**Chemical and Biological Thermodynamics: Principles to Applications**
**Prof. Nand Kishore**
**Department of Chemistry and Biochemistry**
**Indian Institute of Technology, Bombay**

# Lecture - 44
# Further discussion on applications of DSC in thermal unfolding of proteins and protein-solvent interactions

We have been discussing the applications of differential scanning calorimetry to the systems of biological interest. We have taken a few examples and today let us extent our discussion on applications of differential scanning calorimetry in thermal unfolding of proteins and protein solvent interactions. In earlier lecturers I have highlighted the significance of differential scanning calorimetry in unraveling the nature of intramolecular interactions which hold the protein together. And I also mentioned that if we want to understand that which forces hold the protein together, it becomes easier to understand if we try to unfold and then see comment upon or try to find out what interactions were holding the protein together.

In that sense studying the effect of variety of solvents on the thermal and conformational stability of proteins has been very important. Therefore, over a large number of years scientist have been focusing on not only on understanding the contribution of different amino acids, in maintaining the conformational stability of the protein which in other words studying the mutant proteins, but lot of efforts have been dedicated towards understanding the protein solvent interactions. The proteins will definitely respond to the solvent environment, under a given solvent environment, at a given pH the protein will have some conformational stability some thermal stability the transition temperature will be some unique value.

When the solvent environment is changed for example, when the pH is changed or when we add some additives like we can add electrolytes, we can add alcohols we can add variety of cosolutes. For example, it is well known that when urea is added to protein, the protein gets denatured when the concentration of urea is higher of the order of 6 molar or 8 molar. Similarly one iridium hydro chloride will lead to denaturation of protein one iridium thus ionate will lead to denaturation of protein. Denaturation or destabilization of the native structure of protein means reduction in the thermal transition temperature. Therefore, application of differential scanning calorimetry can provide a lot of

information on how much protein is thermally getting destabilized under a given solvent environment that is at a particular concentration of the denaturant.

But there are several molecules which imparts stabilization to the protein. We will also take certain examples where the additives can provides stabilization to the proteins, but let us take today an example of how an alcohol can alter the thermal stability of the protein and hence the thermodynamic parameters associated with it is thermal unfolding.

(Refer Slide Time: 04:46)

**Thermodynamic parameters of lysozyme denaturation in various propanol-buffer mixtures at pH 2.0**

| 1-propanol (M) | $T_d$ (°C) | $\Delta H_{cal}$ (kcal mol$^{-1}$) | $\Delta H_{vH}$ (kcal mol$^{-1}$) | $\Delta H_{vH}/\Delta H_{cal}$ | $\Delta C_p^d$ (kcal K$^{-1}$ mol$^{-1}$) |
|---|---|---|---|---|---|
| 0 | 52.03 ± 0.52 | 91.2 ± 1.9 | 89.5 ± 1.0 | 0.983 ± 0.014 | 1.55 ± 0.12 |
| 0.67 | 47.44 ± 0.50 | 92.6 ± 1.6 | 88.9 ± 1.3 | 0.971 ± 0.013 | 1.24 ± 0.14 |
| 1.34 | 41.64 ± 0.54 | 95.4 ± 2.7 | 89.9 ± 1.3 | 0.945 ± 0.021 | 1.30 ± 0.15 |
| 2.00 | 33.36 ± 0.61 | 94.9 ± 0.7 | 89.9 ± 1.4 | 0.937 ± 0.015 | 1.10 ± 0.31 |
| 2.67 | 25.44 ± 0.37 | 77.1 ± 1.1 | 81.5 ± 0.8 | 1.060 ± 0.010 | 1.02 ± 0.48 |
| 3.34 | 15.69 ± 0.51 | 59.0 ± 1.7 | 64.4 ± 2.1 | 1.091 ± 0.006 | 1.13 ± 0.15 |

Alcohol: $C_3H_7OH$

N = D

Two-state unfolding

Let us go to the slide and look at the data presented in this table. The data presented in this table corresponds to the thermal denaturation of lysozyme in various propanol buffer mixtures at pH 2. Lysozyme exhibit is sufficient thermal stability at pH 2. If we look at the very first row, propanol concentration 0 means we are talking about lysozymes in the absence of any additive. And at pH 2 the thermal unfolding temperature or transition temperature is 52.03 degree Celsius. Please note here that in literature different symbols have been used to represent transition temperature T m, T half or to T d, but we should be able to recognize that these are transition temperatures.

