Bio-Physical Chemistry
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Lecture – 41
Infrared Spectroscopy of Proteins

So, we will start our class today, last class if you remember we were discussing you know quenching of fluorescence and how it can be useful to figure out certain conformations of proteins where the tryptophan is more exposed where the tryptophan or any other chromophore is less exposed right.

Now, before ending the class, I also told you that we will be taking a break from fluorescence; that means, we have done a little bit of fluorescence and the last topic in fluorescence that we want to discuss or I want to discuss rather is something known as fret fluorescence or faster resonance energy transfer which is very useful in studying conformational distributions or confirmations in proteins because it looks at the distance between two labeled sites or ends.

So; however, before you know going into that I think that would be the last topic we would cover in this course. I would like to digress and look at some other techniques right or other spectroscopy tools. So, one of the tools that is very much used in investigating proteins is Infrared Spectroscopy.

Now, before going into the actual protein scenario let me give you a brief introduction about vibrational spectroscopy I am sure many of you know many of you have already done it, but just to brush up your memories; we look at the theory.

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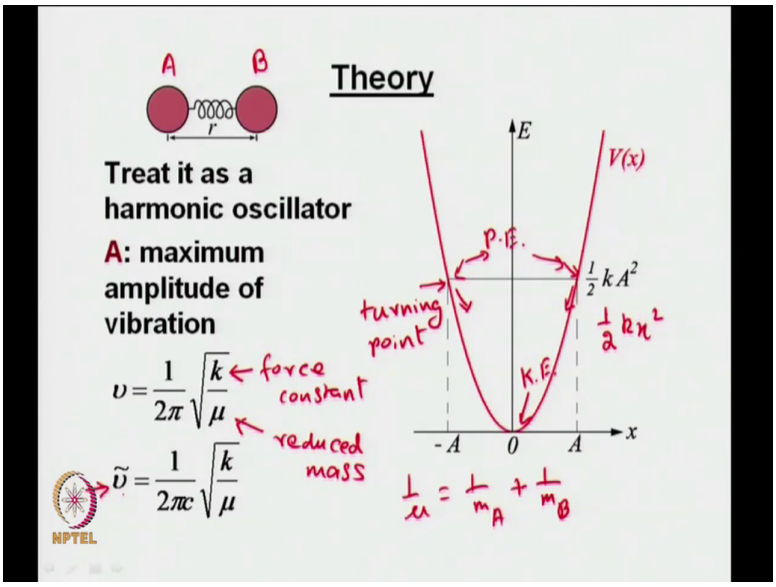

So, what we do is when we talking about vibrational spectroscopy we will talking about vibration of water bond between 2 atoms ok. So, this is say atom A and this is say atom B A and B right. Now, what we do is, because it is vibrating because a bond vibrates we treated as a spring because we treated as a spring you know springs follow Hooke's law then we have a certain potential energy curve, by the potential energy curve you can see it is given by half k A square you can also write as half k x squared.

Now, what is this x? x means the amount of stretching or compression I am doing with respect to the equilibrium bond distance or the equilibrium nuclear geometry so; that means, if I have 2 atoms having a bond in between say this is the odd 0 which is the equilibrium distance that is what the 0 in this graph signifies. So, this is the equilibrium geometry or the equilibrium internuclear distance now what happens is, I can stretch the bond. So, once I stretch the bond

what will happen is the distance is increasing and that displacement from the equilibrium position is being denoted as x ok.

And you can see this red curve this corresponds to half k x square right and what does A represent out here? You will soon see what A represents. See, if we go forward what we do is, we treat this spring oscillation as a harmonic oscillator; that means, the spring is oscillating and vibrating and we treat as a harmonic oscillator.

So, harmonic oscillator means you can see how symmetric this curve is on both sides of 0 right; the middle axis the middle y axis I mean. Now, what is A then? See A is the maximum amplitude of vibration of the spring so; that means, it goes to one position the spring it vibrates and it comes back and goes to another position, but these are the two maxima. Now, at the maximum what happens is at the maxima because doing the movement the spring would be having both potential and kinetic energy at the maxima which are its turning point; that means, because they are maxima they have to come back so; that means, if a spring vibrates and reaches this amplitude it cannot go beyond. So, it has to come back from here and it also has to come back from here.

So, at these two points at these two points at these two points the energy is fully potential and at this point 0 the energy is fully kinetic right. So, then what does half k A square mean? Half k A square means where A being the maximum amplitude of vibration this is the maximum potential energy, you can have for the given spring given the fact that A is the maximum amplitude of vibration.

So, you can understand at the turning point. So, this is a turning point. So, this is a turning point this means that the spring cannot vibrate beyond this, at the turning point because the energy is totally potential; that means, E is equal to half k A square right and at the bottom where you have totally kinetic energy then E will be equal to the kinetic energy and in between it is a mixture of both ok.

Now, this is a very important characteristic parameter which is known as the vibrational frequency that is nu. So, nu is given by 1 by 2 pi root over k by mu where k if you remember

is referred to as the force constant and mu is referred to as the reduced mass. And what does reduced mass refer to?

So, reduced mass is actually a combination of both the masses m A and m B or m 1 and m 2 that is some it is an expression. So, mu can be given by an expression which involves both the masses right or we can write 1 by mu is equal to 1 by m A plus 1 by m B right. So, this is how mu or the reduced mass is given. So, this is the characteristic vibration frequency.

Now, mostly when we are talking about vibration bands we do not express in terms of frequency rather what we do is we express in terms of wave numbers right. So, wave number is given by this expression. So, this is the corresponding wave number this is the frequency this is the wave number right is equal to 1 by 2 pi c root over k by mu.

Now; obviously, we will understand that say that this wave number which is in the centimeter inverse that is the unit we mostly use. We still refer to it as frequency ok. Though it is not absolutely right in terms of the nomenclature, but it does not change much because if you would keep in mind energy is equal to h nu or delta E is equal to h nu right or we can also write delta is equal to h c nu bar; that means, in both cases energy is proportional to the either the frequency or the wave number.

So, anyway the bottom line is that if you are looking at any vibrational spectroscopy, you will see that the x axis is in terms of wave numbers, but still we end up calling it as frequency ok.

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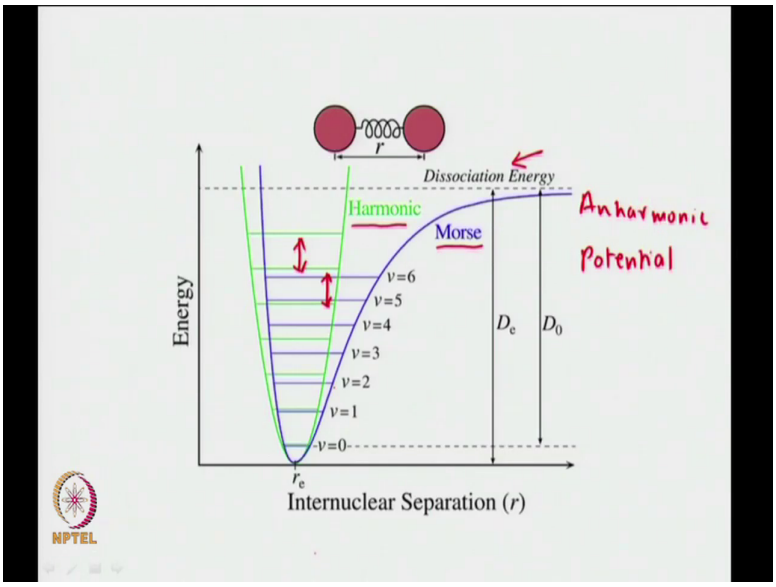

So, this was a very brief intro about introduction about this vibrating bond, now just one more thing before we start looking at vibrations. So, you can see this is another plot where you have two potentials shown. One is a harmonic potential which is given in green and the other one is a morse potential which is also known as an anharmonic potential. So, this is also known as an anharmonic as an anharmonic potential ok.

Now, what is the difference as you can see the harmonic potential is absolutely symmetrical the anharmonic potential; however, is not because if you go to very high internuclear separation that is what I said internuclear separation if we got to very high internuclear separation which means that your stretching the bond like anything.

See you do not expect the bond to remain stretched and nothing happen even if you do it for a huge distance which means that after a certain distance the bond will snap or break. And

because of bond will snap or break, you can see what happens, this is something referred to as a dissociation energy; that means, what it says is if you go to this energy because energy which was on the y axis if you go to this energy the dissociation energy if that if that is the extent of vibration energy you are putting in and if the molecule is there in terms of it is vibration energy that mean it is vibrating so fast it is vibrating with so energy.

So, what will happening; what will be happening is the bond will break and the molecule or whatever this bond will dissociate right that is what it means; however, if you look at the harmonic potential you do not get this bond dissociation picture. So, this is so, this morse potential which is also the anharmonic potential is the real potential that a bond would face or experience during vibration ok.

Now, there are other implications to this for example. I will just give you one thing before I move out of this slide. So, for the harmonic potential if you see the energy levels are all equispaced. You can see the energy levels for the harmonic potential which are in green are all equispaced if you can see. However if you look at the anharmonic potential which are given in blue so, you can see the energy levels start there is a wide gap from bottom.

