NMR spectroscopy for Structural Biology
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Lecture: 37
NMR Analysis of Protein Dynamics - 1

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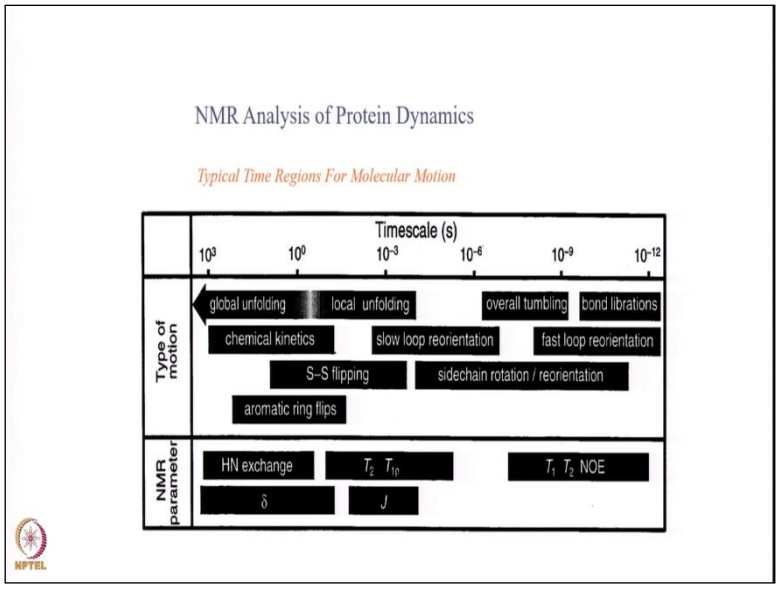

So, we have been talking about some aspects of protein dynamics. We said in proteins the dynamics can occur at various time scales, which I am showing this slide once more. Various time scales are there for protein motions. They range from picoseconds to the minutes and hours. And of course this protein dynamics itself is a huge subject one can talk about it for several hours, but here we are going to take a few of those topics and discuss in some detail.

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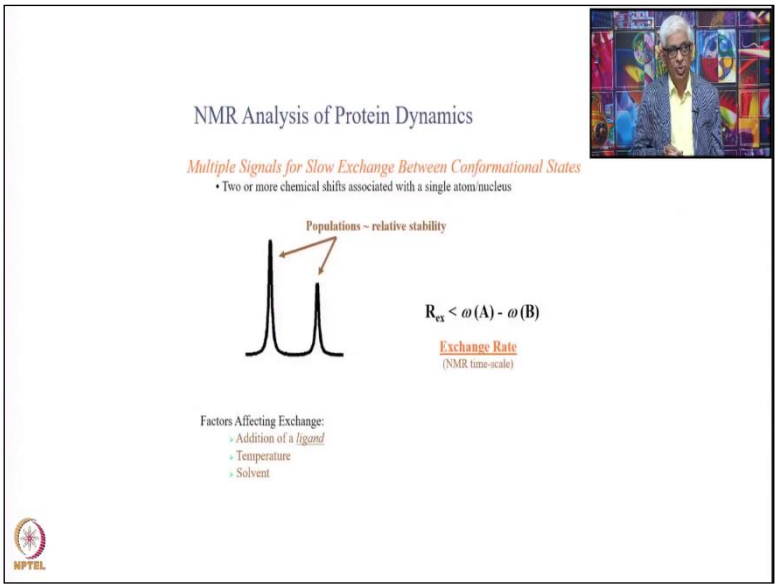

And I already talked about the particular aspect of millisecond to microsecond time scale motions. These are in conformational exchanges, two-site chemical exchange.

