**Chemical and Biological Thermodynamics: Principles to Applications**

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**Lecture - 53**
**ITC in Drug-Protein Interactions**

In the previous lectures we have discussed the principle of isothermal titration calorimetry, differential scanning calorimetry and how to design experiments on both this calorimetric techniques in details.

Then we also discussed that depending upon lot of literature over several years in the past, the guidelines have suggested that in order to engineer binding affinity in a molecule, the polar groups need to be introduced at right position. This is because the trends over a number of years have suggested that the drugs from the first in class to the best in class need to have more affinity, but the binding the recognition should be more exothermic in nature. That is why studying drug protein interaction with a variety of drugs is very important and it is expected to derive guidelines or it is expected to give information on how an existing molecule can be improved and what kind of functionality should be introduced in the novel drug molecules.

Today we will discuss the use of isothermal titration calorimetry in drug protein interactions. How to analyze the data and how the data obtained in terms of signed and magnitude give information about intermolecular interactions.

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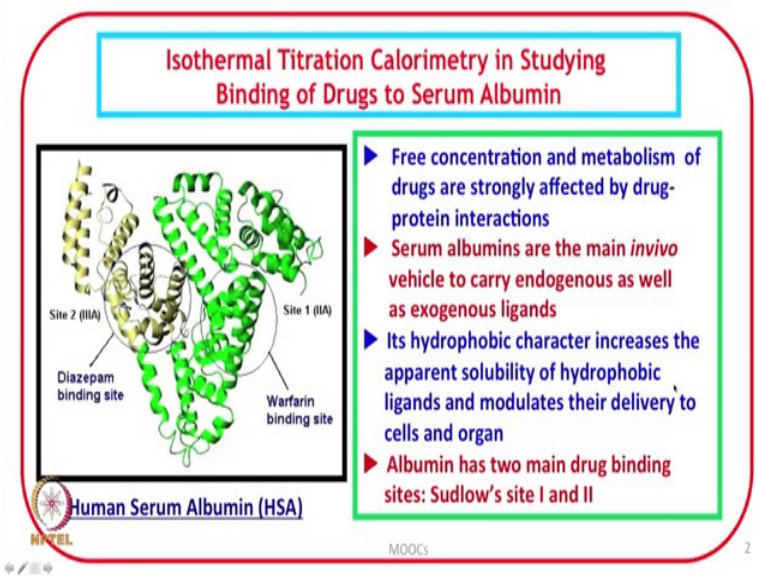


Let us take a look at this slide. To begin with let me again take the examples of serum albumin. Let us take a look at the structure of serum albumin. This is the structure of human serum albumin, and as it is structure itself suggests a variety of molecules can bind to it, and once again recalling my discussion in the previous lectures. That serum albumin human serum albumin is a main human drug transport protein in the living system. It carries a variety of drugs to the target sites. And therefore, in order for the delivery of the drug at the target site the binding affinity or the binding of the drug molecule with the serum albumin has to be optimum.

Because if the binding is very, very tight it may not be even delivered or if the binding is very, very loose it may be delivered on the way before it reaches the target sites. Therefore, tuning the binding affinity once again is very, very important. And that is the reason we need to study the binding of a variety of drug molecules with the proteins the carrier proteins So that we can get some guidelines towards the rational drug design. Now let us take a look at the figure again the comments here I have highlighted in my previous lectures, and tell about the significance of studying the bindings of drug molecules with the serum albumin. But I would like to notice the last comment once again over here that albumin has 2 main drug binding sites sudlow site one and sudlow site 2.

Sudlow site one which is also known as warfarin binding site and sudlow site 2 which is also known as diazepam binding site, because warfarin and diazepam bind at these 2 sites respectively. These are the 2 main bindings sites where variety of drug molecules have shown association.

