globule state the traditional molten globule state for which the definition adopted is intact secondary structure compared to that of the native state, and lost tertiary structure. It did not show an endotherm in the differential scanning calorimetric profile. Now let us discuss that

what leads to the formation what leads to an endotherm in the differential scanning calorimetric profile.

The folded form of a protein or the native structure is stabilized by secondary structure interactions, and the interaction which are responsible for holding the tertiary conformation intact. Secondary structure largely has hydrogen bonding. And after that in the tertiary fold in addition to hydrogen bonding you have hydrophobic interactions and some other ionic interactions. Now out of these 2 which one is a cooperative process? The endotherm which is observed in the differential scanning calorimetric output profile is a cooperative transition. And that cooperative transition is absorbed when the tertiary structure is lost. What is meaning of a cooperative process? A cooperative process is the one in which the one step facilitates the formation of the next step. In other words if one bond is broken it facilitates the breakage of the next form, next bond and vice versa.

So, when a protein is heated when it is starts unfolding. The unfolding cooperative process is due to the loss of the interactions which hold the tertiary structure of the protein intact. And if in a protein the tertiary structure is completely lost therefore, it is not expected to show a cooperative thermal transition. And based on these arguments these authors let us look at the figure, these authors claim that the apo alpha lactalbumin in which the tertiary structure you see is lost look at in the CD spectra secondary structure is intact it is not expected to show a cooperative thermal transition, whereas the holo alpha lactalbumin which has intact tertiary structure should show the cooperative thermal transition. And after this report several other papers appeared in literature, which showed that the molten globule sate in several proteins does not show a cooperative thermal transition in differential scanning calorimetric profile.

So therefore, if we do not see an endotherm in the differential scanning calorimetric profile; that means, if it is just a flat is a straight line, and then we should not conclude that the protein is already denatured, because the protein may be in some intermediate form. Let us take a look at the figure again. If we get a flat line like this then immediate conclusion should not be drawn that it is that the protein is in the denatured form. As demonstrated over here there is no thermal transition observed here, but still the protein has secondary structure which is almost same as that of the native state. But has poorly defined or lost tertiary structure.

So therefore, absence of a cooperative thermal transition in DSC should also be interpreted as that it could be the protein could be in an intermediate structure and it should be verify simultaneously by the application of spectroscopic methods, at one such spectroscopic method which gives direct information on the secondary and tertiary structure of the proteins as we discussed earlier is circular dichroism spectroscopy.

So, what we discussed in this lecture is that floriations and circular dichroism spectroscopy and I am sure some other spectroscopy can also give information about the formation of partially folded states of the protein. But most of the information which is available based on the spectroscopic methods is qualitative in nature. Now we have started discussing the quantitative aspects. First we discussed that the literature shows that the molten globule state shows a cooperative transition, but some author showed that the molten globule state which fit into the definition of no tertiary structure, but intact secondary structure it does not show a cooperative transition.

So therefore, we can interpret this controversy in the sense that a state in which the secondary structure is still intact or more than the native state, but the tertiary structure is lost is not expected to show a cooperative thermal transition. We still have to discuss that how to get more quantitative information about this partially folded states. And those quantitative thermodynamic informations give what kind of information about the common structural features or the structural features of these partially folded states.

We will discuss these matters in some details in the next lecture.

Thank you very much.