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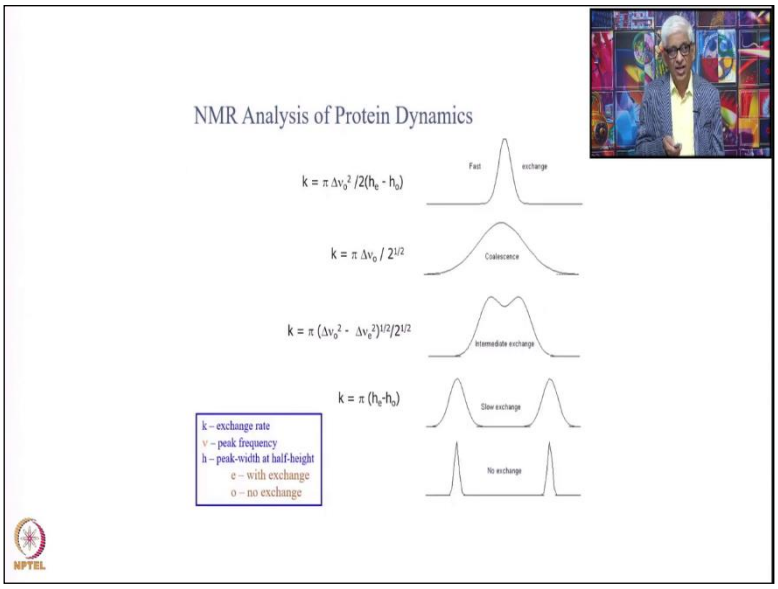

This we talked about last time and how depending upon the exchange rate and the chemical shifts of the individual sites the lines will change in character, the line widths will change, how they merge depending upon the exchange rate vis-a-vis the chemical shift difference between the two.

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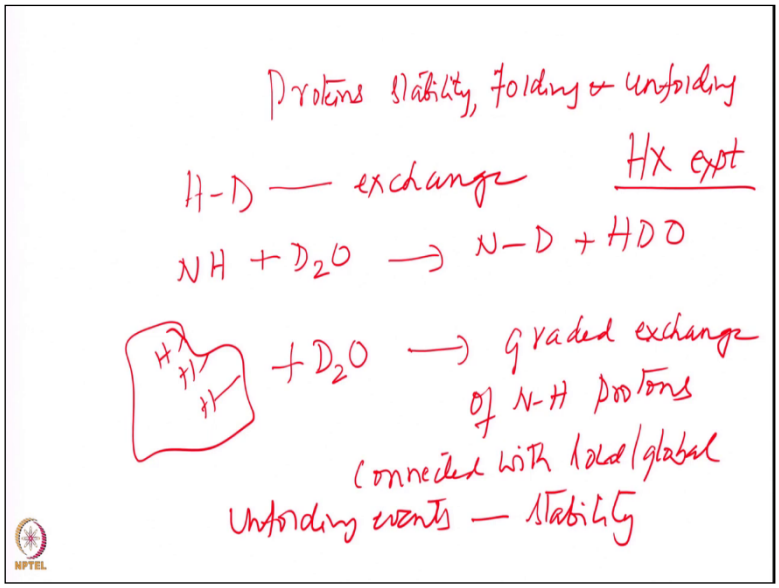

We are going to look at one more aspect of this time scale motion, which is on the minutes to hours timescales and this particular thing is also related to protein stability and folding-unfolding. So, this has implications for protein stability, folding and unfolding and what are you talking about; this is the motions on minutes to hours timescales and sometimes even seconds to you can take means time scales.

And this is what is called as NH deuterium-hydrogen exchange, this is H-D exchange. What is exchanging? These are the NH protons. The amide protons, they exchange with D_2O when you put D_2O, this forms N-D + HDO. This is what we are talking about. So, you have a protein, let us say various kinds of a protein here and there are various protons sticking around here, proton here, proton here, proton here, and this is the folded protein; some hydrogens are on the surface, some are in the interior.

And then when you put D_2O in it, some of those which are on the surface they will exchange and the ones which are in the interior will take time. So, there is a graded exchange of NH protons. This is intimately connected with local/global folding-unfolding events, therefore this has implications for protein stability. So, this became an important technique, hydrogen exchange, this is also called as HX experiments.

So, the idea is that you monitor the exchange of the amide protons with D_2O and that has implications or it will indicate the relative stabilities of different portions of the protein.

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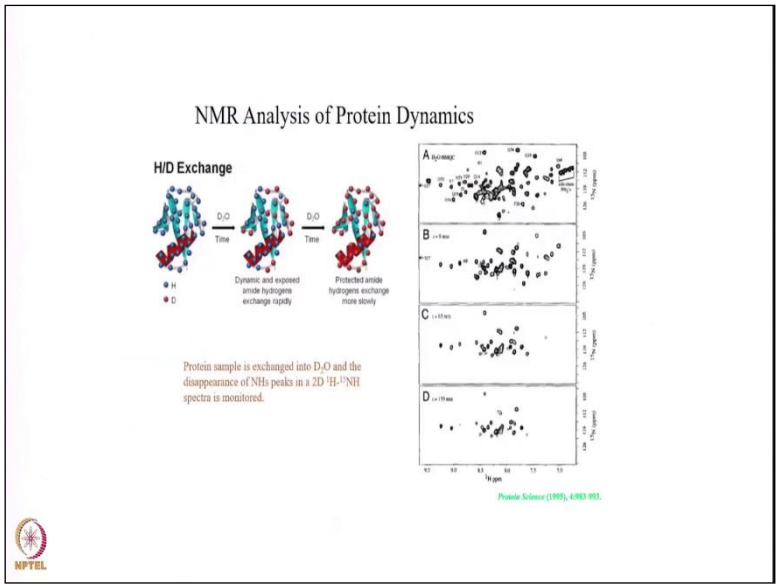