So, in the absence of propanol the transition temperature of lysozyme is 52 degree Celsius associated with a calorimetric enthalpy of 91.2 kilo calorie per mol a vanthoff enthalpy of 89.5 kilocalorie per mol and delta Cp of 1.55 kilocalorie per mol. And when we are reporting the data it becomes very important to also associate this standard deviations with the data. And here we see the standard deviation reported in transition

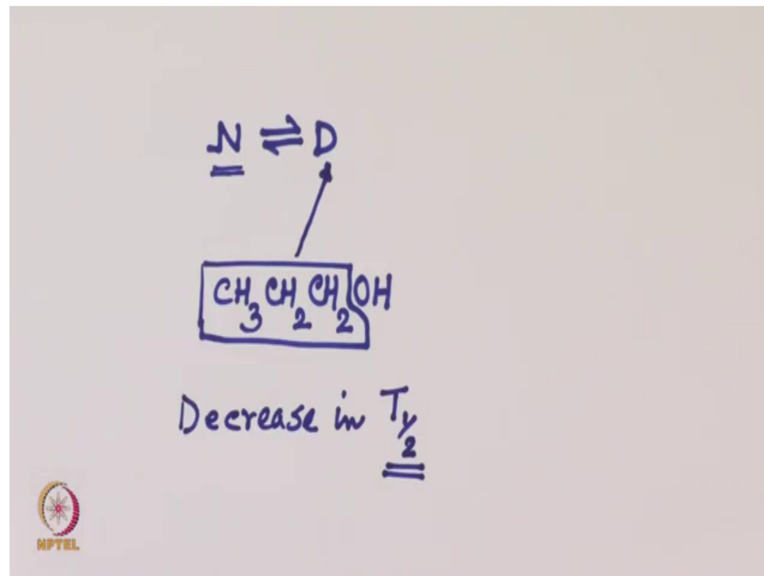
temperature is of the order of 0.52 degree Celsius and the standard deviations in other thermodynamic parameters have also been reported.

When alcohol is added now we go down the column first column, you see when the concentration of one propanol in the mixture is 0.67 molar. The transition temperature decreases from 52 to 47. There is a drop in the thermal transition temperature of the protein. And if you see the calorimetric enthalpy is slightly increased over here rather than decrease there is a marginal increase in the calorimetric enthalpy. Vanthoff enthalpy can be calculated, I will comment on the vanthoff enthalpy a bit later. And the associated change in heat capacity has also gone down a bit. And let us make some more observations in this table, that when the concentration of propanol is further increase to 1.34 molar. Although the transition temperature is again further decreasing, but the calorimetric enthalpy has shown a little bit more rise and the delta Cp value is not very largely effected.

Now, further addition of propanol in the mixture see from 2 if you take to 2.67 to 3.34 molar, the transition temperature is sharply decreasing 33.36, then 25.44, then 15.69. And from 2 molar onwards there is also a sharp decrease in calorimetric enthalpy. Whereas, the delta Cp is not very largely affected. So, the conclusion that can be drawn from this table is that one propanol D leads to thermal destabilization of the protein because the transition temperature is decreasing from 52.03 to 15.69 when the concentration of the alcohol in the mixture is 3.34 molar another observation is the ratio of vanthoff to calorimetric enthalpy is more or less close to 1 in each case.

If the ration of vanthoff to calorimetric enthalpy is almost remaining 1; that means, the thermal unfolding of the protein is obeying a 2 state unfolding mechanism. By a 2 state unfolding mechanism means either the native state is significantly populated or the denatured state is significantly populated without significant population of any intermediate state. Let us now discuss what could be the reason for the decrease in thermal stability of the protein and how the calorimetry has helped us in understanding that.

(Refer Slide Time: 11:10)




We are talking about this equilibrium; native sate of the protein in equilibrium with denatured state of the protein, when there is no alcohol present the equilibrium is in largely favour of native state. And when start adding any additive any kind of additive. That additive can interact with N and also can interact with D it is the preferential interaction which will decide in which direction this equilibrium move.

For example, if any additive interacts preferentially with N native state will shift the equilibrium towards N and it will lead to the thermal stabilization of the protein; that means, the transition temperature will increase. And if any additive which interacts preferentially with the denatured state in that case the equilibrium will be shifted towards the right and the transition temperature will decrease. Now here we are talking about propanol, we are talking about CH 3 CH 2 CH 2 OH, and if we look at the molecular formula of propanol you have a hydrophobic part attached to it CH 3 CH 2 CH 2. And now you want to decide that whether this hydrophobic group will interact more with the native state or it will interact more with the denature state.

