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

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**Lecture - 57**
**Estimation of Binding Constants in Strong to Ultratight Protein-Ligand Interactions Using Differential Scanning Calorimetry**

This lecture is a continuation of the previous lecture in which we were discussing estimation of binding constants in strong to ultra tight protein Ligand interactions using differential scanning calorimetry and since this lecture involves lot of derivations; part of the derivations were covered in the previous lecture and rest of the discussion, we will continue in this lecture.

So, just as a recap to what we did in the previous lecture was that; we discussed that the optical methods or isothermal titration calorimetric methods are very good in a certain range for the determination of binding constants and when it comes to determination of the binding constants of the order of 10 raised to the power 20; 10 raised to the power 30; 10 raised to the power 40 or even higher; then those methods cannot be applied.

Differential scanning calorimetry offers a very good mode for the estimation of the binding constants in this high range associated with the binding of ligands with the biological macro molecules. With this background, we started discussing 1 is to 1 binding mode interaction and developed certain equations. Now let us take a look at the slides.

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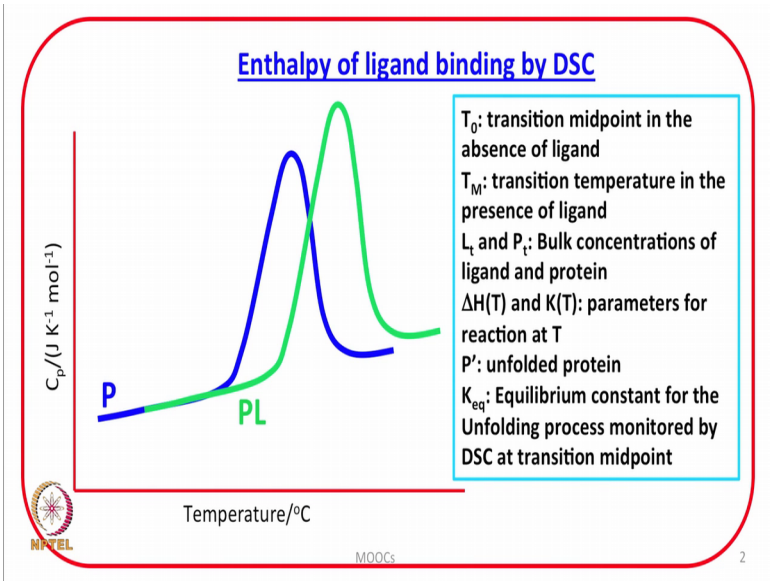


So, what we discussed was that we should thermally unfold a protein and as well as thermally unfold protein ligand complex; by the use of differential scanning calorimetry and obtain various thermodynamic parameters such as transition midpoint in the absence of the ligand transition temperature in the presence of ligand; total ligand and total protein concentration that we already know and we defined the delta H T and K T are the parameters for a reaction at temperature T and prime; we used for the unfolded state of the protein.

And wherever we used equilibrium as a subscript we said that it will be equilibrium constant for the unfolding process monitored by DSC at transition midpoint, then we went ahead and used the Van't Off equation 1 can use any of these 2 forms to arrive at the final 2 equations which are mentioned in this slide.

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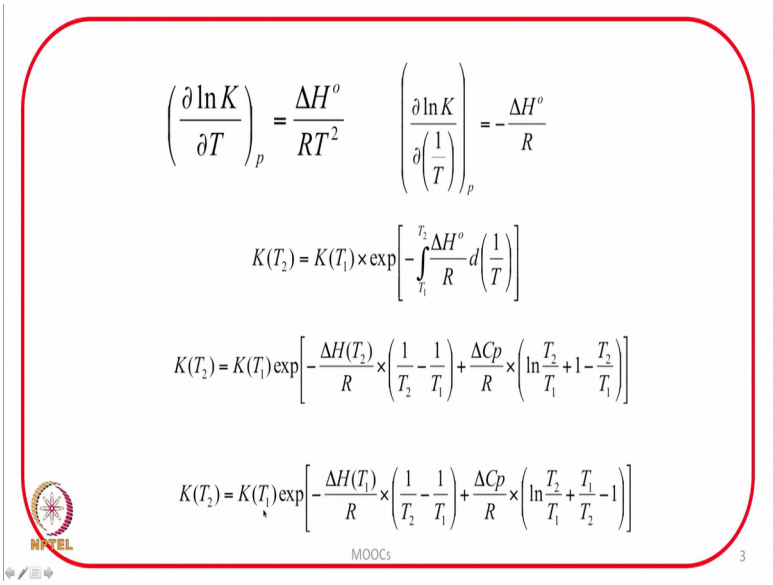