I will show you this here. Suppose you have a HD exchange, you have here a protein, which is like this it, has a helix here, and it has the β-sheets and there are a lot of protons sticking around. Now here these the red ones are the deuteriums and these ones are the protons. Now if you put D_2O to a solution in water, you have the protein dissolved in water. So, therefore initially all the protons are in the H-state. You can lyophilize it. So, when you lyophilize and make it dry powder then it is all HNH.

Now we dissolve it in D_2O, what will happen, the ones which are on the surface of the protein they will exchange immediately. So, you start getting here these red balls, the red balls are the deuterium and this depends upon time. How much time you give for this, you keep D_2O inside this. You monitor as a function of time. The ones which are on the surface will immediately go away, while which are in the interior they are not ready to exchange immediately.

It requires a certain process for them to become accessible, then you go on with the time. So, more will get deuterated. So, this will actually reflect in your NMR spectra. How do you monitor? Where do we monitor? We monitor in the HSQC spectrum, ^{1}H-^{15}N-HSQC spectrum or the HMQC spectrum. We can use either the HSQC spectrum or the HMQC spectrum, it does not matter.

So, this is the initial state of the protein where it is all protonated, this is not deuterium. Now you see as a function of time, you add D_2O to it, as a function of time you can see here this is 9 minutes, this is 63 minutes, and this is 133 minutes, you can see how this number of peaks

starts going down. Why do they start going down? Because we are looking at ^{1}H-^{15}N-HSQC spectrum.

So, wherever there is a proton, it will show a signal. If there is a deuteron, it will not produce a signal. The ones, which are exchanged out with the deuterium, all these red ones, will not show you a peak. So, as a function of time, if you look at the spectra you see the peaks will start disappearing more and more, showing that these ones are in the interior. Some of these are still there everything has not gone.

Even after this many minutes; this is after 133 minutes, this is almost more than two hours, all of them have not gone. So, slowly as you increase the time more and more are disappearing. From here to here some disappeared, here to here more disappeared, here to here more disappeared, it keep going. But in certain proteins, the proteins are so stable, it will take years for them to completely exchange.

What is required here is that there has to be some local unfolding events, because the protein is in a folded state. If the protein is in a folded state, the NH proton is not accessible and this has to become unfolded, then after that it can exchange with D_2O then it became ND. So, there is a process here, there is a folding-unfolding process. So, depending upon how the local folding-unfolding events are happening, you can have different kinds of exchange rates.

And this is the process that happens, that is why you see some peaks are remaining and more and more disappear as you give more time. So, local folding-unfolding events continuously keep happening although the protein is not totally unfolded, it is still a folded protein.

But there are local dynamic changes that are happening inside make the proton accessible to the D_2O which can actually penetrate the protein structure and then it will start an exchange process.

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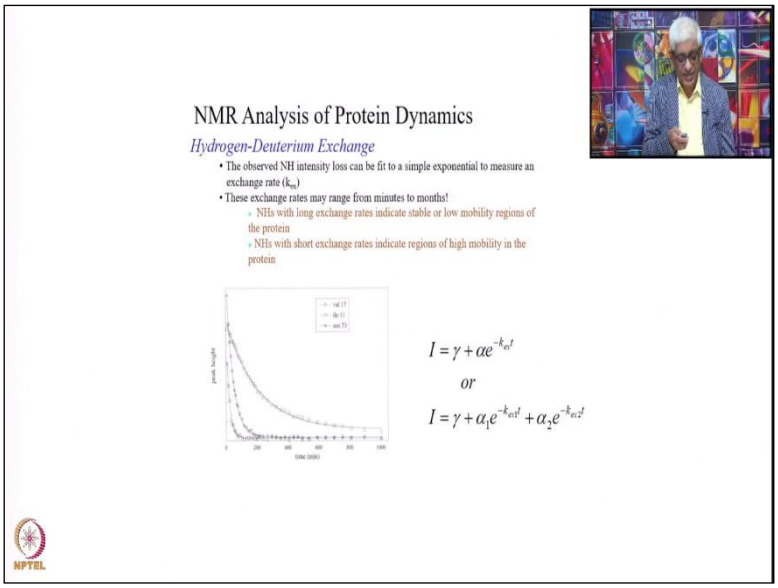