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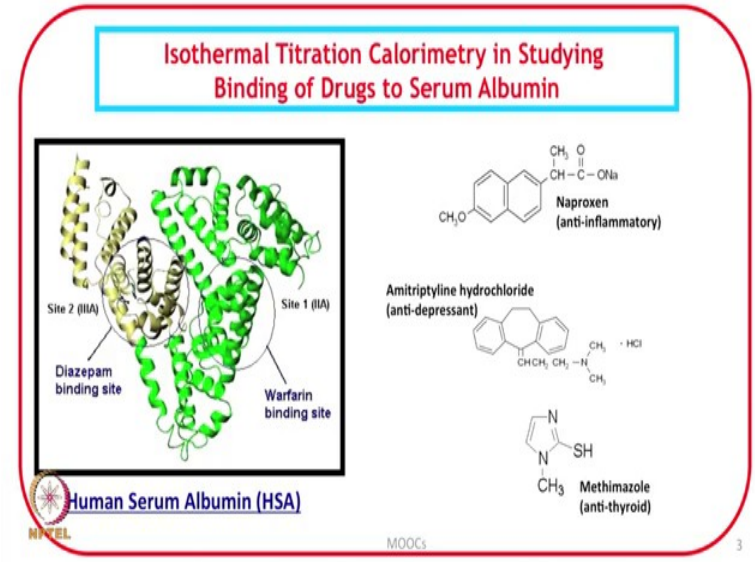


In today's lecture I will take example of 3 drug molecules. The first drug molecule is naproxen. Naproxen is a anti inflammatory drug molecule. Let us take a look at it is molecular structure. It has hydrophobic content, this part forms hydrophobic content and it also has an ionic character, because COO minus growth will impart negative charge at the end.

The second example that I am going to take is that of amitriptyline hydrochloride. Amitriptyline hydrochloride is an anti depressant drug molecule, and once again taking a look at it is molecular structure. It suggest that the hydrophobic content of amitriptyline is higher than the hydrophobic content of naproxen. And the third drug molecule that I will use in discussion today is methimazole, methimazole is an anti thyroid drug molecule. The reason for choosing these 3 molecules for discussion is because of the difference in their structure, the difference in their hydrophobic and ionic content.

Isothermal titration calorimetry, can be used in studying the binding of these drugs with serum albumin; now when we want to do an experiment on isothermal titration calorimetry. As we have discussed several times that the solutions need to be prepared in

buffer, and the buffer has to be added desired pH. So therefore, one of the requirements in isothermal titration calorimetry is that the drug molecules should have sufficient solubility.

Now if one takes a look at the literature information. Isothermal titration calorimetry has also been used in the study of binding of the drug which have very low solubility in aqueous solution. In that case several research groups have used either DMSO or chloroform in order to dissolve the drug molecules. The percentage of chloroform or the percentage of DMSO which has been used in literature, in isothermal titration calorimetric experiments varies from 5 percent to 25 percent.

A word of caution when one uses these kind of non aqueous solvent at such a large percentage in the drug binding studies is, that simultaneously one must evaluate the effect of these non aqueous solvents on the conformational stability of the protein. Why I am saying that? We must be worried about checking the conformational stability of the protein is, that if these non aqueous solvent alter the conformational stability of a protein then the integrity of the binding site will also be affected.

And therefore, it will also affect the binding ability of the protein molecule. So therefore, if the drug molecule has sufficient solubility in aqueous solution it is well and good, but if one has to use some percentage of non aqueous solvent in making the solutions care must be taken or simultaneously one must check that the conformational stability of the biological macromolecule under investigation is intact in that percentage of the non aqueous solvent.

However let us now come back to this slide, all these 3 drugs naproxen sodium salt, amitriptyline hydrochloride and methimazole had sufficient solubility in buffer to permit isothermal titration calorimetric experiments accurately. Let us first discuss the interaction of naproxen with serum albumin. So, I am will be discussing this example form the literature, in which the isothermal titration calorimetry yielded the data given in this table.