And these equations basically connect the K at a certain temperature with the knowledge of a K at a reference temperature or in other words K at temperature T 2 with K at temperature T 1 with the knowledge of the enthalpy change at that reference temperature and the corresponding heat capacity change along with the temperatures 2 and temperatures T 1.

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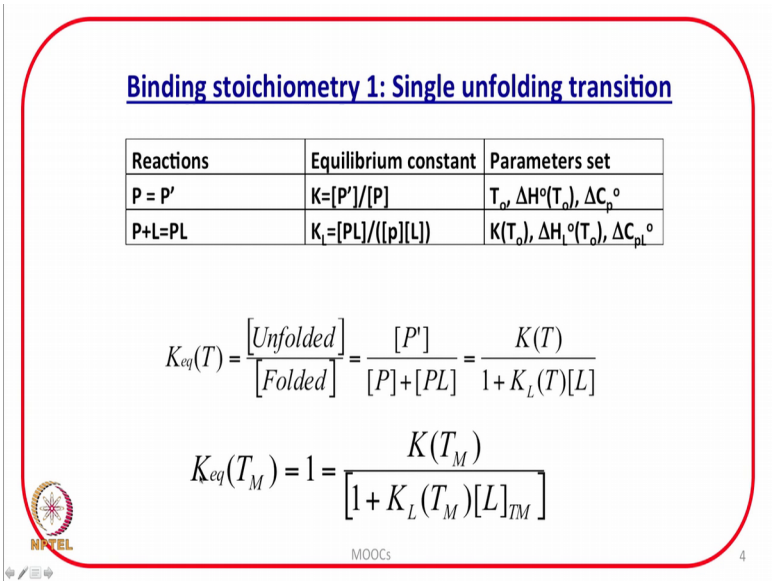


These; we discussed in the previous lecture, then we started with the binding stoichiometry for a single unfolding transitions and introduced these equations along

with the expressions for their equilibrium constant and parameters set and we arrived at these 2 equations for K. K equilibrium at a given temperature T and K equilibrium at T M which is equal to 1 and then finally, we also came up with an equation which is for K L T M and that K L T M that is the binding constant that we are interested in because K L T M can further be connected to K L at any temperature T.

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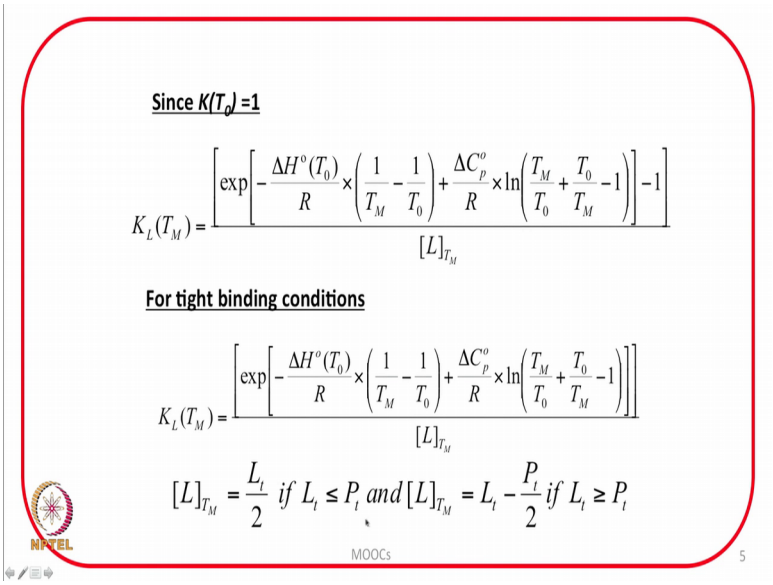


Now, let us discuss further since we know K T 0 is equal to 1; why K T 0 is equal to 1 because T 0 is the transition midpoint and if the unfolding is 2 state then the value of K at transition midpoint is equal to 1; that we have discussed several times. Now when we choose T 0 as the reference temperature, please listen to this carefully, if we choose T 0 as the reference temperature and substitute this K T 0 equal to 1 in one of those 2 equations; which we just discussed; the equations which connect K T 2 with K T 1.