So, then what you do is and as you also keep looking at this even here you see these intensities of the particular peaks. So, even those peaks which are present here their intensities will keep decreasing as a function of time. We do it for many times, we have shown only the four time points here. But you do it for many time points, you can monitor the intensity changes of all of these peaks how they eventually disappear.

So, if you plot these intensities this is what is shown here. So, as a function of time you plot the intensities of the peaks. This is shown for few residues here. There are three residues which on a particular protein, it does not matter which kind of what protein it is, but this particular proteins are shown, you see this one slowly exchanges, these ones are very rapid, this is the most rapid, after that this one, after that this one.

So, there is a clear variation in the exchange rates of these ones. This section is very rapidly very likely this is on the surface the one which is here, and this one is probably slightly in interior it takes more time, initially all of them will start at the same point and then this one is very slow. So, therefore this is interior. Now what you do, you fit these to functions of this type, exponential functions.

Fit this to an exponential function, you can fit either single exponential function or a bi-exponential. So, this will indicate the number of processes that are happening in the process of exchange of the protons. So, this is the single exchange here. So, it gives you, there is one exchange rate indicating one process, here there are two exchange rates indicating two processes, this may be local followed by global and things like that.

So, all of such things can happen and that will result in different kinds of fitting parameters and these ones are important. Obviously these exchange rates have information about that about the structure of the protein.

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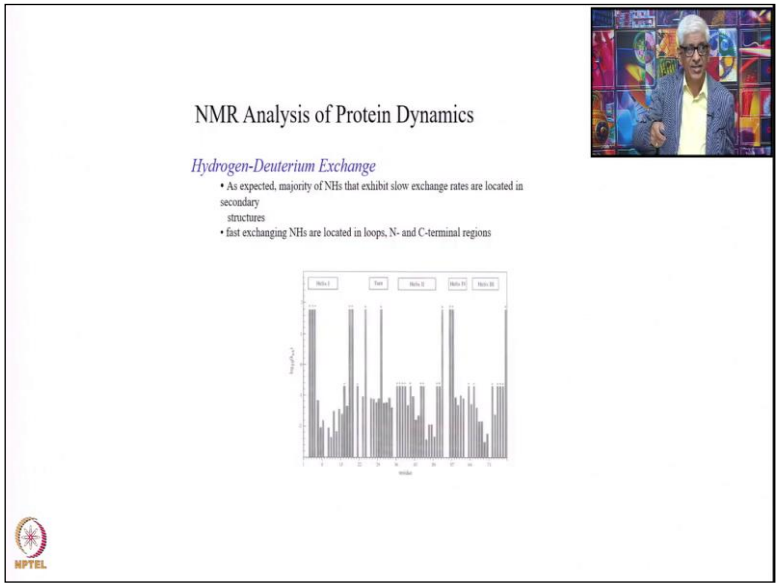

You see here, this is for a particular protein here. What is plotted here is the log of the exchange rate versus the residue for a particular protein. Now these ones are very fast exchanging, exchange rates are very high. Then you have these slow ones here and you see a variation in the exchange rates and notice the ones, which are in the N-terminal, or in the loop area these are the secondary structure elements in the protein.

You have a helix one here, there is a turn here, there is a helix, there is a helix, and there is a helix, if these are secondary structures and they are connected by certain loops and turns. Where there is a turn, the possibility of its getting exposed to the solvent is more, therefore they will exchange very rapidly. Therefore, the exchange rate is high for those ones and when there is a regular secondary structure then you see that the exchange rates are relatively small.

Therefore this is indications about the structure of the protein, the stability of the protein, different areas of the protein, even in the different area in the folded areas, there is a variation in these exchange rates. So, that will tell you how the protein can sequentially unfold and we can measure the equilibrium constants for the unfolding reactions in this plane. That indication is already there.