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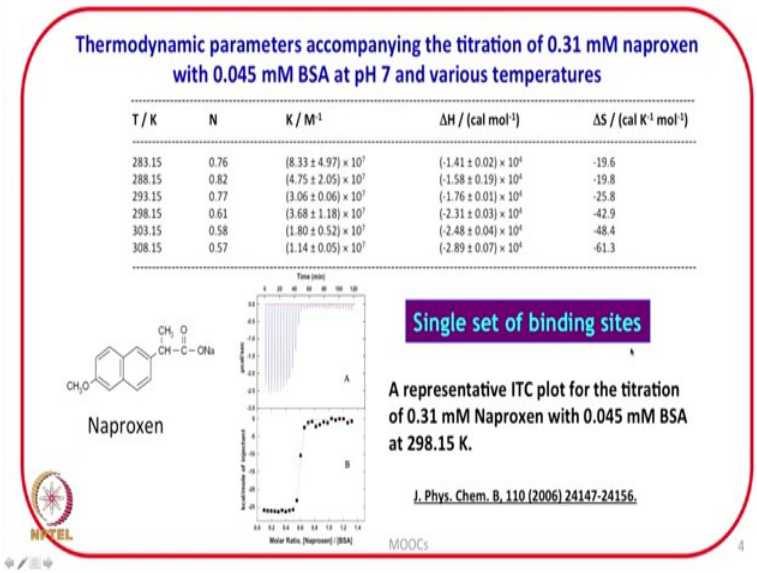


In the design of this experiment naproxen at a concentration of 0.31 millimolar was taken in the syringe. And bovine serum albumin at 0.045 millimolar was taken in the cell. Many times the protein is expressed in terms of micromolar or millimolar, and several researchers like to express the protein in terms of milligram paramol.

So, with the knowledge of the molecular weight of the protein one can convert the molarity into milligram paramol. And this was studied at p H 7 at various temperatures. When you look at the data which is obtained by use of isothermal titration, calorimetry the isothermal titration calorimetric profile is shown in the following figure. Even if one does not take a look at the table to begin with if one just looks at shape of the isothermal titration calorimetric output.

It is very clear that the binding is highly cooperative and the binding is very tight because you see the slope here is very high, the binding is very, very tight. And that is what is seen in this table if you see if I pick up the data at t equal to 298.15 kelvin which is 25 degree celsius. The binding of naproxen with serum albumin at 25 degree celsius is an companied with an n value of 0.6 k value of the order of 10 raise to the power 7, that is what I was commenting upon 10 raise to the power 7 is high binding.

It is also associated with an enthalpy of minus 23.1 kilocalorie per mole. And an entropy change of minus 42 calorie per kelvin per mole. K value which is of the order of 10 raise to the power 7 suggest. That the binding is tight the binding enthalpy is exothermic in

nature and entropy change is also negative. Now let us discuss about what could be the meaning of these thermodynamic data. First quantity that will listed in this table is the temperature, the second is n, n is the stoichiometric number stoichiometric ration. And as I discussed in one of my earlier lectures that if all the concentration all the protein molecules in the cell active volume of the cell are active, then n value can be taken as the stoichiometry of the reaction.

If n is equal to 1 that is one mole of the drug molecule binds with one mole of the protein one is to one ration. If n is equal to 2 according to a single site binding model; that means, 2 molecule or 2 moles of the drug binding per mole of the protein. And if n is equal to 0.5; that means, one drug molecule is shared between 2 protein molecules. Once again first of all we have to establish that whether n is equal to or n can be taken as the stoichiometric number stoichiometric ratio. As I said that can only be taken if all the molecules in the cell active cell volume of ITC are fully active, and then appropriate interpretation can be assigned to the value of n. The second thing which we observed in this table in the binding of naproxen to serum albumin is the delta H is negative and negative value of delta H clearly demonstrate clearly suggest that the interactions are polar in nature, this could be a combination of ionic interaction and hydrogen bonding, these are the possibilities which is also supported by the negative value of the change in entropy.

Now let us go back to the slide. And take a look at what more we can discuss based upon this example taken from the literature. Let us keep in mind the molecular structure of naproxen which will help us in understanding the further discussion, now it is important as I said earlier to choose a suitable binding model and the data which is presented in this table is based upon the fitting of a single set of binding sites model.