So, nu is equal to 0 nu is equal to 1, but as you go up what is happening is the gap in between the energy levels is decreasing. And once you reach the dissociation energy the gap is really small because when you dissociate, when you dissociate what happens is you are almost in the continue of energy levels because there is almost no quantization now that the particle is free right.

So, I am sure these things you already knew it was just a briefly recap of what you have done in your spectroscopy course.

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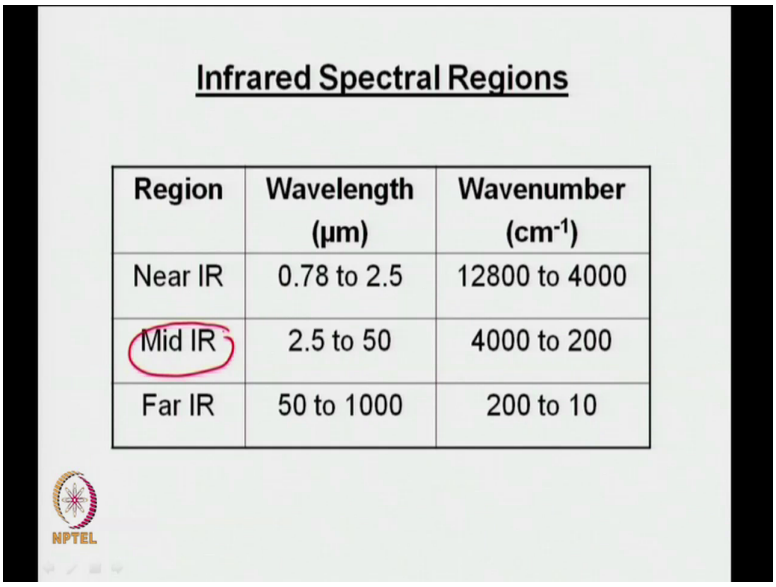

So, let us quickly look at the different infrared spectral regions that we have. So, one is the near IR the wavelength from 0.78 to 2.5 micron and the wave number. This is the frequency we actually refer to is 12800 to 4000 centimeter inverse. The mid IR this is the one we are going to concerned with it is 2.5 to 50 micron in terms of wavelength and 4000 to 200 centimeter inverse in terms of the wave number and there is a far IR too which is 50 to 1000 micron or 200 to 10 centimeter inverse. So, this is the one the mid IR which we are going to focus on mainly.

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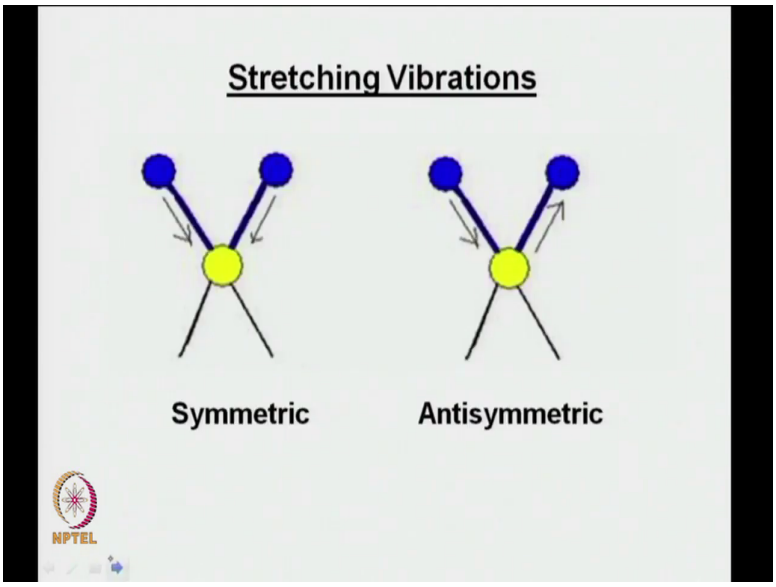

Now, let us look at some vibrations. So, this is an example of two types of stretching vibrations, one is a symmetric stretch because you can see both the arrows are pointing towards the same direction. The other one is the antisymmetric stretch because in one case you are compressing the bond in one case you are stretching the bond. So, that is why these are called symmetric and antisymmetric stretching vibrations.

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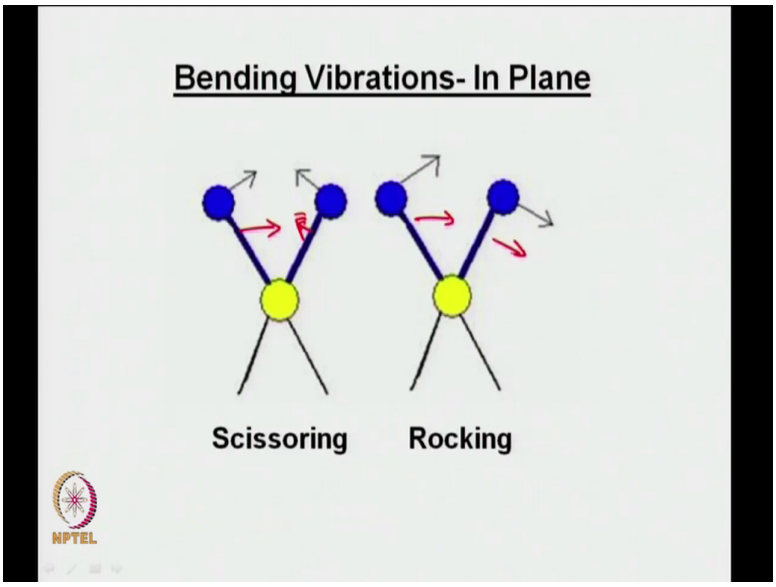

Then there are bending vibrations stretching is not the only thing you can do a bond can even bend. So, you can see what happens is this is all in all these bending vibrations are in plane; that means, occurring in the same plane. So, scissoring means so, scissoring means so, this is as you can see this is moving towards like a scissor this is moving towards this direction and this bond is moving towards this direction. So, it is like a scissoring effect.

The rocking is while in scissoring both the bonds were moving towards each other like a scissor as I use scissor to cut something. In the rocking you can see both the bonds are moving in the same direction so; that means, it is like a rocking chair your rocking your moving back and forth, but remember all the both these things are in plane.

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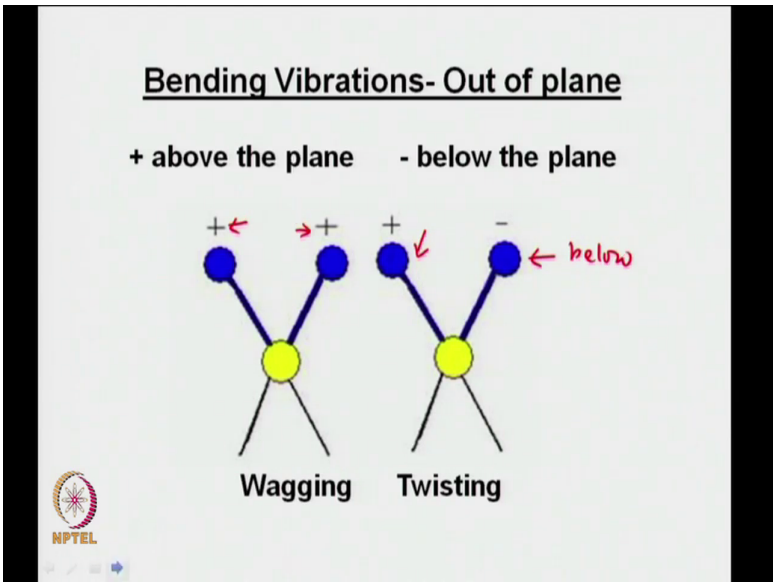

And what about then if you know you have a set of bending or in plane bending vibrations, then you are sure going to have some out of plane bending vibrations. So, what do if you look at this figure what do the plus and minus have do? Plus means your vibrating above the plane and minus means your vibrating below the plane.

So, if you look at this so, the first one this is plus right and this is also plus means both are above the plane. So, it is like wagging of a tail. So, you are wagging remember this is the out of plane your coming out of plane, but both the bonds are moving in the same direction right.

Now, in the case of twisting; however, the opposite thing is happening, one bond you can see it is moving out of plane; that means, above the plane and this bond is moving below the plane right. So, this is a case of twisting. So, these are different out of plane bending vibrations.

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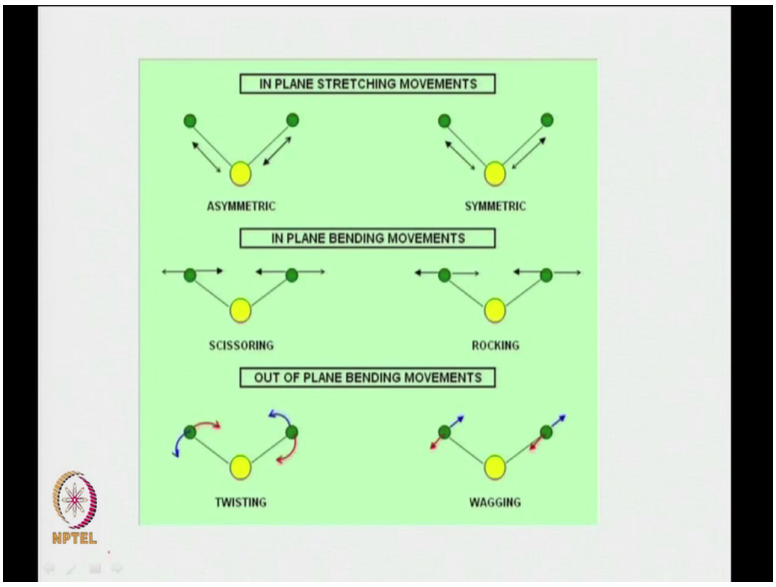

So, here again we look at them together. The first two you can easily recognize asymmetric and symmetric. The next two in plane bending movements scissoring rocking and the next two twisting wagging which are out of plane bending movements the thing we just discussed.

