Chemical and Biological Thermodynamics: Principles to Applications

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Lecture - 52
Quantitative Thermodynamic Characterization of Partially Folded States of Proteins

We have been discussing the use of calorimetry in addressing the partially folded sates of proteins. We earlier discussed that a qualitative characterization of the partially folded states can be very successfully achieved by using fluorescence spectroscopy or circular dichroism spectroscopy. Then, we also discussed that differential scanning calorimetry of the partially folded states will either give an endotherm which has a lower calorimetric enthalpy and a lower transition temperature compared to that of the native state and if the conformation of a protein strictly falls into the category of the proteins in which the tertiary structure is lost and secondary structure is intact, one may not even get an endotherm in differential scanning calorimetric profile.

Then, while discussing the application of fluorescence spectroscopy, we discussed that the molecules like ANS 8-anilino-1- naphthalene sulfonic acid have provided valuable information, valuable qualitative information about the partially folded states.

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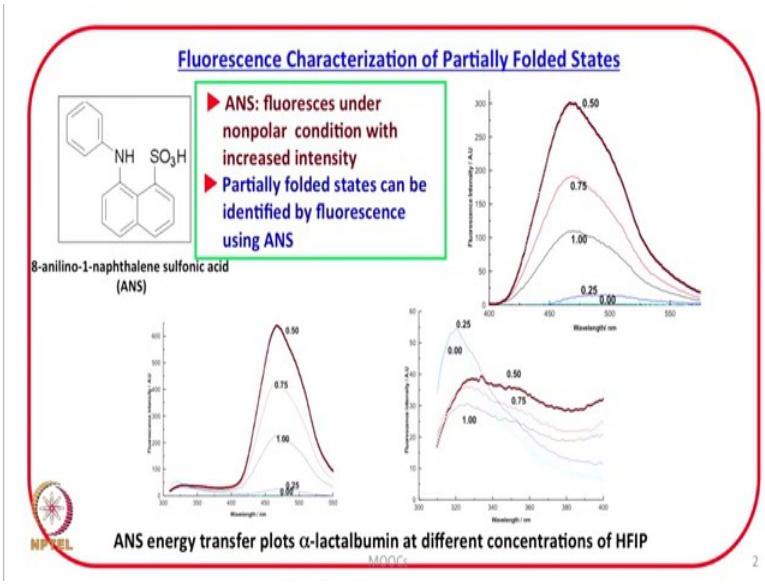

Let us now take a look at the slide. This is what I was talking about that ANS has provided good qualitative information about the partially folded states of protein. The key in its interaction was that ANS fluorescence under non-polar condition with increased intensity and this intensity increase is happening because of its binding under the non-polar environment.

Now, when it comes to the binding isothermal titration calorimetry can provide valuable information about the binding interactions because isothermal titration calorimetry in one experiment will tell you the number of binding sites. That is how many moles of ANS are binding with how many moles of the protein. It will give you information about the enthalpy of interaction, entropy change of interaction and the binding affinity when we talk about the numbers. Now, when I say N delta H delta S delta GK, we are not only talking about the signs of these thermodynamic quantities, we are also talking about the magnitude, we are talking about the numbers. That means, we are now making the process understanding in a quantitative manner and isothermal titration calorimetry is an answer to these types of questions. Let us take a look at.

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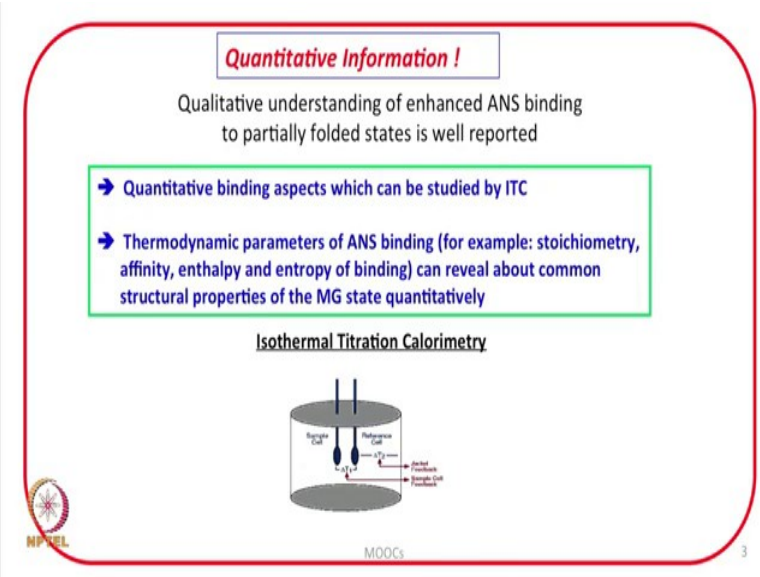