Now here K T 1 will be K T 0 and we will substitute equal to 1. So, what we will get K L T M is equal to this expression; this is by using one of those 2 equations. Let me just go back and highlight that by using one of these 2 equations and also by using this equation what we arrive at is K L T M is equal to exponential of minus delta H naught T 0 divide R into 1 by T M minus 1 by T 0 T M is the transition midpoint in the presence of ligand T 0 in the transition midpoint in the absence of ligand delta C P 0 is the change in heat capacity and this concentration of L at T M is the concentration of the ligand at transition midpoint.

Now, here we are talking about the binding which is very strong the binding constants which are of the order of let us say 10 raised to the power 20; 10 raised to the power 30 to 10 raised to the power 40; that means, the binding is relatively strong if the binding is strong; obviously, the transition temperature transition temperature of thermal unfolding in presence of ligand and associated bindings will also be high. So, what is observed if the binding is very very strong the; let us take a look at this slide this entire quantity within this bracket is much higher than minus than 1. So, 1 can be easily ignored because exponential of this factor is going to be much higher than minus 1. So, therefore, if you ignore minus 1 for tight binding conditions we end up with this relation K L T M is equal to all these factors.

Now, let us move ahead; let us look at these conditions. According to these conditions, the concentration of ligand at transition temperature is total ligand concentration divided by 2; if the ligand concentration is less than the total protein concentration.

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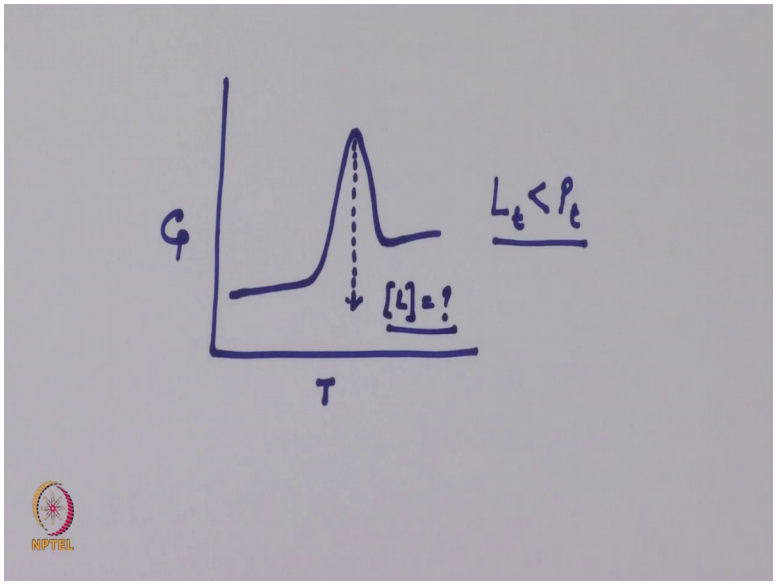


How do we interpret that? Let us say; we have; this is the thermal unfolding of the protein in the presence of ligand. Now if total ligand concentration is less than total protein concentration; that means, there is no more ligand freely available because the binding is very tight; no more ligand freely available in solution. So, therefore, when we have a transition temperature right K; K is concentration of denatured state divided by the concentration of native state right and therefore, if we write about the concentration

of the ligated complex at denatured state and at the native state; obviously, both the concentrations are going to be half and half.

And that is why the comment made over here that total ligand concentration at transition temperature the; at transition temperature is equal to the total ligand concentration in solution divided by 2; that is we are talking about; let me go to this slide; we are talking about; what is the concentration of ligand at this point; obviously, because K is equal to 1 and therefore, the concentration of the ligand at T M is also expected to be half of the total ligand concentration.

Now let us take a look at the next comment that total ligand concentration at T M is equal to L T minus P T divided by 2; if total ligand concentration is much higher than protein. Now here the concentration of protein is much lower and it is fully complexed and therefore, when you again allow the protein to unfold; again the transition temperature case going to be equal to 1 and therefore, the concentration of the ligated form denatured form and concentration of ligated in the native form are going to be same.