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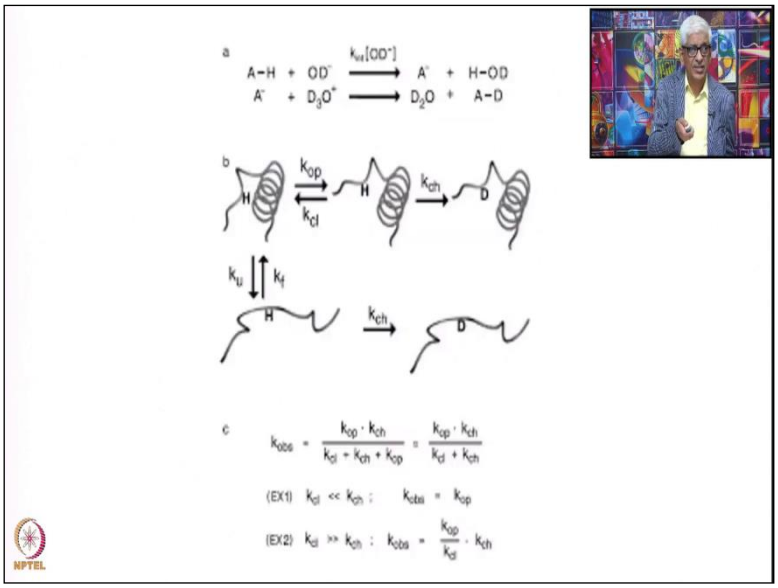

Now the same thing I am going to explain here to you. This is the A and the H this probably A will be the amide proton here and this exchange can be catalyzed by certain kinds of catalyst and so you have the water which is coming the D_2O and you have the OD^-, the A^- + HOD and A^- + D_3O^+. This is a particular pH, you have the hydronium ion here, the deutronium ion here, you get D_2O^+ and this is how the exchange process happens.

And that is indicated here in a schematic manner with respect to the protein structure. You have a helix here, and then you have a loop somewhat hanging around here, and there is a proton here. Now you see the chain unfolds here. This portion remains the same, this unfolds the proton is now becoming free. So, there is an equilibrium here, the opening rate and the closing rate, there is an equilibrium here.

And then once it is in the open state, then it will immediately exchange, this exchange rate is very high compared to this closing rate. Therefore, once it is here, this exchange will happen in this manner. Therefore, it is a relative importance of this rate versus this rate, which determines how fast the system will exchange with D_2O. Therefore, obviously it has to do with the equilibrium between these two here.

So, if you have the unfolding like this, local unfolding, this is unfolding rate and this is folding rate, and then you have this once it is unfolded here, this exchange will happen immediately with the D_2O, you get an unfolded protein. If you have an unfolded chain here, this is a partially unfolded here. So, you have a certain segment, you can also have situations like this, it can go

from here to here, from here to here, and then here to here, or it can go directly from here to here.

So, therefore you have the measured exchange rate is quantified in this manner. So, there are two kinds of mechanisms which are indicated here as EX1 and EX2. EX1 this typically this closing rate is much smaller than the exchange rate, that is this rate is much smaller than the closing rate in which case that is called as the EX1 mechanism. Once the protein comes here this will immediately exchange.

So, that is this situation as well. On the other hand there is the EX2 mechanism, this rate is much larger this rate, therefore the protein would like to remain in this state as long as possible. Therefore, occasionally it will exchange because there is always a possibility there is a certain chance. This is all kinetics, therefore one knows this there are always a certain probability.

And then the measured exchange rate will be given by this.

$$k_{Obs} = \frac{k_{op} * k_{ch}}{k_{cl} + k_{ch} + k_{op}} = \frac{k_{op} * k_{ch}}{k_{cl} + k_{ch}}$$

This rate depends upon what you have, what relative magnitudes of this and this you have, so, you have here different situations.

For the EX1 mechanism, $k_{cl} \ll k_{op}$ then you can ignore this k_{cl}. Then it will simply become $k_{obs} = k_{op}$; that is this one. So, immediately goes here and then goes there.

On the EX2 mechanism, when $k_{cl} \gg k_{ch}$ then the $k_{obs} = \frac{k_{op}}{k_{cl}} k_{ch}$.

Notice here this is your equilibrium constant k_{op}.

Therefore, there is information about the equilibrium constant here for particular residue. Depending upon which residue you are monitoring, you have the information about the equilibrium constant for that particular event. So, therefore you can translate that into free energy and it gives information about the stability of the protein. So, therefore this is enormous possibility of determining the stability of the protein using the dynamics.