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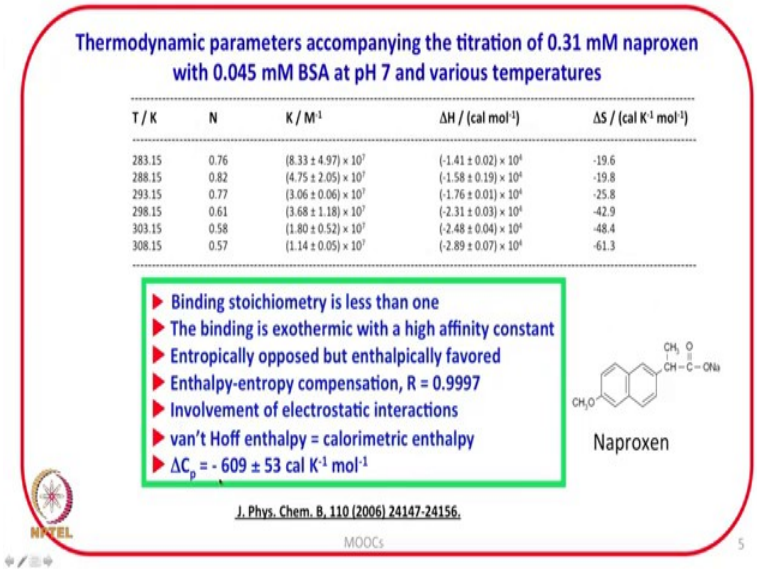


Now, let us take a look at what kind of observations are made and what of kind of conclusions can be drawn. Let us take a look at binding stoichiometry is less than 1. Now if the binding stoichiometry is less than 1, then appropriate interpretation must be assigned.

Second binding is exothermic with a high affinity constant, improving the affinity has been a priority in industry. And binding is exothermic means it establishes it demonstrates that the interaction of this drug at the binding site involves polar interactions. Third one entropically opposed and enthalpically favored; that means, when you talk about the standard reaction gives energy change that is having a favor from enthalpy change, but entropy change is opposing the negative sign of delta G naught. Next comment enthalpy entropy compensation is completely observed in this data that is when you plot delta H against t delta s, you have linear regression coefficient of 0.9997 suggesting that there is a good enthalpy entropy compensation.

The next comment is involvement of polar or electrostatic interactions are suggested which is based upon the negative value of delta H. Vanthoff enthalpy is equal to calorimetric enthalpy, how do you calculate vanthoff enthalpy? We use this temperature dependence of binding constant and calculate the vanthoff enthalpy. In this particular case what was observed is the vanthoff enthalpy calculated from the temperature dependence of binding constant is equal to calorimetric enthalpy at a given temperature.

And that means, this establishes that the binding is taking place only with the native state of protein. The next comment delta Cp is minus 609 plus minus 53 calorie per kelvin per mole. This also I touched upon one of my previous lectures. That when you study the binding as a function of temperature, we also get another thermodynamic quantity delta Cp. And in this case delta Cp is minus 609 calorie per kelvin per mole; that means, the binding of naproxen to serum albumin is leading to strengthening of the protein structure protein conformation strengthening, in the scenes that the hydrophobic groups are slightly getting buried leading to a negative change in the value of heat capacity.