We are interested in getting the quantitative information as we just discussed qualitative understanding of enhanced ANS. Binding to partially folded states is well reported and the effort need to be made to make the process in more quantitative manner. The availability of high sensitivity isothermal titration calorimetry started giving some

answer to this. So, what kind of experiments need to be designed? First of all, one needs to generate a molten globule state under equilibrium conditions and in the previous lectures, we have discussed how to generate a molten globule state or partially folded state under equilibrium condition. We can try variety of solvent conditions and then, what can be done is, you take the molten globule state of the protein in the cell of isothermal titration calorimeter and ANS can be taken in the syringe and the interaction can be studied through titration.

Now, let us take a look at the slide. The comment is quantitative binding. Aspects can be studied by ITC and the next comment is thermodynamic parameters of ANS binding. For example, stoichiometry affinity enthalpy and entropy of binding can reveal about common structural properties of MG state quantitatively and I would rather further add if any. That is the question is the molten globule states obtained from different proteins, do these states show similar binding characteristics because that can then give information whether they share the common structural features or not. Isothermal titration calorimetry can provide an answer to such questions. Now, let me present some data from literature and start trying answering to these questions. Let us take a look at the slide.

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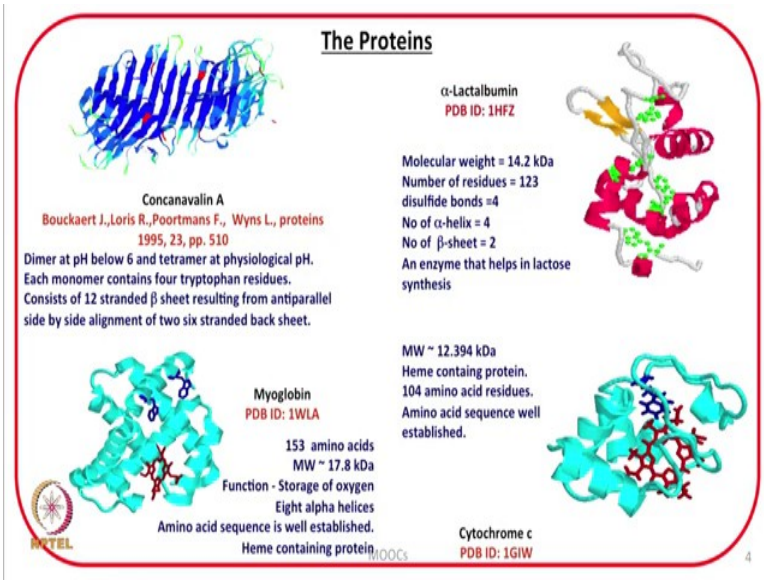

Literature suggest that the proteins like concanavalin A, Myoglobin, Cytochrome C and Alpha Lectalbamin can easily be pushed into a molten globule state under certain solvent

conditions. We have earlier discussed about the alpha lactalbumin. Alpha lactalbumin, a milk protein having calcium, it can easily be pushed into a molten globule state by removing calcium that is by changing the holo protein to an apo protein and the apo protein is also called AS states of the Alpha Lactalbumin and earlier I also took example of Cytochrome C, that is if you reduce pH3 about 1.5 or 1.8 and then, introduce some large amount anions, this protein can also be pushed into molten globule states and there are other example also with these two and few more proteins also.

Now, let us discuss isothermal titration calorimetry in identifying the molten globule state. So, what kind of experiments now can be designed? Recall the circular dichroism spectroscopic experiments or the fluorescence spectroscopic experiments. What you do in circular dichroism spectroscopy is, you keep on increasing the concentration of the additive and monitor how the secondary structure is changing, how the tertiary structure is changing and in the fluorescence spectroscopy, what we were observing is that when ANS is added to the native state of the protein or ANS is added to the denatured state of the protein, the fluorescence intensity is different than the fluorescence emission intensity of ANS when it binds to the molten globule state. Maximum emission intensity is observed, when ANS binds to the molten globule state. Now, these results suggests that we can design isothermal titration calorimetric experiments in the following way.

Experiment number 1 to study the binding characteristics of ANS with the native state of the protein. Second study, the binding characteristics of ANS with the denatured state of the protein and third study, the binding behavior of ANS with the molten globule or so-called partially folded states of the protein and then, make comparison.