So, here we are talking about the dynamics right, the kinetic exchange and that is a dynamic process happening at a millisecond or seconds to hours to sometimes even years timescales.

Some of them remain in a particular state, in BPTI, for example the protein stays for several couple of years without getting exchanged. There are some very stable proteins like that.

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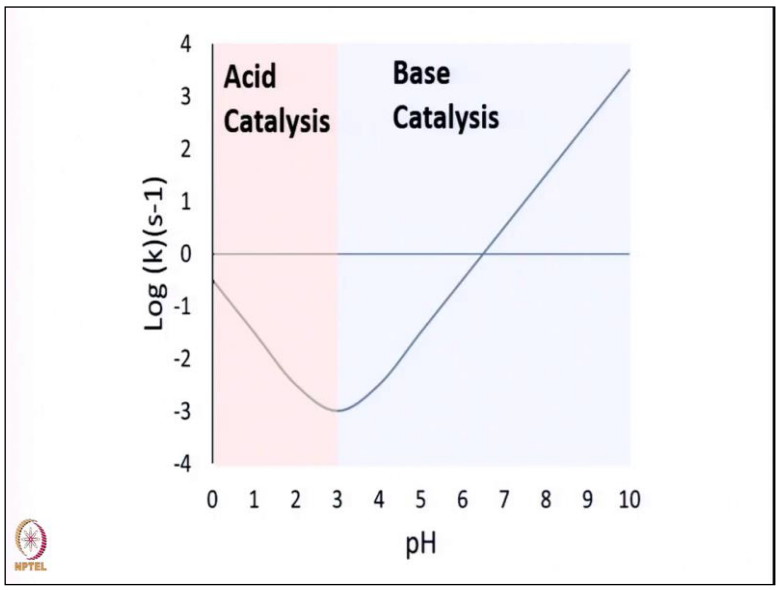

Now this exchange is a catalytic process and depending upon the pH of the solution the exchange rate can be different. Now you can have catalysis, the process can be catalyzed either by the acid catalysis or by the base catalysis, this can happen and in either case therefore you see there is a high exchange rate here and then you have a minimum somewhere here. Of course it can vary from protein to protein. This is the schematic, this is an indicator.

You will have some below this use acid catalyzed process, which is the k_{ch}. So, it is acid catalyzed and this here is base catalyzed and now here you have the lowest exchange rate. We are going to use this. This strategy one can use to get information about the protein folding-unfolding events.

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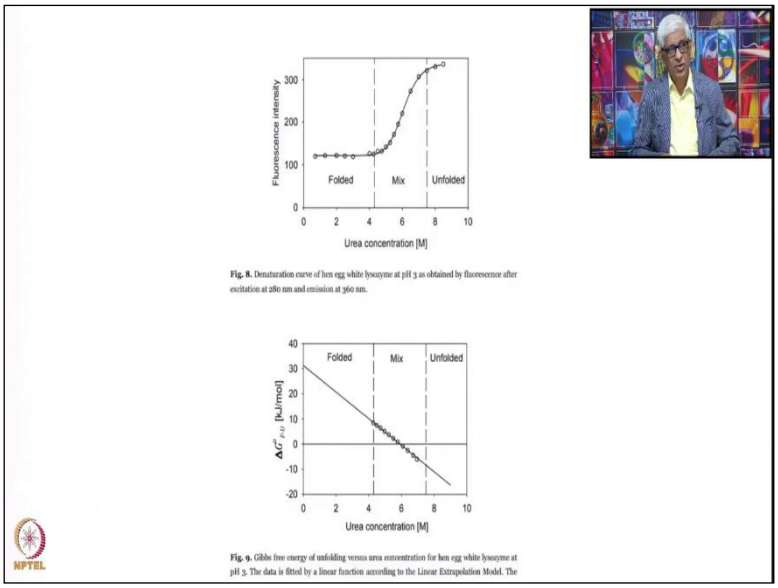

Now when you want to do a protein folding study, so either you can start with a folded protein and slowly go into the unfolded state or you can start with an unfolded protein go into the folded state. How do you do this? So, you do this by using one of the denaturants. So, what is the effect of the denaturants? So one of the denaturant which is commonly used is urea and you can monitor the protein folding by measuring the fluorescence of the particular tryptophans or something inside your protein.