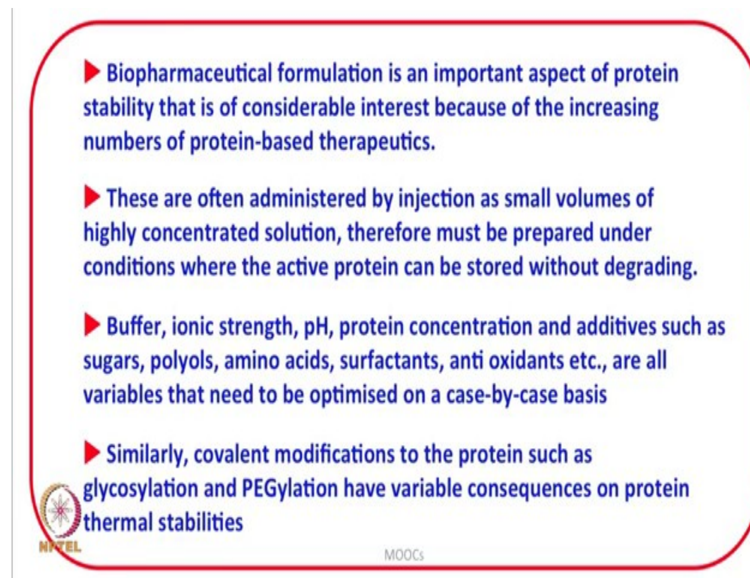
Obviously, when protein is unfolded is undergoing partial unfolding or unfolding the hydrophobic groups are getting exposed an alcohol can interact more with the denatured state compared to the native state. So, the interaction is preferentially more with the denatured state and therefore, it is predicted that the interaction of propanol with protein will shift the equilibrium towards the right side, and therefore, what we will expect is a

decrease in the value of T half. And that is what is exactly seen now let us go back to this table. And that is what we see that as the concentration of propanol increased the equilibrium is shifted more and more towards right. And hence the transition temperature keeps on decreasing and that is what is commented over here that alcohol will preferentially interact with denatured state it is interaction will be more with the denatured state. Therefore, the equilibrium will be shifted towards the right and hence you see the decrease in the transition temperature.

The data here does present some sought of complexity here. In this case because the calorimetric enthalpy is slightly increasing in increase in concentration. And this aspect I will discuss a bit later there are several alcohols which are non to disrupt the tertiary structure of the protein, but strengthened the secondary structure of the protein. Disruption of tertiary structure of the protein and maintaining or strengthening the secondary structure of the protein leads to formation of a conformation which is called molten globule state. And I will discuss about this molten globule state a bit later. So, the way the interaction of cosolute or solvent with the protein may affect the thermal transition temperature or the other associated thermodynamic parameters can initially be predicted based upon the nature of the molecules.

Or in other words the data obtained can be interpreted in terms of it possible intermolecular interaction. And we can appreciate like the data which is presented in this table that differential scanning calorimetry will quantitatively enable us to understand these interactions therefore, the applications of differential scanning calorimetry to the systems of biological interest can give very, very important further guidelines.

(Refer Slide Time: 17:02)