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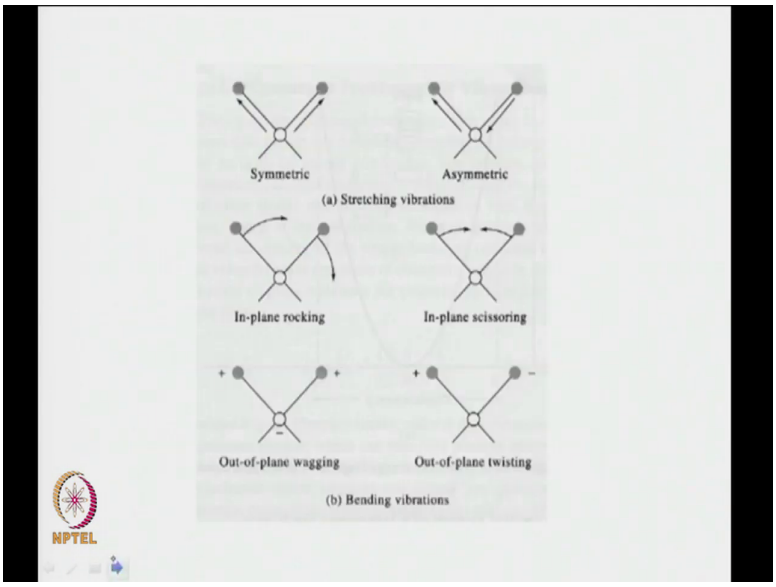

And to stress the fact again you can see this is another similar figure which again enforces the fact what are the differences between the different types of vibrations the symmetric, asymmetric, in - plane rocking, in - plane scissoring; that means, this is the in plane bending vibrations, then out-of- plane wagging and out-of- plane twisting right. So, the last two are bending vibrations, the first two are I mean the last four are bending vibrations, the first two are stretching vibrations are shown out here ok.

So, whenever we talk about a vibration right whenever talk of a vibration, these are you know broadly defined the different types of vibrations that can be present. Now, when we go to a protein molecule all these types of vibrations would be there. But what are the types of vibrations we would be really interested in if you want to look at a protein folding, unfolding or different conformation aspects that is what we are going to focus on ok.

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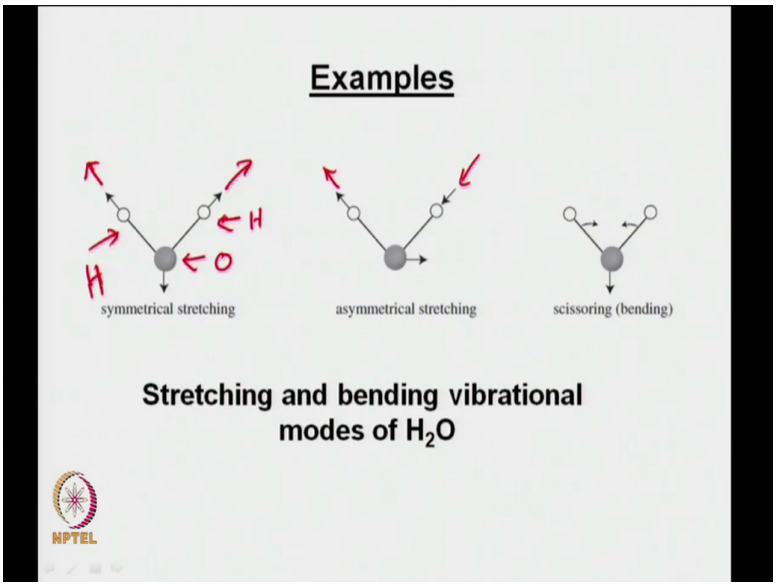

So, this are some examples of molecules before we move into proteins. So, for example, this is for H 2 O right. So, this is the oxygen; this is the oxygen; obviously, and these are the two hydrogens right. So, this is also hydrogen.

Now, you can see this is symmetrical stretching the first one, the second one is asymmetrical stretching and you can see why it is so. In the symmetrical stretching both these arrows in the symmetrical stretching both the arrows a pointing in the same direction right, in the in the antisymmetrical stretching it just a reverse. So, this arrow is pointing in this direction, this arrow is pointing in this direction; that means, in one case you are stretching the bond the other OH bond is getting compressed and this is the scissoring which is a bending vibration, but still in plane right.

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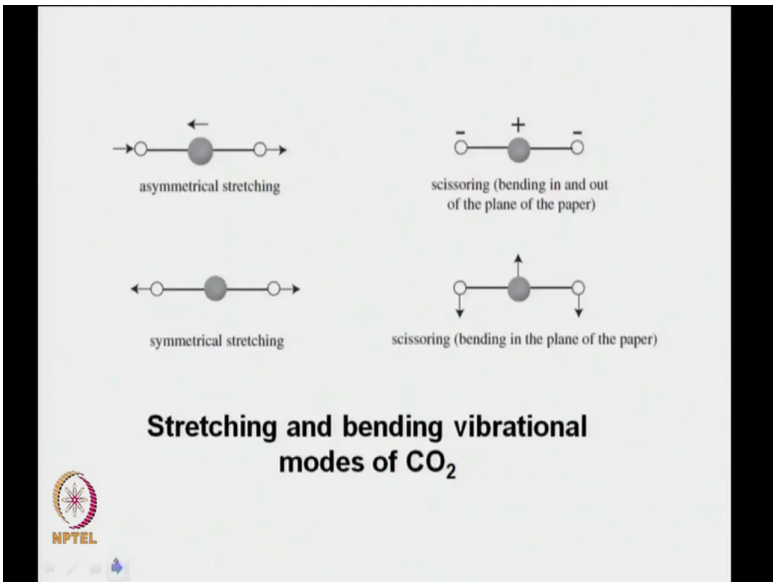

Now, the other one or the other example is for CO 2, you can see the first one is an asymmetrical stretching; the second one or well yeah the other second one is a scissoring or bending in and out of the plane. So, the plus and minus remember what we talked about then, just below the bending in and out we have the scissoring which is the bending in plane and to the extreme left bottom is the symmetrical stretching. So, these are the different vibrational modes of CO 2.

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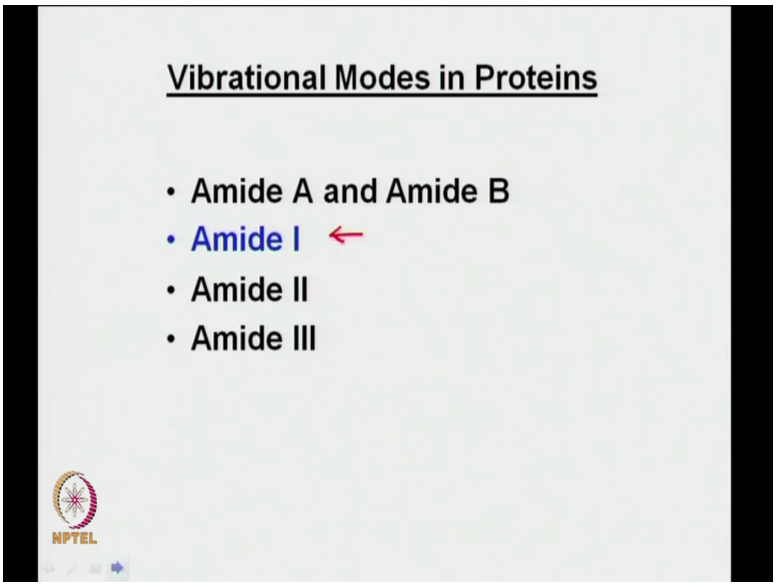

Now, what about the vibrational modes in proteins, now because you know this course we have mainly interested in the proteins how to study their different conformations and many other aspects.

So, what are the vibrational modes and I will you know tell you what they what they are and what I mean by these. So, the first one is referred to as amide A and amide B these are different vibrational modes. The second one is amide 1 and this is in a different color because this is the one we will be most interested in and we will be most discussing about, the third one is amide 2 and then you also have amide 3.