And since here the protein is in small amount, it is fully complex. The total ligand concentration is going to be total ligand concentration at transition point. Let us take look in the slide; is going to be total ligand concentration in solution minus total protein concentration and solution divided by 2 if L T is greater than or equal to P T; I repeat the ligand concentration at transition midpoint is L T by 2 L T is the total ligand concentration in solution if L T is less than or equal to P T and the concentration of ligand at T M is going to be L T minus 0 T by 2 if the total ligand concentration in solution is greater than or equal to the total protein concentration in solution.

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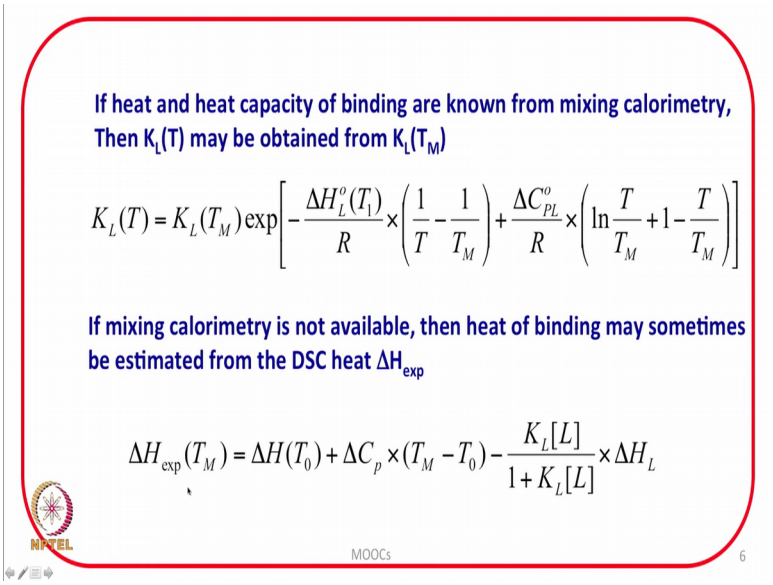


Now, if the heat capacity of binding is known from mixing calorimetry, then K L T can be determined form K L T M. This is the comment; how it can be determined. Let us take a look at this again; I am using one of those 2 derived equation where the final temperature I am keeping as T and initial temperature I am keeping at T M and we are applying this for the ligand binding. So, K L at a temperature T is equal to K L at a temperature T M into exponential minus delta H naught L at T 1 by R into 1 over T minus 1 over T M plus delta C P L in standard state divided by R into log T by T M plus 1 minus T by T M.

Now, the question is for this; we need to know the enthalpy of binding and heat capacity of binding and if these 2 quantities are known from mixing calorimetry; suppose if we do not have you know; the instruments which give both the K and delta H very very accurately, but we have an instrument like I T C can give you very correctly the value of enthalpy of ligand binding, even if the binding is very very very strong. So, if we can determine delta H L from there from mixing calorimetry and delta C P; obviously, can obtain if you determine the temperature dependence of delta H L, then we can fix over here, but suppose if missing calorimetry is not available, we do not have an I T C and we have only a DSC then how do we get let us take a look at the equation.

If the mixing calorimetry is not available, then heat of binding may sometimes be determined from DSC and how we get delta H? This is what we are interested in delta

either we say exp or we can write H L no problem this equal to the written equation how it is let me explain that in one of the previous lectures we discussed.

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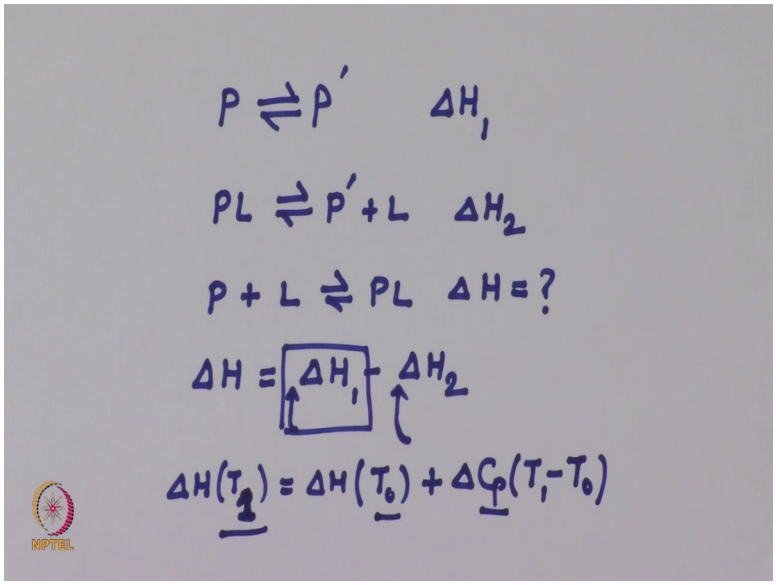