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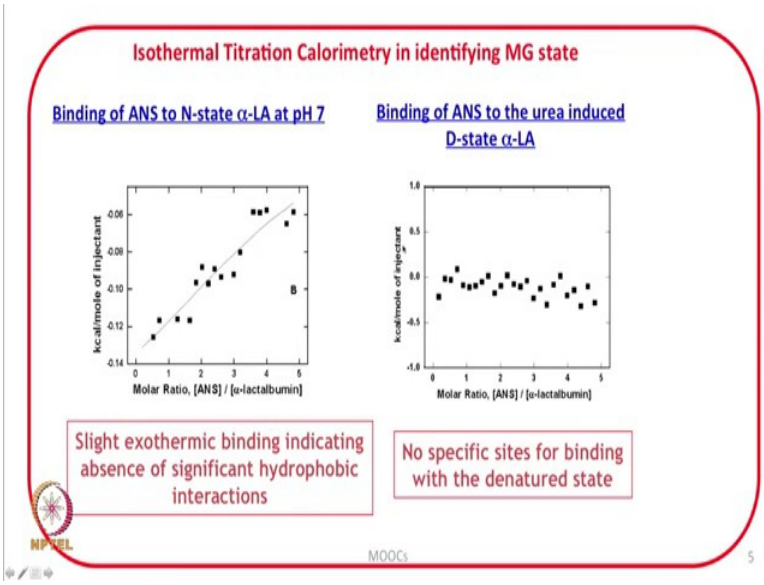

Let us take a look at the slide. This figure from literature suggests that the binding of ANS with the native state of alpha lactalbumin at pH7 is very weak.

Earlier I have discussed that what kind of shape represents weak binding, what kind of shape represents strong binding. This is the binding profile kilocalorie per mole of injectance versus the molar ratio ANS to alpha lactalbumin suggests that the binding of ANS to the native state does take place, but it is very weak. It is slightly exothermic binding and slightly it indicates absence of significant hydrophobic interactions and as I said that the next experiment that should be designed is the binding of ANS to the denatured state of a protein. You can denature the protein by adding urea. So, this figure represents binding of ANS to the urea induced denatured state of alpha lactalbumin and in this we do not observe any binding kind of behavior, any binding profile and you see the amount of heat absorbed or liberated is almost 0. That means, ANS is not showing any binding ability towards the denatured state of the protein.

Remember that all these isothermal titration calorimetric profiles which are being shown over here have been appropriately dilution corrected. The next experiment that can be designed is to study the binding of the ANS with the molten globule state of the protein. Now, keeping in mind that the fluorescence emission intensity of ANS increased many fold, when it is interacted or binding with the molten globule state. Similarly, we expect that the isothermal titration calorimetric profile should also be significantly different,

different than as seen in this figure, as seen for the binding of ANS with the native state and the binding of ANS with the denatured state.

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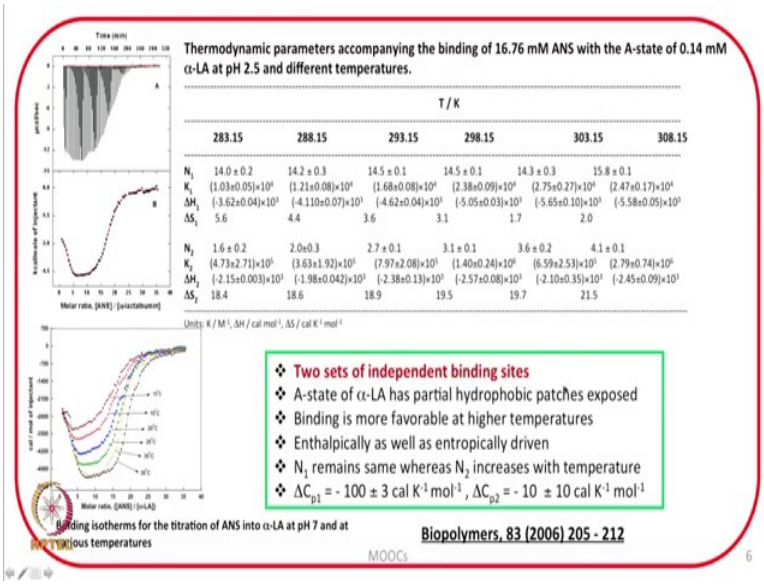

Let us take a look at what kind of profile is obtained. This figure represents the titration of ANS with a state of alpha lactalbumin or the molten globule state of alpha lactalbumin as expected. The binding behavior is different than that observed with the native state and the denatured state. We get a valley kind of isothermal titration calorimetric profile.