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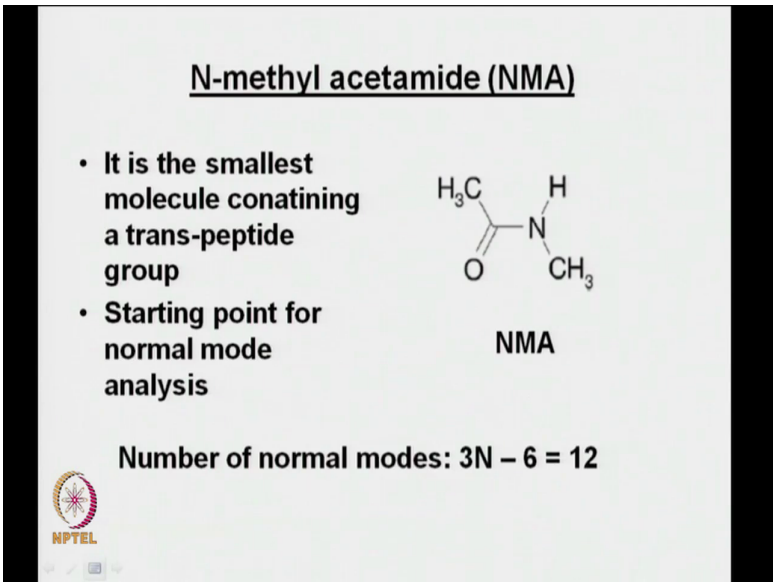

So, these are the different significant modes of vibration which are present in proteins and as I said or as I told you the amide 1 is the one we are going to spend some time on.

Now, if you would like to model the vibrations then you have to start from a very simple molecule right. So, the simple molecule we start with is called N-methyl acetamide and you will see why it is the one which is being used because you look at it Apeptide has many many amino acids right, but in this case you can see N- methyl acetamide. It is just one small unit and if you are going to do calculations on this then you will be having very accurate calculations.

So, this is called N- methyl acetamide, it is the smallest molecule containing a trans - peptide group and it is a starting point for normal mode analysis. Now if you would remember normal modes like if you have if you have these translational you know the translational modes,

rotational modes and then the other modes of vibration, people do normal mode analysis right. You must have done during your; you know group theory course.

Then for N-methyl acetamide the number of normal modes is given by 3 N minus 6 which is equal to 12. Now, this we know the 6 comes from the fact that you have 3 translations x and y and z and 3 rotations x y and z again. What is N, N is the total number of atoms I have in the molecule N methyl acetamide ok. So, here what we have out here is it is 3 N minus 6 which is equal to 12.

Now, moving on so; that means, in N methyl acetamide. We still will be having 12 normal modes not 1, not 2, but 12 normal modes ok.

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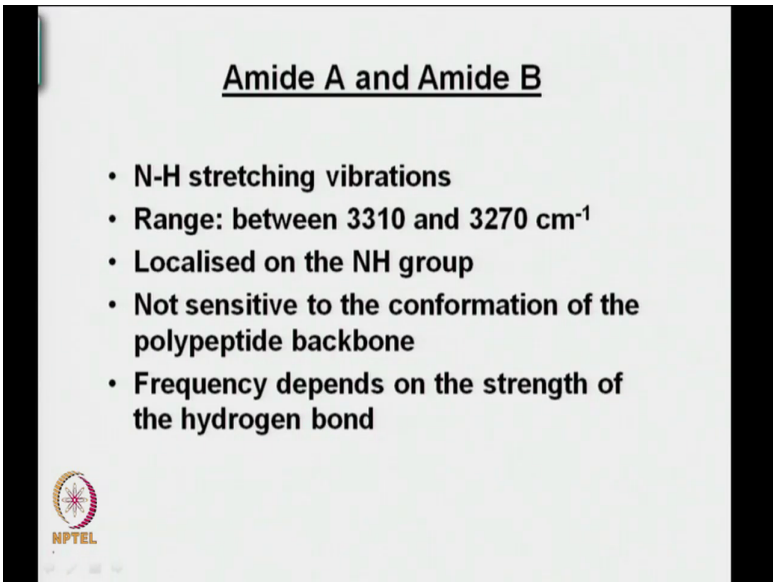

What about amide A and amide B? See amide A and amide B are essentially N-H stretching vibrations right. So, the range between 3310 and 3270 centimeter inverse, they are localized primarily on the N-H group; N-H stretching vibrations that is what they are. It is not sensitive to the conformation of the polypeptide backbone.

Now, this is a problem because see if you are in proteins you mostly are interested in changes in conformations which is the backbone you know alpha helix possibly changed to beta sheet or alpha helix changed to a random coil a beta sheet is changed to alpha helix or random coil and if this mode is not sensitive to that change then you would not be looking at this mode right to see what is going on with the protein conformation. And the frequency; obviously, depends upon the strength of the hydrogen bond because it is an N-H stretching vibration.

So, you can understand if you the change solvents, it would be it would be affected by the solvent because that is how your hydrogen bonding interactions would change.

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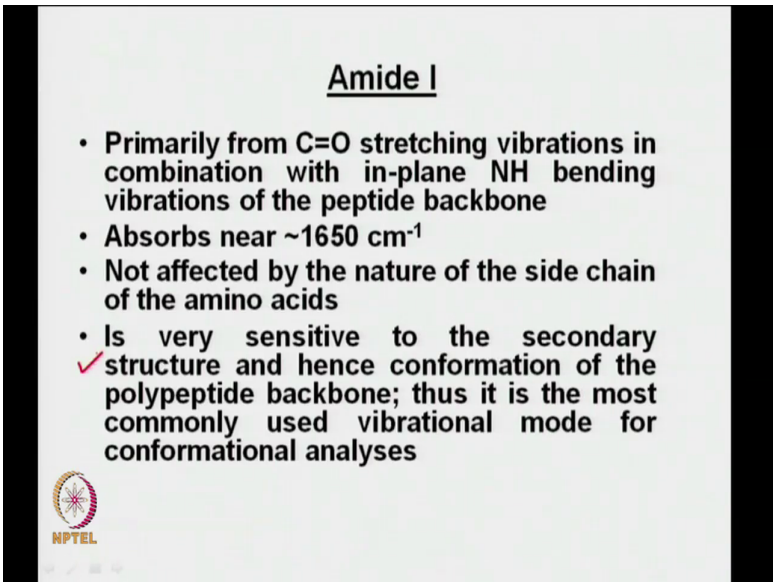

Now, what about amide 1 as I said this is the one we are going to be primarily concerned with. So, amide 1 is primarily from the CO stretching vibration remember the peptide bond we have a carbonyl group. It is primarily CO stretching vibration which constitutes the amide 1 band, but along with that there are small combinations from in-plane NH bending vibrations of the peptide backbone ok.

The CO stretching vibrations it will you know this absorbed near about 1650 centimeter inverse in proteins or peptides. These are not affected by the nature of the side chains of the amino acids. However, this is a very important point the last point is very important, why is it important see what it says these stretching vibrations are very sensitive to the secondary structure and hence conformation of the polypeptide backbone ok. Thus it is the most commonly used vibrational mode for conformation analysis.

Now, see what it means. What it means is this aspect was not available for the NH stretching or bending vibrations right. But when we are coming to the CO which is the amide 1 mode, the CO stretching which primarily constitutes the amide 1 mode. What we are saying is it is very sensitive to the conformation of the polypeptide backbone. Then; obviously, you can understand that if it is so sensitive to the polypeptide backbone then; obviously, this is the one we should be looking at or one should be looking at if you have to do some protein conformation analysis ok.

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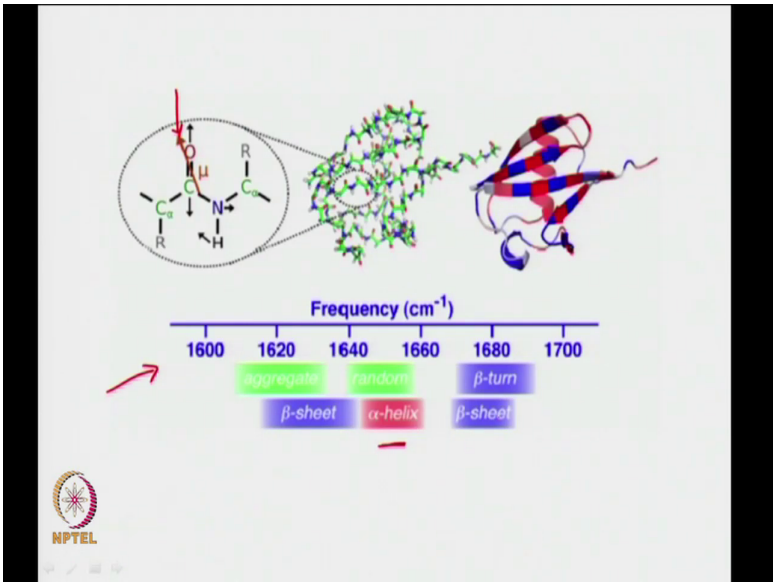

So, this is again what we are talking about. So, if this is a certain protein right and you can see this is kind of a beta protein mostly. Now you look at this mu if I can show you with the arrow; this mu is your corresponding amide 1 transition dipole moment.

Now, see though I said it is CO it is not exactly along this aligned along this C O; however, it is at a certain angle because you have some contributions from this NH in-plane bending modes right. But one is that so, this is the type of dipole moment you are looking at, but the second issue is you look at this scale. Now this is very important see, proteins will be having different conformations right either alpha helix or beta sheet combination of both random coil if you would denature your protein or if you take a taking any unfolded protein, then using the amide 1 band can I try to figure out which one is which.

So, this is what it says. You can see the red one is the alpha helix you can see alpha helix is in between 1640 1660 centimeter inverse then you have the random coil which is kind of in the same region. Then you have the beta turn which is at 1680 beta sheet and beta sheet you can see beta sheet occurs at two places because it has two antiparallel beta sheet when you are looking at aggregates it has essentially two bands one at about 1620 1625 centimeter inverse and the other one at about 1680.