And as a protein unfolds the fluorescence will change. So now here you see this is the urea concentration you plot, you monitor the folding-unfolding process. You have the folded protein here your particular fluorescence intensity and the unfolded state you have the fluorescence intensity here and this is the transition zone. Typically you have the midpoint is somewhere here, but sometimes of course this is a very schematic.

It can be different for different protein, different places, this is at a particular pH. This is for hen egg white lysosome at pH 3, but for different proteins, it can be different. The midpoint can be at 4, it can be at 3.5 and so on and so forth at urea concentration in molar. The point is it has a kind of a sigmoidal behaviour here in this state. Typically, if you are at lower urea concentration reasonably wide range of urea concentration the protein can remain in the folded state.

We are going to make use of these to study the folding pathway of a particular protein. And from this EX2 mechanism, which I indicated to you earlier, that you can actually calculate the ΔG, the free energy of the folding-unfolding event. So you see, in the folded state the free

energy is very high, this is in kJ/mol. So, you convert that into kcal of course you divide by 4.18.

So, therefore you will have here about 4 to 5 kcal/mol here and here it will be much less and this will be even much less here. So, therefore the folding to unfolding requires a larger free energy and then slowly it goes over into the intermediate free energy, then it is a very low free energy for the state. So, therefore this is typically when it is in this state, the protein is able to exchange with D_2O much more rapidly.

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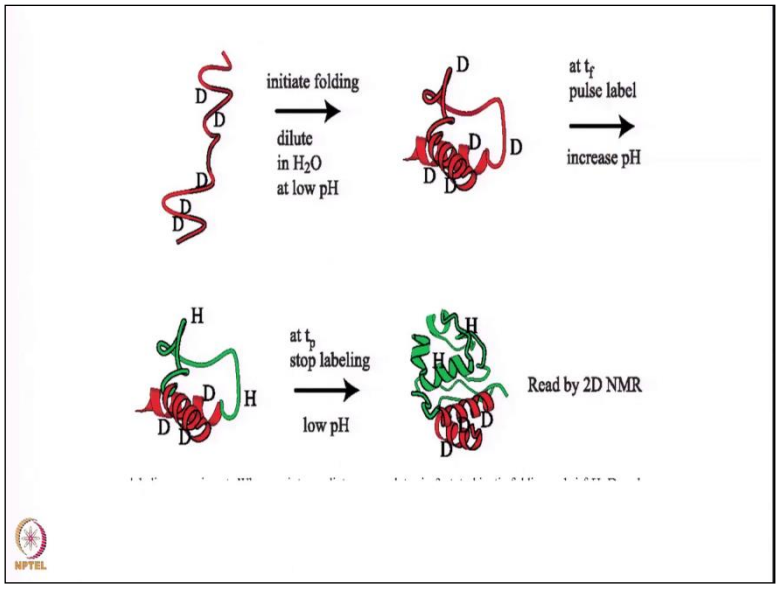

How do we use this? We use for studying the protein folding pathway. What you do is, you create an unfolded state by adding urea or something, and then here the protein is completely deuterated. What you do? You prepare a buffer in D_2O in urea and then of course you and lyophilize the sample. So, then you get a powder and then you have a protein which is deuterated but unfolded.

Now what you do? Suppose I have urea as 8 molar urea. Now I have to initiate the folding process, this is an unfolded state, I want to go from here to the folded state. How do I initiate the folded state. Now I add in H_2O to it, dilute in H_2O. You add something like about 20 times dilution.

Suppose you had 8 molar urea, this is the unfolded state, and let us say you do 20 times dilution in H_2O, but at low pH and what pH? This is at pH 3, somewhere around pH 2.5 to 3. So, then

20 times dilution, what will be the concentration here? So, this will be like 0.4 molar urea. So, 10 times will be 0.8 molar, 20 times will be 0.4 molar area.

Now you see at 0.4 molar urea, the protein is not unfolded. So, it is in this condition only. Now it can in principle fold. So, this is what the initial folding event, you start with an unfolded state. Now you initiate the folding by 20 times dilution with H_2O, that means you are providing a condition for the protein to fold, but you are not providing a condition to exchange,

because you put a low pH. Therefore, the protein starts to fold, you give a certain period of time, it has folded to some extent but it is still D_2O. It is still deuterium because you have put the pH very low. So, when the pH is very low, there is the minimum exchange rate. So, it is still in the D_2O. Now you put a pulse label, what is the pulse label, here you put a pulse of water H_2O.