That if you thermally denature a protein; let us say the corresponding value of the delta H is delta H 1 and if I take P L and we form it P prime plus L then I say delta H 2 and if I am interested in the enthalpy of this reaction; then this is simply delta H is equal to delta H 1 minus delta H 2, but here you remember that we have to multiply by the fraction of the protein; which is in the denatured state or fraction of the protein which is ligated; if we are assuming that completely denatured; completely ligated, then we put 1 over here otherwise it has to be accounted for.

Now, for this I can write delta H at temperature whatever it is here, if I am saying in the absence of ligand then it will be equal to delta H at another temperature or let me put this as T 1 that will be easier if I put T 1. This will be at delta H T 0 plus delta C P into T 1 minus T 0. So, at any temperature, we can connect it the enthalpy with the reference temperature and delta C P. Now let us go back to the slide and take a look at this is what is done in this part; just take a look at delta H T 0 plus delta C P into T M minus T 0. This is delta H 1 and for the ligand binding part when we talk about the unfolding of the ligand bound protein then the fraction of the protein which is ligated is K L into concentration of L plus divided by 1 plus K L into concentration of L multiplied by the enthalpy of ligand binding.

Let me go back to this slide; I was saying that we need to put fraction over here and fraction over here and here we are assuming that the protein is completely unfolded and here.

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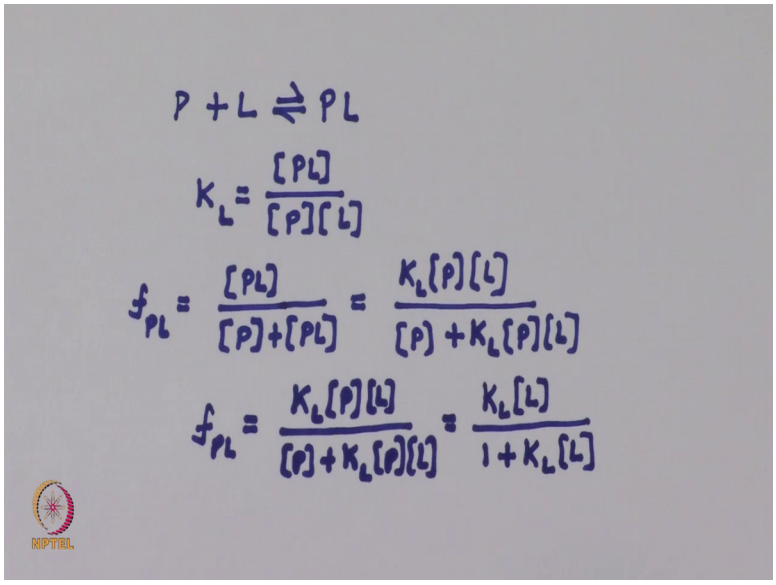


We need to put the protein; the unfolding of the protein; the fraction which is ligated; how do we get that? We have P plus L is forming P L and K L is equal to concentration of P L divided by concentration of P into concentration of L. How do we calculate the fraction of the protein is ligated? If I represent that as f P L; this will be P L divided by concentration of P plus concentration of P L because this is the concentration of protein which is ligated. This is the total concentration of protein whether it is in free form or in the ligated form.

So, this is equal to for P L; I can write from here; this is K L into P into L; this, I am using P L from here substituting over here and down there P plus again for P L, I will choose to write from here K L into concentration of P into concentration of L. Now, I can get rid of concentration of P which is a common factor over here. So, fraction of the ligated protein is equal to then K L into concentration of P over; let me rewrite again P plus K L into P into L. Let us remove P. So, it is K L into L over 1 plus K L into concentration of L.