Now, all these different patterns or all these different bands are characteristics of these proteins different proteins whatever structure you are referring to so; that means, what you are trying to do is if you have an helical protein you would be having a certain IR signal or spectrum. If you having a beta protein you would having a certain signal, if you having aggregates you know protein aggregates are the root causes of many neurodegenerative diseases like as Alzheimer's, Parkinson's.

Now, if you want to look at the aggregates protein aggregates how they are aggregating; if you want to look at the protein aggregates in kinetics, then in those cases you are the bands you would be looking at are two one is the 1625 centimeter inverse which is the pretty well developed band. And then there is a very small band at 1680 centimeter inverse its characteristics of antiparallel beta sheet aggregates ok.

Now, to look into it a little a little more detail the first question that comes to your mind is I understand that my amide 1 mode is. So, sensitive to the protein secondary structure changes

why is it so or how should I understand or how should I try to understand it is sensitivity to such changes in structure or conformation ok.

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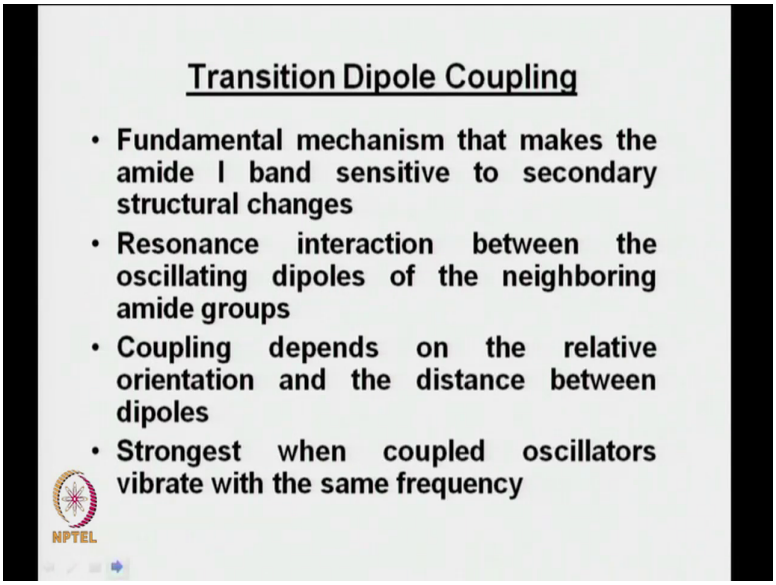

So, let us look at this first, the transition dipole coupling is the fundamental mechanism that makes the amide 1 band sensitive to secondary structural changes so; obviously, you think about these three words transition dipole coupling. We have already looked at the transition dipole in the previous slide which was mu which means is once you have a transition dipole this dipole has to couple with other dipoles.

Now, where will these other dipoles come from? Very simple. So, when we are talking about N methyl acetamide we have just only one unit and we have one dipole right, but when we are talking about a polypeptide or a protein we will be having many many amino acids depending upon how big or small the protein is.

So, now what will happen is, all the individual transition dipoles of these CO groups would be coupling with each other to give rise to the actual spectrum you have seen and that is what brings around the sensitivity this is the root cause of the sensitivity of your of this amide 1 band to conformational changes; that means, all the transition dipoles couple with each other remain delocalize over the whole protein moiety or molecule and they change or they are sensitive to any conformational changes which are occurring.

Going further what is it arising from as I just said it arises from resonance interaction between oscillating dipoles of neighboring amide groups, what it means is see we will be having one dipole say number 1, number 2, number 3, number 4. So, on number 1 interact with number 2, number 2 interact with number 3, these are adjacent number 3 and number 4 like that, but see number 1 is also interacting with number 2, 2 is interacting which is in this you know in once is, 1 is also interacting with 3; that means, every dipole is getting affected by the other dipole to some extent or the other. But, how; however, what will coupling depend upon as you can understand the neighboring groups would be having the maximum you know kind of interaction or coupling.

So, coupling depends upon the relative orientation; obviously, how oriented they are in terms of the geometry angle and the distance between the dipoles, say the dipole distance if you would recall you know the type of you know this Van der Waals coupling or dipolar interactions we are looked at before always we had this term this dependence upon the distance between the dipoles right. So, in this case too if you are talking about dipole or coupling then you have to talk about distance and what it means is the larger the distance the lesser is the coupling or the smaller the distance is the more is the coupling. Then; obviously, it is strongest when coupled oscillators vibrate with the same frequency you know that make sense.

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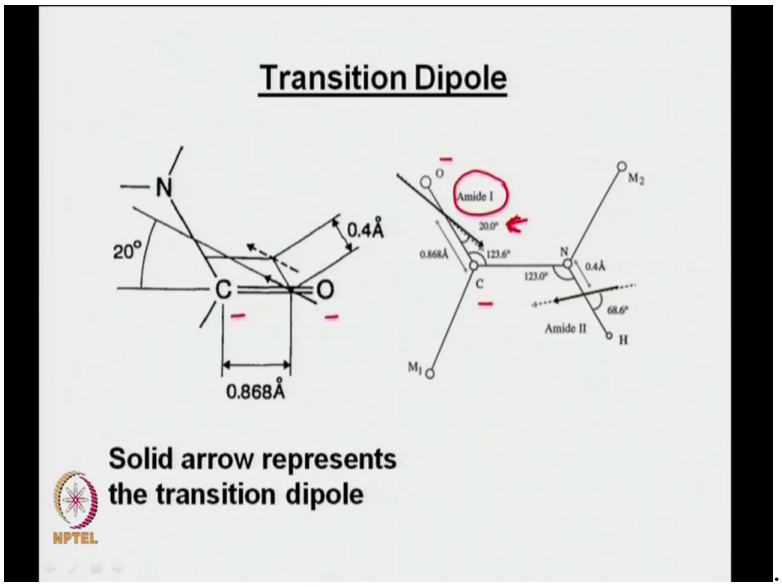

Now, let us look at the transition dipole again. So, here this is a zoomed into picture now you can see how the transition dipole is. So, this is the C N bond, this is the CO bond you can see the arrow of the transition dipole, do not worry about the broken arrow it is not of much consequence out here, but this arrow this is typically how the transition dipole is aligned right.

So, the solid arrow represents the transition dipole and if you would just reorient; that means, if you would just change the orientation of the molecule and this is how your transition dipole would look at. And please make sure that this is what we are talking about is the amide 1 right this is the amide 1 band; we are or amide 1 vibrational mode we are talking about ok.

Now, here we can understand in this case. So, this is C O. So, this one is CO right here; this one was CO we have just changed the orientation of the molecule, but look at this you look at this angle this angle of about 20 degrees. So, what it is saying is sorry. So, what it is saying is

that the dipole is so oriented it is about an angle of 20 degrees with the CO axis ok. So, it is just not perfectly aligned along the CO axis because it is minor contributions, but though minor these do change the orientation from the NH in plane bending modes and others ok.

So, this is the transition dipole on 1 unit; that means, on 1 peptide unit on 1 amino acid I mean now you know think about this because you have a sequence of amino acids all or each and every amino acid will be having this you know CO NH bond with the neighboring that is what it will have on the peptide backbone; that means, there would be so many and each of these would be coupling with each other. So, that the whole affect is kind of a delocalization of these interactions over the protein molecule.

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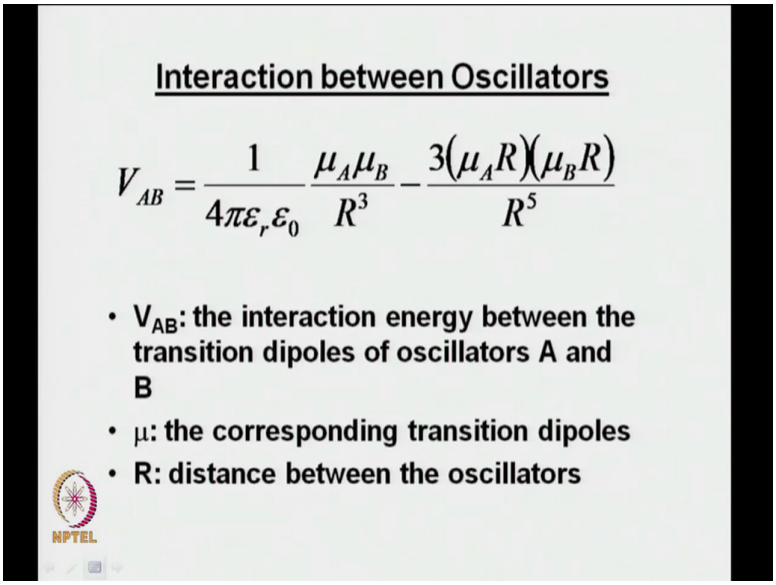

So; obviously, when we are talking about the CO NH bond then as I said we are going to talk about interaction between the oscillators because that is what dipole coupling is all about and

just to take a quick look and this you have looked at before. So, this is the interaction potential or the interaction energy V AB you can see what it is depending upon it is depending upon the mu A mu B; obviously, which are the dipoles associated with their oscillators and look at R you have this R cube dependence ok.