So, then you put the pulse, it means you increase the pH at this point of time, that means it is a kind of a pulse. You allow the system to start exchanging. So, at low pH it is not exchanging, you increase the pH at time t_f. Until time t_f you allow the protein to fold, but it has not exchanged, but at this point you increase the pH of the solution by adding a certain amount of hydroxide or things like that NaOH or something like that. That means you have provided the condition for the protein to exchange from deuterium to proton.

Therefore, now you see whatever is exchangeable at this point it will exchange. Now this will become what is in the surface here, suppose it has folded like this; whatever are the deuterons here they will exchange to protons here. Now we want to monitor this. So, what you do, I want to monitor which deuterons have exchanged into protons.

Therefore again I stop labelling that means I lower the pH once more. I immediately lower the pH, so that the exchange process is quenched. So no more exchange happens at this point. So, therefore what I have done, I allowed the protein to fold for a period of time t_f, then increase the pH suddenly allow the exchange to happen for a certain time period and this also can be varied and then at this point at t_p you stop the exchange process.

So it suddenly lowers the pH. So, during this between t_f and t_p the deuterons are exchanging into the protons. Now you monitor this low pH, and whatever has happened here, you can read

by two-dimensional NMR in the HSQC spectrum. You will start seeing whatever changes have happened here. So, that is how you get the information about stepwise folding you can keep varying this t_f and t_p.

So, if you give more time for this more protons will get exchanged, less time less protons will get exchanged. Therefore you have to vary this. So, there are two things which one has to vary; one is the extent of folding that is governed by this t_f, how much time you give, and then for how long you will keep the pH increased at a higher value, so that the exchange happens and for how long you will keep it depends upon how many protons will appear as NH protons and non-deuterions.

Therefore you can monitor this by a 2D experiment in the HSQC spectrum. Because in the HSQC spectrum you will only see which are exchanged, you do not see the other ones.

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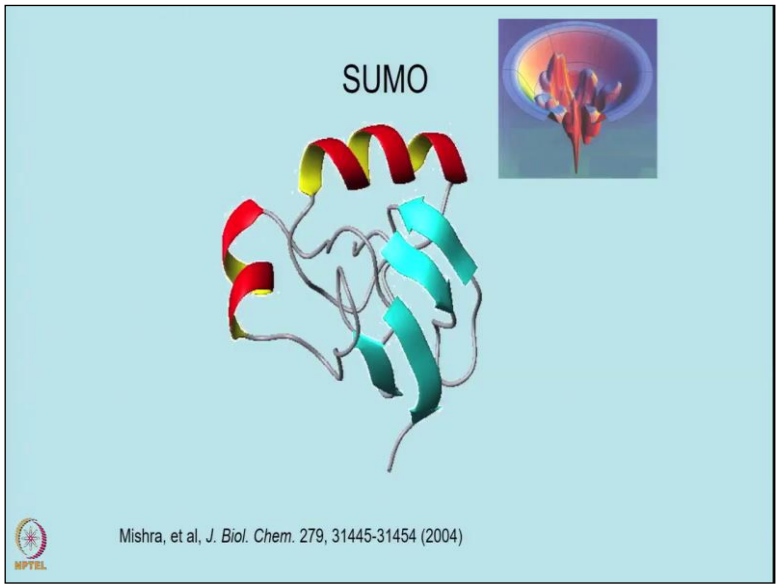

So, that is how you monitor the proteins folding process. The folding process, you first denature the protein and initiate the folding process by diluting the denaturant and you allow it for a certain period of time and then you change the pH condition. This of course has to be done very rapidly. Inside the solution itself, you have to design a certain hardware also for doing this because you have to give a pulse.

Suddenly add an NAOH and the stiring mechanism has to be introduced, so that the exchange process starts and then after that you stop the exchange and monitor how many deuterons have exchanged into protons. You can keep varying these times, t_f and t_p. So, this is how the

hydrogen-deuterium exchange can be used to study protein folding, protein stability and this time scales can be in seconds to hours timescales.

So, now we will use another strategy to monitor the folding of the proteins. This will be again start from the denatured state and we go over by monitoring systematic changes in the conditions of the solution with regard to the urea concentration and that will be demonstrated with regard to the particular protein called SUMO, and what is shown here is the folding funnel and we will talk about that in the next class.