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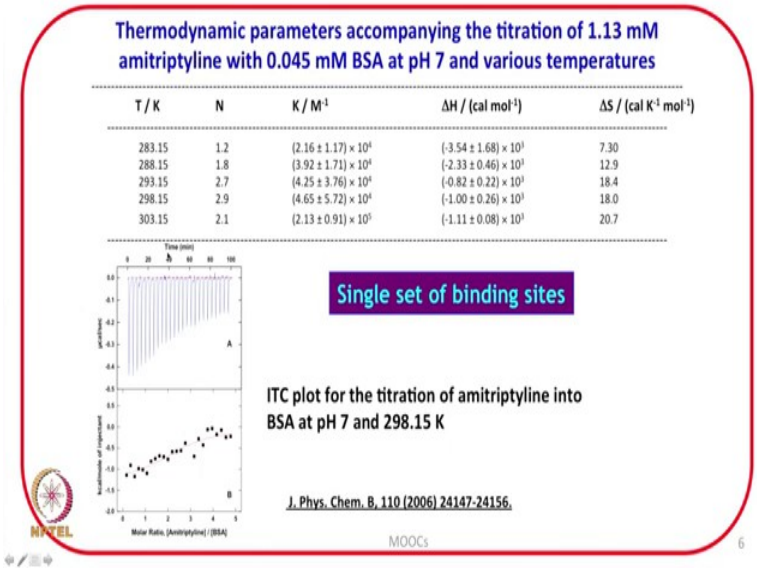


Now, let us take another example, example of the drug amitriptyline. And the conditions which were use for designing this experiments are listed in this title that is the protein concentration was forty 5 micromolar or 0.045 millimolar and the amitriptyline concentration was 1.13 millimolar and the observed isothermal titration calorimetric profile is shown on this figure. Now first thing is again as discussed earlier that even if we ignore the numbers given in the table we just take a look at the figure, the ITC output clearly suggest that the binding must be weak because the cooperativity in binding isotherm is very, very weak. And then if you look at the table the values reported in the table the k values are of the 10 raise to the power 4. In case of naproxen we observed the k values of the order of 10 raise to the power 7 and the binding profile was very cooperative, here it is not that cooperative and the binding affinity is of the order of 10 raise to the power 4.

At the same time if we know delta H though the values are negative, but the extent of exothermicity is also less now compare to naproxen. Whereas, delta s has now become positive in the case of naproxen the values of delta s were observed to be negative. Once again here a single set of binding sites model was used to extracts these thermodynamic quantities.

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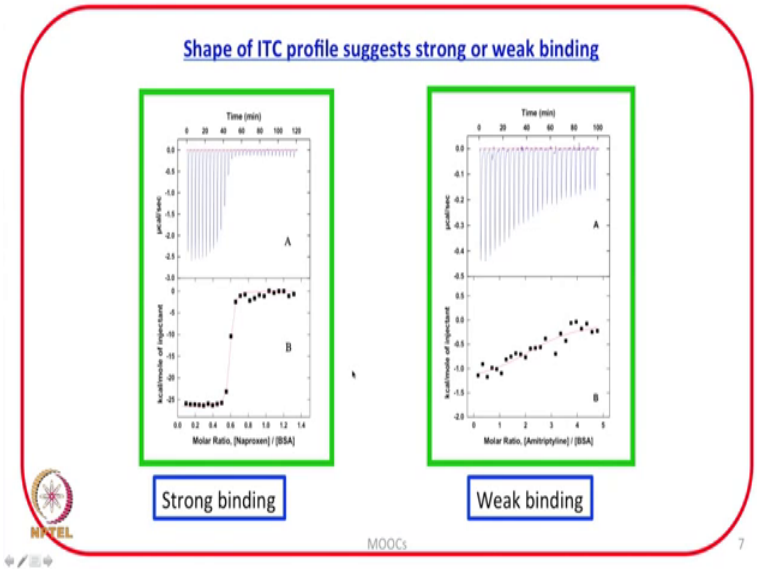


Let us take a look at the next slide. This was the shape observed with binding of naproxen with serum albumin, clearly suggesting that the binding is very, very strong. And this was the shape observed with the binding of amitriptyline, and you see the cooperativity the cooperativity is high over here the cooperativity is low over here; obviously, this is strong binding this is weak binding. So, just by looking at the shape of the ITC profile the first immediate conclusion that comes in that in which case the binding is strong and which case the binding is weak.