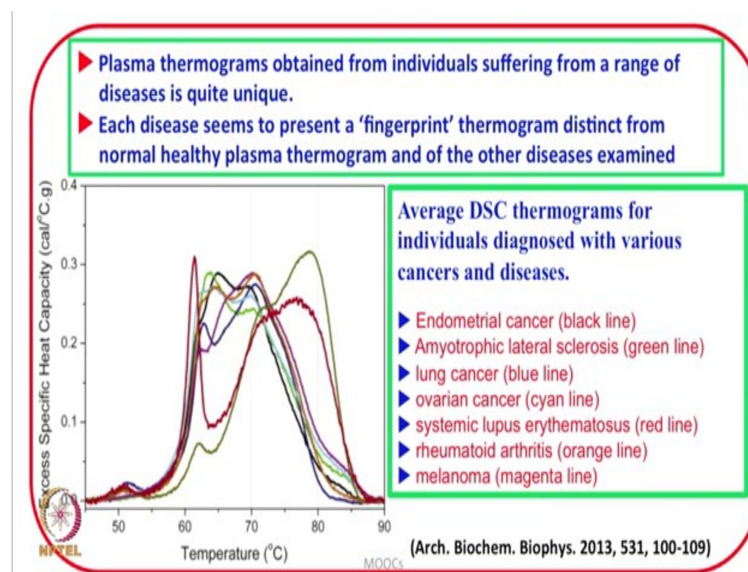

For example, biopharmaceutical formulation is an important aspect of protein stability that is of considerable interest because of the increasing number of protein based therapeutics, and these formulation are often administered by injection as small volumes of highly concentrated solution, and therefore, must be prepared under conditions where the active protein can be stored without degrading.

Let us emphasis on this word this sentence where the active protein can be stored without degrade. So, therefore, how can we check whether the protein has degraded or not how can we check whether the protein has undergone some denaturation differential scanning calorimetry provides an immediate answer to it. Buffer, ionic strength, pH, protein concentration and additives such as sugars, polyols, amino acids, surfactants, anti oxidants, etcetera are all variables that need to be optimized on a case by case basis. As we just discussed that alcohols in general will lead to decrease in the thermal stability of the protein and as we will discuss a bit later sugars polyols amino acids can even lead to increase in thermal stability of the protein, and all these changes in thermal stability can be assessed by differential scanning calorimetry in a quantitative manner because differential scanning calorimetry will not just give you the changes in transition temperature, but it will also give you the changes in the calorimetric enthalpy. It will also give you the values of vanthoff enthalpy, and we have discussed the wider significant of the comparison of calorimetric and vanthoff enthalpy.

Similarly, covalent modifications to the protein such as glycosylation have variable consequences on protein thermal stabilities. And we have already discussed about the mutation aspects. This discussion suggests that differential scanning calorimetry is not just of academic interest because it just does not give you the numbers or the signs, but it gives us many directions. For example, we just talked about the storage of the proteins we just talked about the folding unfolding of the proteins the use of proteins in therapeutics. And therefore, differential scanning calorimetry has become a very popular method of choice in pharmaceutical industries. This can also play an important role in establishing that how the proteins in humans which have varying degree of health for example, health if we compare the proteins taken from the healthy human being and a human being who is effected by some decease, how do these proteins show up when subjected to differential scanning calorimetry.

So, let me show you one example, where plasma thermograms were obtained from individuals suffering from range of deceases and what was observed is that the thermograms when compared with that of obtained from a healthy human being are different from those who are suffering from different deceases. So, then the question is that can the thermogram, which is associated with each decease can it be used as a fingerprint. In fact, in that publication those authors mentioned that each decease seems to present a fingerprint thermogram which is distinct from normal healthy plasma thermogram and of other deceases examined. Let me show you though thermograms.

(Refer Slide Time: 22:24)

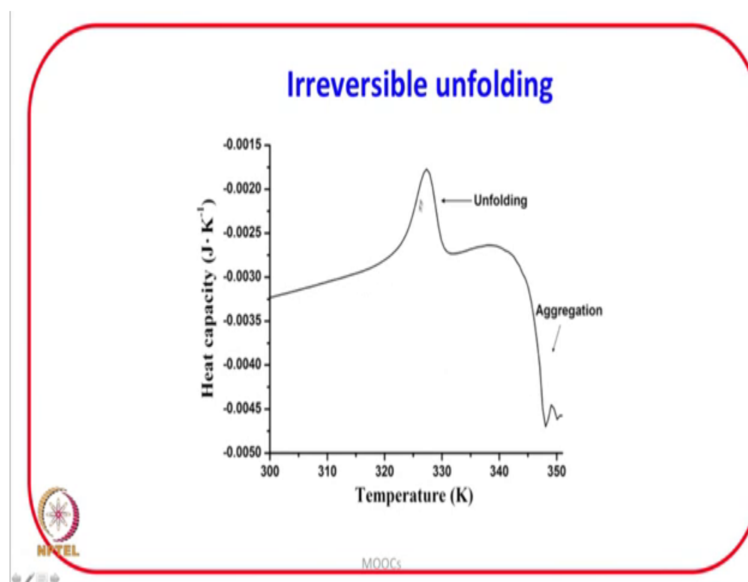


Which were obtained these are plasma thermograms in this figures from individuals varying from a healthy human being to the person suffering from different deceases for example, each thermogram is not that of a single human being the authors collected the samples from various different healthy human beings who had no deceases listed in this table and varying from 8 minimum 8 persons to more number of persons the data presented here in the form of thermogram is an average data.