So, just to explain it a little more; V AB it is interaction energy between the transition dipoles of oscillators A and B right. It is s two different oscillators like two adjacent amino you know acids or two neighboring CO NH groups I mean. Mu the corresponding transition dipoles of A and B the oscillators and what is R, R is a distance between the oscillators ok.

So, this is the typical interaction energy you are looking at, now think about this again there will be so many As and so many Bs not only A you will be having A B C D E F G H depending upon the number of amino acids you have again and all these will be having their own interaction energy right. So, it so; obviously, then it will be a very well developed interaction ok.

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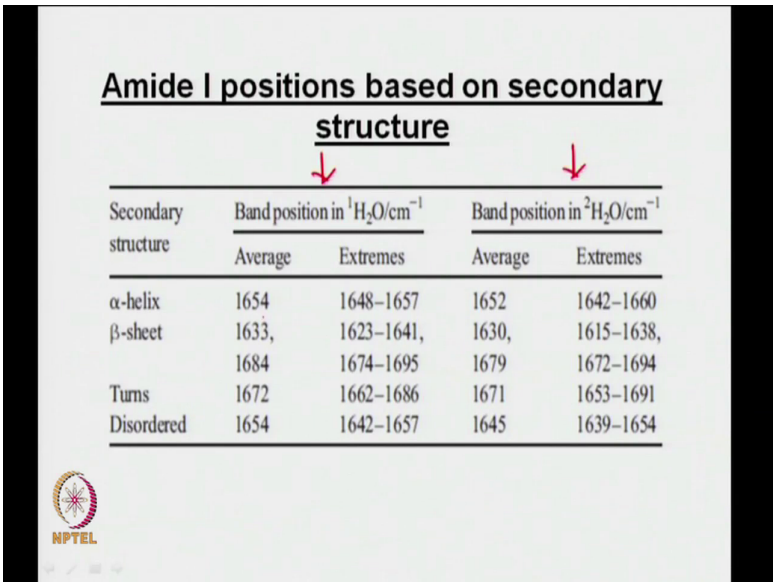

Now coming back to the amide 1 positions again, what the table shows you is the characteristic amide 1 position of your; you know IR spectrum; that means, the peak of your IR band for a given secondary structure. So, look at this for alpha helix now pay attention to this there are two columns out here right, one gives you the band position; that means, your IR band position in H 2 O; obviously, in centimeter inverse the other one is 2 H 2 O which is heavy order which is ditorated that mean D 2 O we can also write that.

Now, while we are talking about D 2 O this will become clear later as we move on with this discussion, but we will first just look at this, for alpha - helix the average is about 1654 centimeter inverse and the extremes are from 1648 to 1657. Now when you go to 2 H 2 O that is D 2 O then the average becomes 1652 and it is goes from 1642 to 1660 ok.

Now, beta - sheet again it will be having 1633 as the average in 1 H 2 O and the extremes are 1623 to 1641. Also you can look at this there is another band as 1684 now this is typically observed in anti parallel beta sheet aggregates right and these are the extremes which are given the second column; obviously, you are giving you the corresponding you know frequencies in D 2 O, then you have turns and then the disordered regions right ok.

So, this is now it is not that you will always have to go by this and this will always be maintained throughout. But at least this is a guideline for you to understand what is going on when you are saying starting with an alpha helical protein, you are denaturing the protein, how your spectrum going to change and all these things this is what is going to let you know ok.

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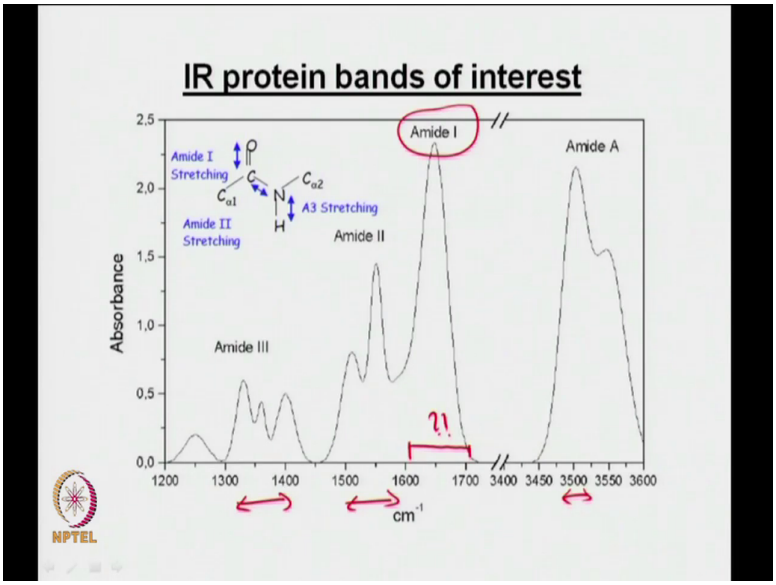

So, here is we are looking at kind of a complete spectrum from protein. So, you are looking at what, you are looking at the amide 1 stretching which is the CO stretching, then there is amide

2 we have not yet talked about and then there is amide 3 stretching as you can see these are mainly the modes which are shown out here CN NH and all these things, but we will look at this later right, but let us talk about the amide 1 mostly.

So, as you can see out here this is the amide 3 this is the range of amide 3 it is given this is the range of amide 3 ok, now this is the range of amide 2 it is coming out here and then amide A remember this is what we started with NH and obviously, the NH. So, this is where it this one comes and then this is the one we are interested in and this is the; this is the region we are mostly going to talk about or focus our discussion on.

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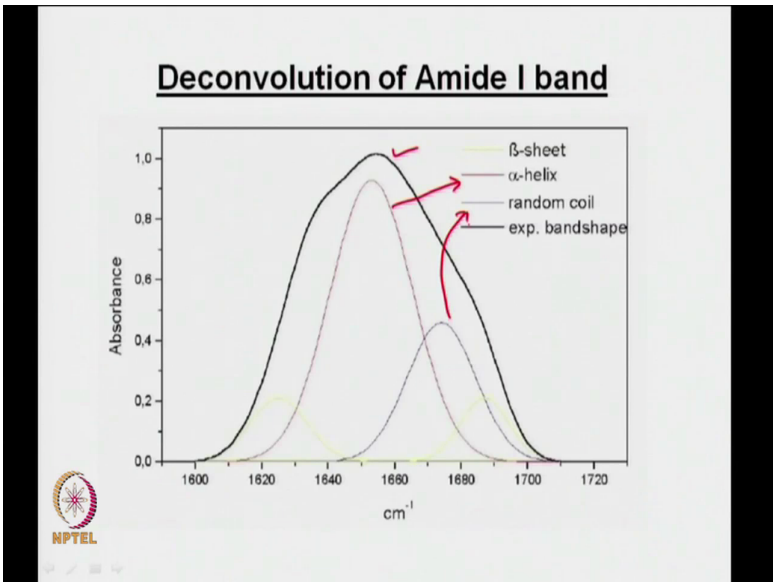

Now, let us look at the typical amide 1 band ok. So, this is the; this is this the black line shows a typical amide 1 band or shape coming from a certain protein ok. We do not know what the protein is, we do not have to know this is just an example so; that means, you have taken the

protein you have taken the IR spectrum now let us right now let us not worry about how you know what are the experimental issues, how should we do this experiment we will discuss those slowly.

But anyway what it says is that if you are given band like this an experimental band now see the experimental band is very broad now why is it broad, it is broad because as you can if you would remember the alpha - helix is the disordered structure is the beta sheet they have many overlapping you know they are very close to each other what you looked at was the central frequencies right.

That means the maxima, but they will be having their own widths right and hence they will be overlapping. So, all these will be merging together. So, how would you extract useful information from a given experimental band the way you do it is to do a deconvolution; that means, you try to deconvolute or try to extract the information out; that means, you try to decongest or deconvolute; that means, you try to take the information out from the given experimental band.

How do you do it, what you do what you do is you fit the spectrum to the side of Gaussian or Lorentzians whatever you want to right and then you try to figure out how much of alpha - helix I have, how much of beta - sheet I have, how much of random coil or disordered structures I have, how much of turns I have like this. Now these are nowadays done very regularly very frequently and you can see what this what you have under this experimental band.

So, under this experimental band shape the black one you can see you have 4 typical Gaussians, now what are these Gaussians corresponding to see. For example, this band corresponds to an alpha - helix so; that means, because this one almost as say the maximum intensity and almost all the maximum area; that means, mostly under these conditions your protein is alpha helical in nature ok.

Then this is the random coil and the rest is the beta - sheet. So, this is how what you have try to figure out so; that means, by fitting your experimental band shape to a combination of these

4 Gaussians, you have try to figure out how much of percentage. I have for belonging to alpha – helix, how much of the protein in terms of percentage belongs to the random coil and how much of the confirmation in terms of percentage belongs to the beta - sheet.

Now, this is typical of any protein you would take mostly you would be getting experimental band shapes which are pretty broad. But because you know because you know from the previous table what the you know central frequencies are for your beta or alpha then you can actually deconvoluted using Gaussians to try to get the percentage information of helices, beta- sheets, random coils.