Now, let us go back to the slide and this is what is substituted over here. We use this fraction into delta H L. So, therefore, what we have is the enthalpy of ligand binding at

any at transition temperature can be obtained now we have this derived this various equations now let us discuss some equations which are required for simulation because the idea is to do simulations to fit the theoretical model to experimental data points. So, that we can extract the values of binding constant that is the main purpose.

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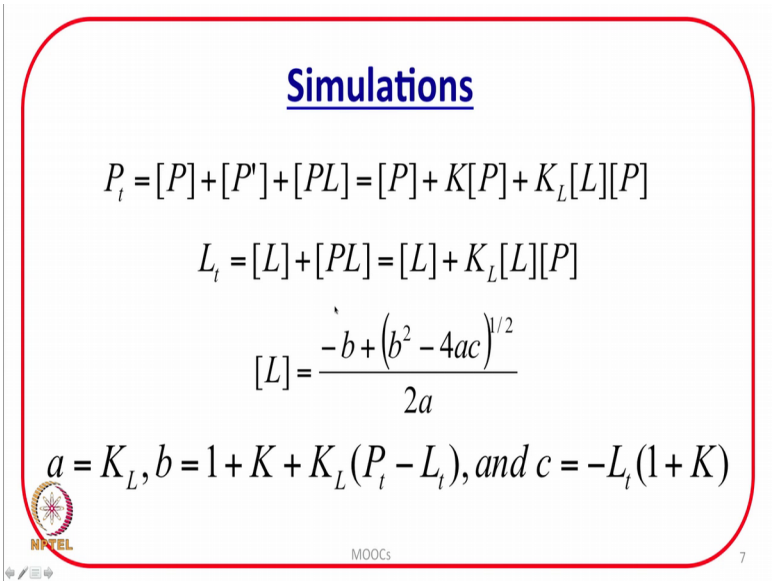


Now let us go back to the slide; total protein concentration in solution is equal to the concentration of protein in the native form which is free the concentration of protein in denatured form and the concentration of protein which is in ligated form.

Now, from the earlier derived equations, we can substitute for concentration of P prime, we can substitute for concentration of P L and we will end up with this equation which is concentration of P plus K times concentration of P plus K L into concentration of L into concentration of P. Similarly the total ligand concentration is some of the concentration of ligand in the free form plus the concentration of ligand in the complex form and again for the concentration of P L by using one of those equations earlier discussed I can write in terms of concentration of L and concentration of P. Now you see, if I know the total protein concentration and if I know the total ligand concentration, then I can get the concentration of L as well as the concentration of P L in the free form.

Now, if you combine these 2 equations, it will take a quadratic form the solution of which can be expressed as the concentration of L is equal to minus b plus minus roots b square minus 4 a c divided by 2 a minus will give an the number which is not acceptable

that is why plus is retained and where a is K L b is equal to 1 plus K plus K L into P T minus L T and c is equal to minus L T plus K into 1 plus K.

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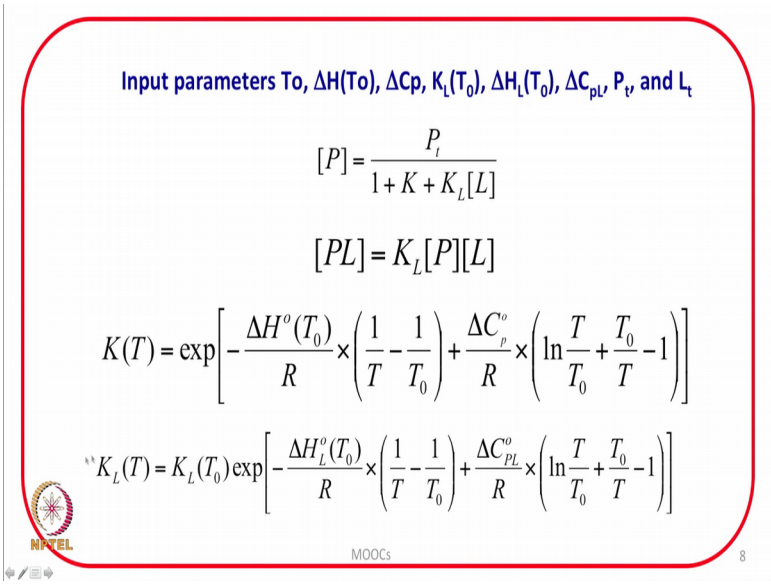


So, this some maths has to be done, therefore, after doing this; what we have the input parameters from the DSC transition temperature in the absence of ligand delta H in the absence of ligand delta C P K L T 0 delta H L T 0 delta C P L P and L T.