How to interpret these kind of profile? It is not a single sigmoidal kind of profile, but it shows a few variations. For example, initially the interaction is towards more exothermic side and then, there is a change and going towards 0. That means, towards the saturation of binding sites whenever get 1, gets this kind of binding behavior. Immediately a conclusion should be drawn that the binding is not taking place in 1 is to 1 manner. There are certainly more than one binding sites on the protein which is making the reversal in the trend of interaction and then, if one studies the binding as a function of temperature, this figure represents the binding behavior as a function of temperature. That means, a suitable analysis of these kind of isothermal titration calorimetric profiles will give you information on the change in heat capacity and as I mentioned earlier, the change in heat capacity is a very important thermodynamic quantity which will help connecting thermodynamic parameters from one temperature to another temperature and not only

that, it will give you some more information about the conformational changes which we will discuss a bit later.

Now, let us take a look at this figure. Once you have the experimental data, we need to come up with suitable binding models. The dots are experimental data points and the fit red line is a best fit to the experimental data points. It turns out in this case that a model representing two sets of binding sites fits the data in the best possible manner and one can list the various thermodynamic parameters depending upon the type of the model which you used. As I mentioned here, the data here was fitted best by a model which is according to two sets of binding sites. So, you have the values of none K1 delta H1 delta S1. That is associated with one set of binding site and then, N2 K2 delta H2 delta S2 associated with the second set of binding site and then, you have all these numbers. What one gets is how many moles of the ligand are binding with the protein, what is the strength of binding in terms of K1 or K2 form, where you can get delta G, also delta G naught and you have information on enthalpies and entropies and the signs of enthalpies and entropy along with the magnitude can be interpreted in terms of the possible intermolecular interactions.

What are the main features, main conclusions from this kind of experiment and data analysis? Let us take a look at what is observed over here that there are two sets of independent binding sites and it is already known that a state or molten globule state of alpha lactalbumin has partial hydrophobic patches exposed that is well known because any intermediate state is supposed to be in a conformation which in between the native and the denatured state. The other observation which is seen in this data is binding is more favorable at higher temperature because K value is increasing with increase in temperature and then, there is information on the number of moles of ANS binding with protein and also, you see there is information on delta CP, both delta CP1 and delta CP2 are observed to be negative. Delta CP1 is minus 100 plus minus 3 calorie per kelvin per mole and delta CP2 is minus 10 calorie per kelvin per mole.

What is the meaning of delta CP positive or delta CP negative? When it comes to binding interaction, we earlier discussed that if in a protein the hydrophobic groups are relatively exposed to the solvent in aqueous environment, then those hydrophobic groups will increase the structure of water and the heat capacity. Overall heat capacity will increase delta CP observed will be positive. Therefore, the negative delta CP in binding

interaction means when a ligand is binding to the protein and that ligand binding is inducing some conformational change in the protein which is leading to burial of some of the hydrophobic residues and since, the hydrophobic residues are getting buried, the exposure to the aqueous environment is decreasing and that is why delta CP turns out to be negative and if delta CP turns out to be positive, that means it is leading to exposure of the hydrophobic residues.

So, what we see in this experiment is that we are getting quantitative information about the binding of ANS to the molten globule state of the protein.

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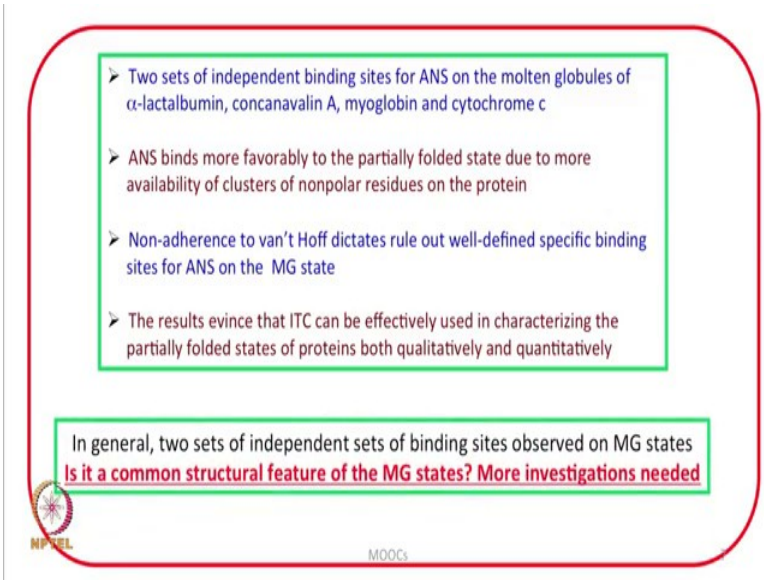

Let us see what more conclusions can be drawn from these type of experiments. One conclusion which comes from this experiment is that two sets of independent binding site for ANS on molten globules of alpha lactalbumin concanavalin A, myoglobin and cytochrome c, this comment is based upon the experiments of ANS with the molten globule states of variety of proteins.