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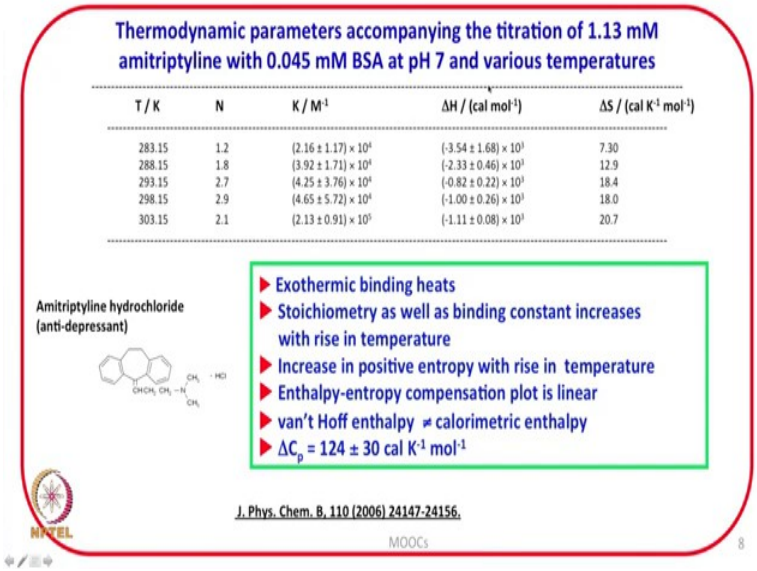


Let us take a look at the next slide. For amitriptyline the binding constant is of the order of 10 raise to the power 4. Enthalpy of binding is much less exothermic. So therefore, what conclusions we can draw conclusion number one, yes the exothermic is binding the binding is exothermic although the extent of exothermicity is small. Next stoichiometry as well as binding constant increases with raise in temperature therefore, suitable interpretation should also be applied or given to it. At the same time there is increase in positive entropy with raise in temperature. Entropy is entropy changes positive. Enthalpy entropy compensation plot in this case is also linear; however, the vanthoff calorimetric enthalpy equality was not observed in this case and delta Cp is one 24 plus minus thirty calorie per kelvin per mole.

Now, if you take a look at the molecular structure of amitriptyline there is a sufficient hydrophobic content in this molecule. And therefore, because of this hydrophobicity the binding site is not able to establish sufficient polar interactions to give a large value of negative delta H. And if the extent of polar interactions in the binding site are not sufficient, then the hydrophobic interactions or the contribution of the hydrophobic packing in the binding site will over way. In this case although if we look at the table although the net effect is still negative; that means, there is a compensation to a larger extent of the polar interactions by the hydrophobic component. And that is also seen in the positive values of delta s.

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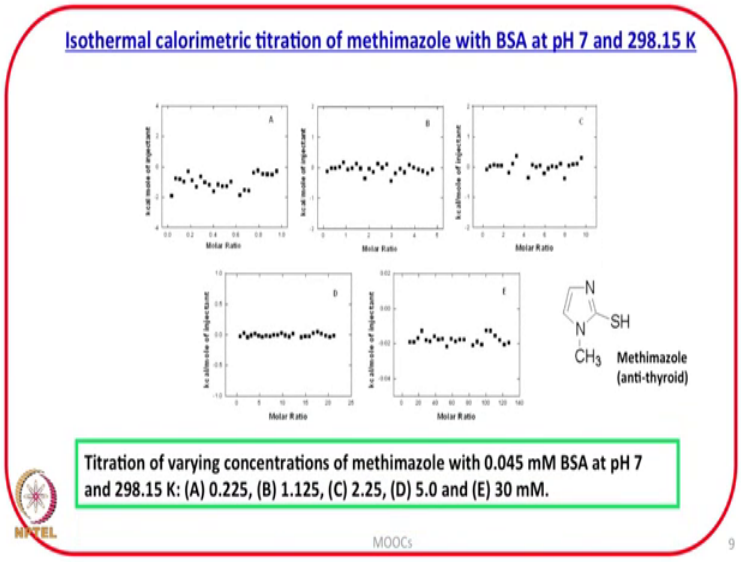


Now, let us take a look at the third drug methimazole. The isothermal titration calorimetric profiles of the interactions of methimazole which is an anti thyroid drug did not show any cooperative ITC profile did not show any cooperative change in the heat profile. It is just flat, which clearly suggest that this drug is not binding now here again let me highlight that rarely it this can also happen that one may not see any change in the heat profile in isothermal titration calorimetry.