Now, a person who is suffering from endometrial cancer, the thermogram is black line you see the black line is quite different from the other thermograms green line, this one green line represents the thermogram from the people who were suffering with amyotrophic lateral sclerosis. And the sample taken from the humans suffering with lung cancer the plasma thermogram is represented by the blue line and similarly if we look at the magenta line that is from the human beings who were suffering from melanoma. So, in other words, what I want to stress upon here that differential scanning calorimetry has a lot of potential in contributing to health assignees as we just saw in this figure that the plasma obtains from people who were suffering from a range of deceases shows different thermograms. So, therefore, there is a lot of potential in this technique in pointing out whether the person is healthy or is having some decease. And that is why this comment was made over here that each decease seems to present fingerprint thermogram distinct from normal healthy plasma thermogram and of other deceases examined.

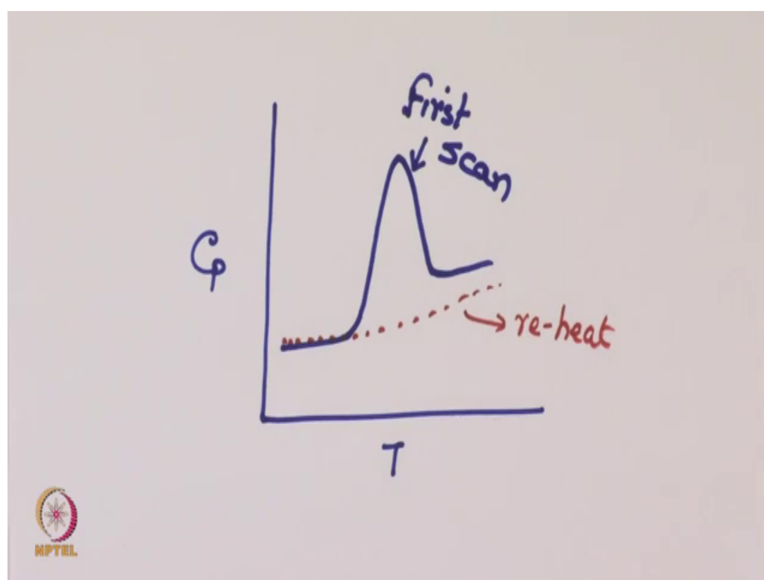
Now, let us discuss other type of thermograms which we obtain from differential scanning calorimetry and what to interpret from that. In general what we have discussed. So, far is on the reversible thermal unfolding of the proteins, but it is not always true that the thermal unfolding of a protein is always reversible. The thermal unfolding can be irreversible also many factors can contributed to the irreversibility of the protein for example, one factor can be when the protein unfolds, and if there is some deamination there is some dehydration or if there is aggregation of the protein after denaturation these are some of the factors which can contribute to the irreversibility in the protein folding unfolding, but how it is detected in a differential scanning calorimetric profile.

(Refer Slide Time: 26:28)




Let us take a look at this figure. If after the thermal transition there is a strong exothermal observe, the strong if I am calling this up overs and endotherm then the downwards will be exothermal this exotherm can begin here also that is as soon as the transition is over or it can begin little later corresponding to the temperature where the event begins. And in such cases the unfolding will more or less always be irreversible. And strong exotherm is associated with the association because association reaction is an exothermic reaction dimerization, trimerisation, oligomerization is an exothermic reaction. So, therefore, differential scanning calorimetry can also establish whether the folding unfolding is reversible or irreversible.

(Refer Slide Time: 27:53)




Because if in the first scan, let us say we have Cp against temperature and this is the first scan and if the reheat is like this. If the situation is like this, if reheat is like this; that means, your endotherm is completely disappearing, then it is hundred percent irreversible.

And let us keep in mind that the loss of equilibrium thermodynamics can be applied to the reversible system. Therefore, it is very important to establish whether the folding unfolding process is reversible or irreversible. And as I mentioned that differential scanning calorimetric can play a significant role in this. So, in this lecture we further discussed the applications of differential scanning calorimetry. There are many more applications of differential scanning calorimetry, which will keep on discussing later on case to case basis by taking various different examples and also in combination with another very important method which is called isothermal titration calorimetry and that we will be discussing in the next lecture.

Thank you very much.