The concentration of P is expressed in this form and the concentration of P L is equal to K L into P into L and K T is given by this equation. These equations, we have earlier discussed and going a step ahead by using one of those 2 equations which I discussed in the beginning; I can write K L T is equal to K L T 0 into exponential all these terms regarding which I have already discussed; how to get this. Now the question is when you want to get K L, we need to know the concentration of P and the concentration of L in the free form and we have just discussed how to get the concentration of P and concentration of L by using the equations under the simulations head.

So, therefore, when you talk about the excess enthalpy relative to P 0, we just discussed you know; sometime how to express the enthalpy fraction of the protein which is in the denatured form into the corresponding delta H plus fraction of the protein; which is in the ligated form into the corresponding delta H and we have discussed; how to get P L; how to get P prime; that we have just discussed in the previous equations. If we can have

an expression for excess enthalpy, we can also convert it to excess heat capacity by its differentiation. So, that is what is mentioned.

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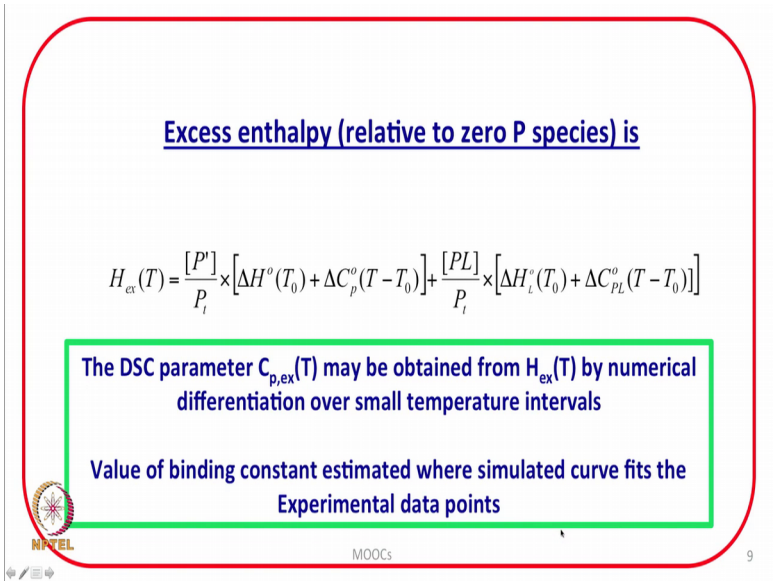


In this comment, the DSC parameter C p ex which is excess heat capacity may be obtained from H ex T by numerical differentiation over small temperature intervals. So, what is done is the excess heat capacity over a range of temperature is simulated from this equation and the data is experimental data is subjected to a fit by this derived equations and for whatever the value of K L; the fitting is excellent that K L value is assumed to be the best estimated value of the binding constant.

And that is what is written in the next comment; the value of binding constant is estimated where the simulated cure fits the experimental data points; although the equations appear to be little bit complex equation, but these are very easy to derive the level of complexity increases when the binding mode changes from 1 is to 1 to 1 is to 2 or higher or if there is instead of one thermal transition; there are more thermal transitions, but never the less; appropriate modeling can be done and for whatever the system is; it is possible to derive the value of binding constants associated with the protein ligand complexation by the use of differential scanning calorimetry.

So, by now, we have discussed the determination of the affinity constant or binding constants from a very low value to a very high value and we also discussed that what range of the values of binding constants are suitable to be determined by isothermal

titration calorimetry and if the values of binding constants are very high to be determined accurately by I T C, one can go to differential scanning calorimetry and estimate the values of the binding constants associated with ultra tight binding process for further information on this topic I refer you to the article published in biochemistry in the year 1990; volume 29, page 6927 to 6940.

Thank you very much.