How do we generalize some conclusions? Generalization cannot be done based upon one experiment or based upon experiments, based upon the observations, made upon the one single protein for this. The system should be tested with the variety of proteins based upon the data obtained from literature on the binding of ANS with the molten globule states observed in alpha lactalbumin concanavaline A, myoglobin, cytochrome c and few other proteins. This comment is made and the comment is two sets of independent

binding sites for ANS on molten globule states of alpha l a con A, myoglobin and cytochrome c have been observed.

The next comment is ANS binds more favorably to the partially folded state due to more availability of clusters of non-polar residues on the protein and this favorable binding is reflected in the increased binding affinity in a good value, in sufficiently high value of binding affinity. Now, once you have t equilibrium constant or binding constant as a function of temperature, the example that I just showed, the data on binding of ANS with the molten globule state of alpha lactal bumin at different temperatures. Once you have the value of k as a function of temperature, I can get Vant Hoff enthalpy and here again Vant Hoff enthalpy and calorimetric enthalpy comparison gives you information about the nature of binding interaction and whether the binding is taking place with only the native state or the binding is inducing some other intermediate state also. In this particular case, the comment made is non-adherence to Vant Hoff dictate. In this case, Vant Hoff enthalpy did not match with the calorimetric enthalpy and the non-adherence of Vant Hoff dictates ruled out well defined specific binding sites for ANS on MG state.

You remember when we were discussing the differential scanning calorimetry, we said if Vant Hoff enthalpy is equal to calorimetry enthalpy. Then, we are talking about two state mechanism and that similar analogy can be extentended over here that if the binding is only taking place with native state and not with any other conformational state of the protein, then the Vant Hoff enthalpy and calorimetric enthalpy will be same and if there are intermediate structures involved in the binding, then this equality will not hold.

Now, let us take a look at the next comment. The results from isothermal titration calorimetry certainly evince that ITC can be effectively used in characterizing the partially folded states of proteins, both qualitatively and quantitatively qualitatively and quantitatively. If i just take this particular example on binding of ANS with MG state, what we observed that the binding is taking place because the isothermal titration calorimetric output profile had a specific binding behavior and this binding behavior was missing with the denatured state and binding was very weak with the native state, that is you know if you just look at, if you just compare the shapes of the isothermal titration calorimetric profile, just comparing the shapes give you a qualitative information on in which case the binding is more tighter and then, the analysis of the isothermal titration

calorimetric profile in terms of various thermodynamic quantities makes the study more quantitatively. It gives more quantitative information.

Now, what general conclusions can be drawn from this kind of studies is, let us take a look at the slide. The examples that I presented in this lecture suggest that in general there are two sets of independent binding sites observed on MG states and since variety of proteins in their molten globule state and here, the molten globule state which I am referring to in this examples are the one's in which the tertiary structure is lost, but the secondary structure is intact.

So, the comment made over here applies to those states that in general there are two sets of binding sites on MG state for the incoming ANS molecule. So, it is a common structural feature of MG states. More investigations are needed. As I mentioned that we cannot draw conclusion based upon 1 protein, 2 protein, 5 proteins etcetera, the experiments should be done with variety of proteins in their molten globule state and also, experiments needs to be done with various partially folded states which are even not fitting into the traditional definition of the molten globule state to draw more general conclusions, but certainly making understanding the process in a more quantitative manner helps us in deriving these kind of general conclusions. Hence, these kind of studies can provide more information on the partially folded states which is important step in understanding the protein folding problem.

So, in this lecture what we discussed is that in addition to the qualitative information on the partially folded state, isothermal titration calorimetry has ability to make understanding of the process in a quantitative manner, the measurement of the various thermodynamic parameters as a function of temperature provides further information on how the binding of these dyes like ANS can affect the conformation of the protein because what is appearing here is the ANS. When it binds, it is leading to change in the negative value of delta CP. That means, it is inducing a little bit more compactness into the protein. So, these kind of examples further demonstrate the ability of the techniques like isothermal titration calorimetry in contributing the area of protein folding. We will discuss more examples of isothermal titration calorimetry when it comes to the discussion on proteins conformation or ligand binding and some more aspects in the future lectures.

Thank you very much.