That is the delta H is zero, but if delta H is equal to 0 can still the binding take place can still the value of delta G naught be negative answer is yes, delta G naught can be negative as long as the delta H naught and delta s naught together impart a negative value to delta G naught if delta H naught is zero; that means, delta s naught should be sufficiently positive should be positive to make delta G naught negative this can also rarely happen.

Never the less let us take a look at this figure this suggest that the methimazole does not bind to serum, albumin whatever concentration of methimazole will take. And then what was observed in this report is that even if you partially unfold, now what was tried in this report is that the conformation of serum albumin was slightly loosened by adding some additive, that is by adding some surfactants, with an idea that if you add surfactant at certain concentration the conformation become slightly loose and it may offer some binding site to the incoming molecule.

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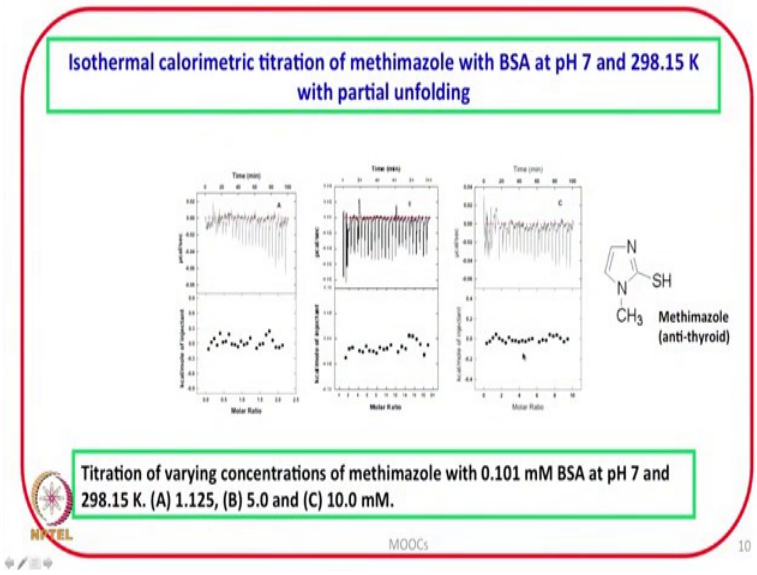


Let us take a look at the slide. What was observed is that even if be surfactant was added at different concentrations, still the binding observed was almost nil, there was no change in the binding profile.

These results suggest that methimazole which is an anti thyroid drug does not bind to serum albumin therefore, serum albumin is not a good transporter or carrier of this drug in the blood (Refer Time: 30:35). So, what we have discussed in this lecture? Is that the data which is obtained from isothermal titration calorimetry, with a variety of drug molecules. First of all the shape itself will clearly give in an indication of the relative strength of pointing. And second fitting suitable binding models will give the thermodynamic data, which help in explaining the mechanism of binding, by mechanism of binding means we establish how many moles of drug are binding to how many moles of protein.

And whether the interaction of the ligand or drug molecule with the protein is taking place majority of polar interactions or nonpolar interactions that gets establish by the values of delta H naught along with delta s naught. And delta Cp naught gives information about that when the drug is binding to the protein whether it is leading to partial stabilization of the conformation of the protein or relatively a little partial destabilization of the conformation of protein. So, here again it is established that the

thermodynamic signatures obtained from the interaction of drug molecules with the protein give a lot of information and help in establishing the mechanism of interactions.

In the next lecture we will discuss how to design experiments to further confirm whether the interactions are ionic in nature polar in nature hydrophobic in nature or involve hydrogen bonding. We will discuss all these details in the next lecture.

Thank you very much.