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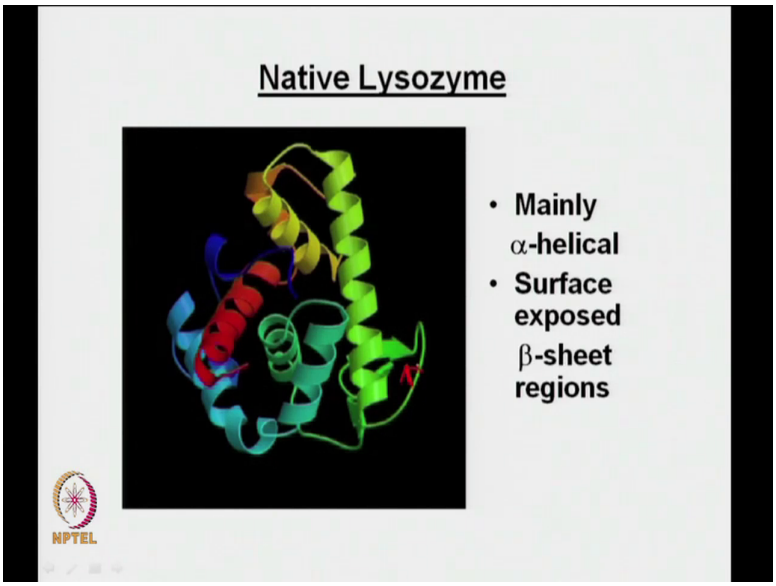

Now, to move on let us look at a typical protein now we are getting talking about a specific protein this is lysozyme and you can see the lysozyme is mainly alpha helical right you can see mostly alpha helices right. And there are some surface exposed beta sheet beta sheets regions

for example, you can see this one this is a beta sheet. So, what it means is you will definitely be having a alpha helices mostly then also be having beta sheet right.

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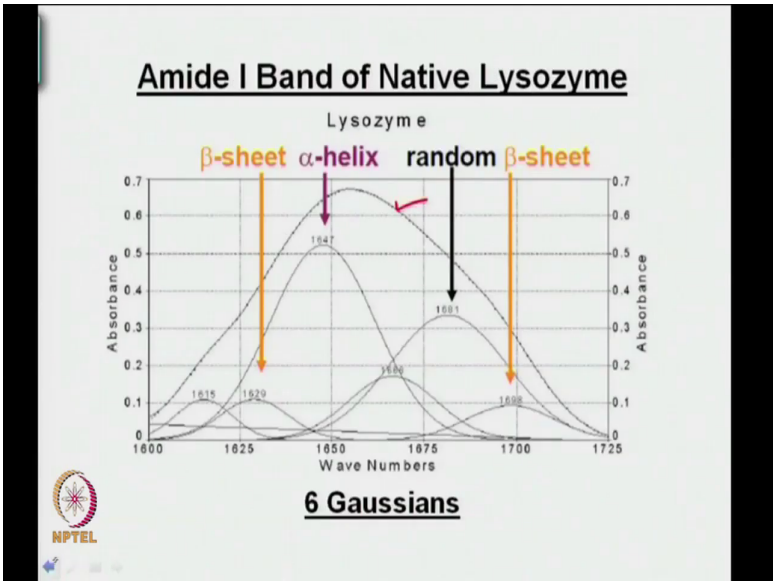

Now, this is the amide 1 band of native lysozyme see how complicated is. you are trying to deconvolute it. So, which was the amide 1 band. So, this black one is the amide 1 band, you are fitting the amide 1 band to 6 Gaussians spectra or 6 Guassians right to sum of 6 Gaussians. What are the different ones? One is alpha helix as I said you are going to expect that because that is predominant in this structure, then you have beta sheet, then you also have the random coil ok. So, that is how you have to you have to try to extract information out of this experimental band shape using this deconvolution method.

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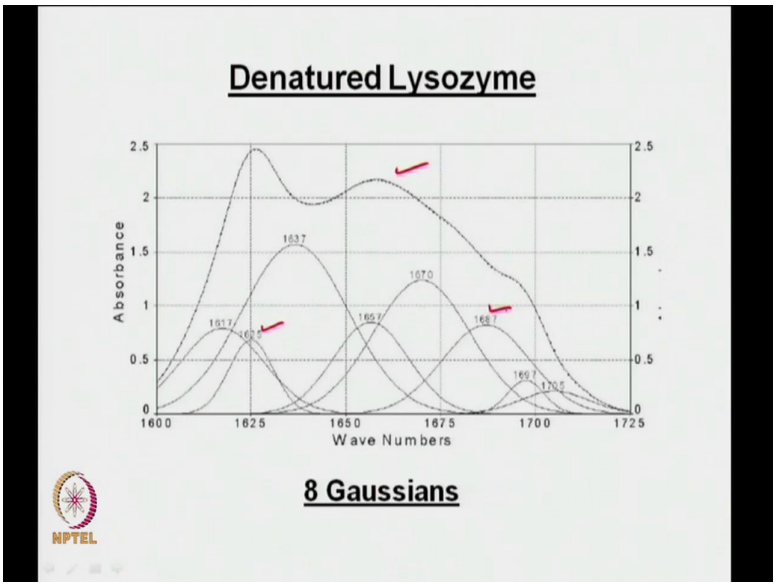

Now, suppose you take a same lysozyme and you denature, now the moment denature what happens is as you can see there has been a change in spectral shape right initially it was like this and the experimental band shape has changed.

Now, because experimental band shape has changed and we have discussed this at some line before there are denatured protein it is much more flexible is much more disordered; obviously, than a native protein. So, you can understand that possibly you will be needing some more Gaussians or more bands to fit this spectrum and that is what you have that is what it has been tried out here and there you can see people are using about 8 Gaussians to try to make some sense or try to figure out or try to kind of get some useable information from the experimental band shape right.

So, the underlying messages is that if you are given an if you are given an IR instrument you do you take an IR spectrum you take an if IR spectrum. Now, once you have taken the spectrum then that is just not the end you will have to do a band analysis, you have to do a decomposition, people also do second derivatives and all these things to get useful of information out. But if you know what you are doing, then in almost all the cases it is very it is you know this thing is kind of very well developed which you know what you are looking for, if you know what you are looking for, then you do not have much more problem you are trying to figured out what different conformational changes I am being having to deal with.

But let me tell you one thing you know proteins aggregate and when you denature proteins because your exposing hydrophobic groups depending upon the tendency of the protein or the you know the number of hydrophobic groups, the protein can either have a smaller tendency to aggregate or it can have a huge tendency to aggregate. The many proteins do aggregate at these denaturant concentrations and what will happen is, if you would increase your denaturant concentration because your exposing more of the hydrophobic groups that protein which has the more of a tendency to aggregate will actually start aggregating. And if they are forming anti parallel beta sheets, then what you will see is, you will see this you can see this 1625 band and this 1687 band you know these are typically coming from anti parallel beta sheet aggregates.

Now, is this solve what it is no many side chains also contribute, but we would not be going into that much details what I will do is, at the end of my discussion on IR spectroscopy I will give you some references. I think these references are extremely useful for anybody working on IR spectra of proteins for them to have a logical interpretation of a logical insight of what we can have under the experimental band shape.

So, again before leaving this slide the denatured lysozyme has an fit with 6 Gaussians right or rather the 8 Gaussians and the native lysozyme has fit with 6 Gaussians ok.

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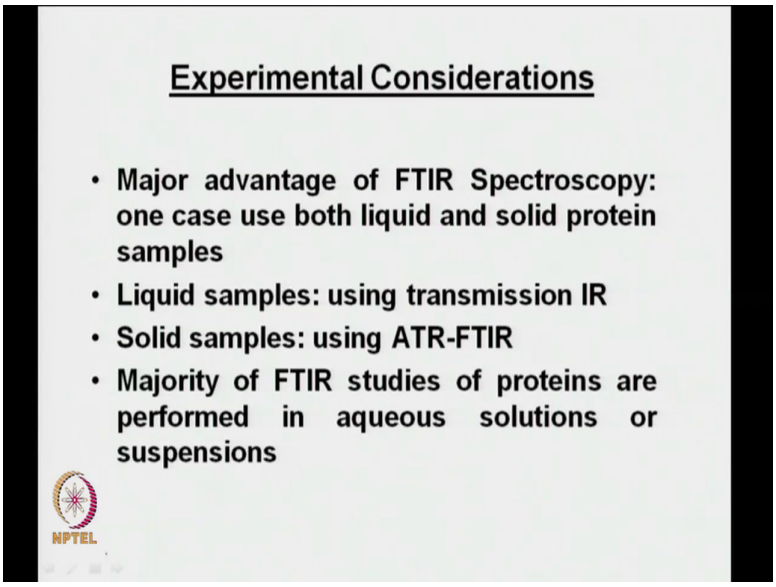

Now, let us move into some experimental concentrations. So, now, what experimental concentrations we are looking for, suppose you are going to do an experiment suppose you are going to measure an IR spectrum with the protein that of a protein.

Now, what is the major advantage of FTIR spectroscopy the one of the major advantages is one can use both liquid and solid protein samples. So, it does not matter So, suppose you know if you have a liquid protein sample good if a solid protein sample which means that your protein is not that soluble or it is hard for you to get a liquid sample whatever, but still you would be able to get an FTIR spectrum.

Now, would that FTIR spectrum of the solid sample be same as the liquid sample. Now that is the different issue all together I am not saying it will be differences are always found there are

reasons for that, but at least you can if you have a solid sample with you would be able to take a spectrum of that.

So, liquid samples can be done using transmission IR. It is like your absorption measurement, here you do a transmission and then you convert into absorption absorbance units. For solid samples you can use ATR-FTIR. So, one is transmission FTIR for liquid samples, the other one is ATR Attenuated Total Reflection internal reflection essentially we will talk about this again very briefly what is ATR technique is and why it is so beneficial.

Now, majority of the FTIR studies of proteins are performed in aqueous solutions or suspensions ok, now it makes sense right because your proteins your physiological environment your bodies water a huge body mass percentages water and; obviously, almost all the proteins are in water right.

So, it make sense then for you to take the IR spectra of proteins in water or if some protein is not soluble in water then you take an aqueous suspensions, but that is where the problem starts.

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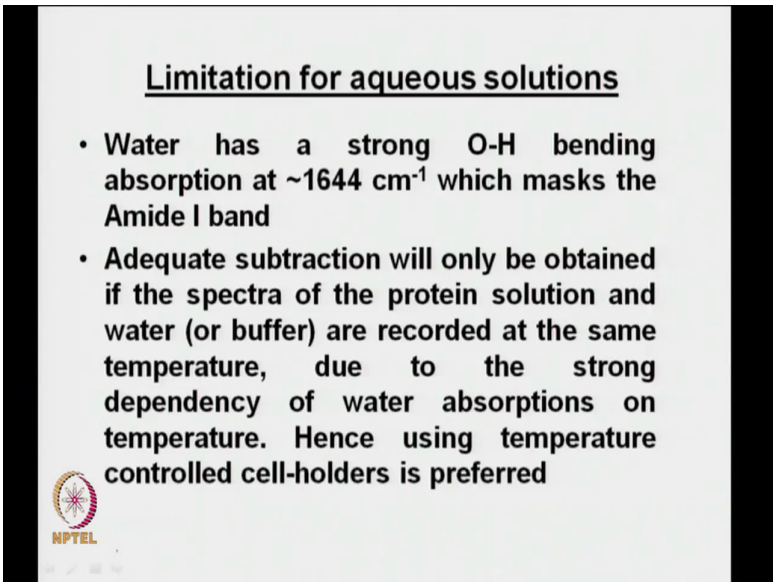

What is the limitation for aqueous solutions, now water has a strong O-H bending absorption at about 1644 centimeter inverse which masks the amide 1 band. Now if you would remember I would show you in the next slide the amide 1 band was kind of centre around 1650 right I mean it is an average position we are talking about.

Now, the O-H bending if it is a very strong O-H if water has a very strong O-H bending in that position which is say 1640 percent centimeter inverse then what will happen, it will try to mask your amide 1 band ok.

So, adequate subtraction so, now, what you can do is you can you can say that I will do a background subtraction and you know make sure that after subtraction the spectrum I have is that because that of the protein only. Now, adequate subtraction look at the second point adequate subtraction will only be obtained if the spectra of the protein solution and water or

buffer in the case of proteins are recorded at the same temperature, due to the strong of water dependency of water absorptions on temperature. Hence using temperature controlled cell - holders is preferred.

Now this makes sense you just cannot take the IR spectrum of the background which is water in this case no protein sample or buffer at one temperature and take the proteins are out of the temperature, because your intensities would be different you cannot do a background subtraction. So, the idea is that is if you are going to do it you make sure that your cell holder is temperature maintained for a proper subtraction.

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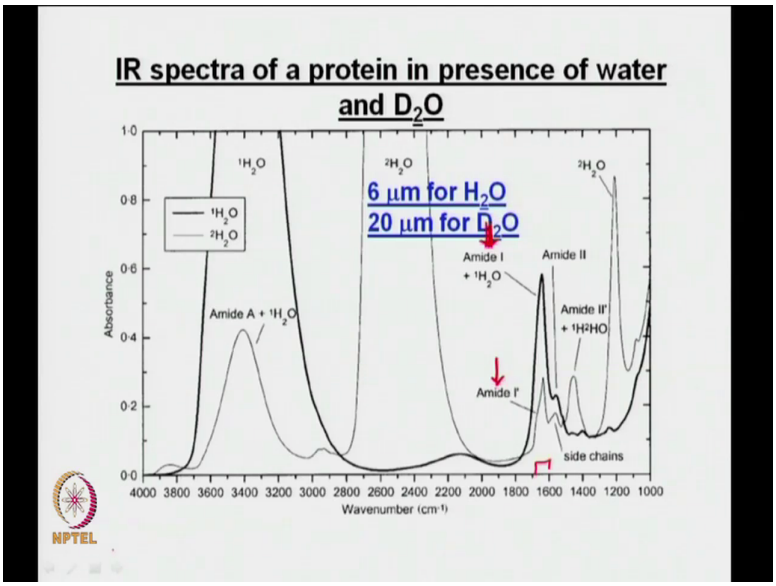

Now, this is the IR spectra this figure shows the IR spectra of protein in presence of water and D 2 O. Now look at this is your amide 1 band right so, this is the region I am I am interested in. So, this 1600 to 1700. So, again this is the region 1600 to 1700 centimeter inverse.

Now, look at this amide 1 plus H 2 O now look at the intensity of this amide 1 plus H 2 O sorry my arrow is becoming too broad you can see what the intensity of this you know band is in terms of is (Refer Time: 44:42) it is pretty high. Now this is for a protein in presence of water. So, the protein also has it is amide band here and it is in addition to the bending that O-H you know a bending mode of water, but look at what happens in the case of D 2 O.

So, when you had when I am talking of this amide 1 prime, amide 1 prime means that in prime means that I have not taken the protein and put it in D 2 O; that means, all the hydrogens have been exchanged replaced by D that is what the significance of prime is. So, if you have do not prime; that means, it is an H 2 O if you have a prime; that means, it is D 2 O ok.

Now see the moment you take it in D 2 O how much the absorbance is increased out there. Now what is the reason, the reason is and I will tell you later that the once you replace H by D then the vibrational that O-H that OD bending mode is far shifted out from the amide 1 region. So, the background from this D 2 O or OD bending is very very low at the amide 1 position.

So, you can understand that if you are going to do a transmission if you are going to do a transmission and if you would be having say a long path length, smaller protein concentration you would rather is D 2 O than H 2 O. Because for H 2 O, the longer the path length we use the more will be the absorbance and the more will be the masking were the amide 1 band and in that case your subtraction is not always proper ok.

So, what are the path lengths used? So, let us look at this, the for taking the water spectrum the path length was 6 micron right and for taking the D 2 O. So, this was transmission for taking the D 2 O the path length was 20 micron ok. Now, again look at this your water was just 6 micron. So, almost 3 times less than that of path length of D 2 O, but still see the intensity of the amide 1 band, which means this amide 1 band already has a huge amount of contribution from the O-H bending.

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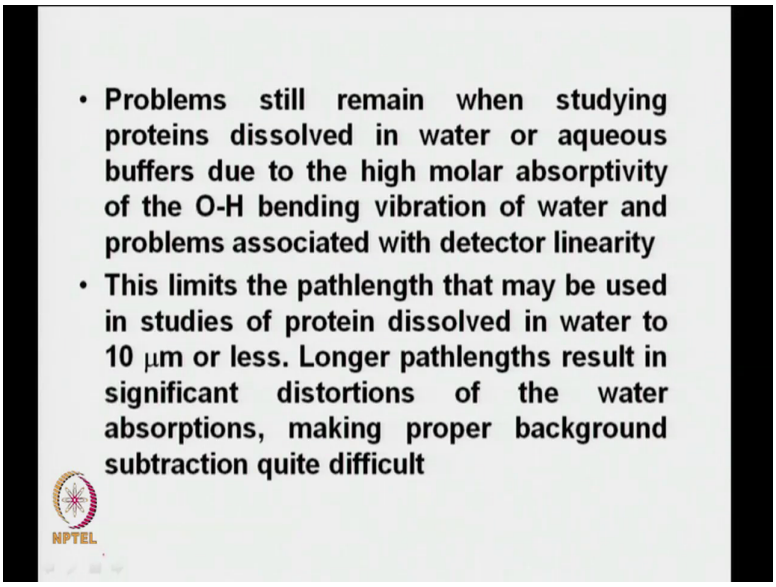

Now as we were discussing problems still remain when studying proteins dissolved in water or aqueous buffers due to the high molar absorptivity of the O-H bending vibration of water and problems associated with detector linearity.

Now, you might think that it does not matter, I will take only the water and I will take only the protein, I will take for the spectrum of water. I will take for the spectrum of protein, I will do a proper background subtraction and it will not be a problem. But the thing is that your background subtraction might not be a problem because the detector responds to the water concentration or to their high absorbance might not linear and then; obviously, your background subtraction is not the way you want it to be.

So, this limits the path length that may be used in studies of protein dissolved in water to 10 micron or less. So, you can understand if it is more than 10 micron what is going to happen,

the detector will not be giving a linear response to the change in water absorbance and if it is not going to give you a linear response then how would you have a proper workable background subtraction you cannot have it.

So, longer path lengths result in significant distortions of the water absorptions making proper background subtraction quite difficult this is what we just said. So, as you can see if you are going to use protein in water you make sure that the path length is really small and the protein concentration is high, because if the only if the protein concentration is high would in the amide 1 band be a huge amount of protein contribution.

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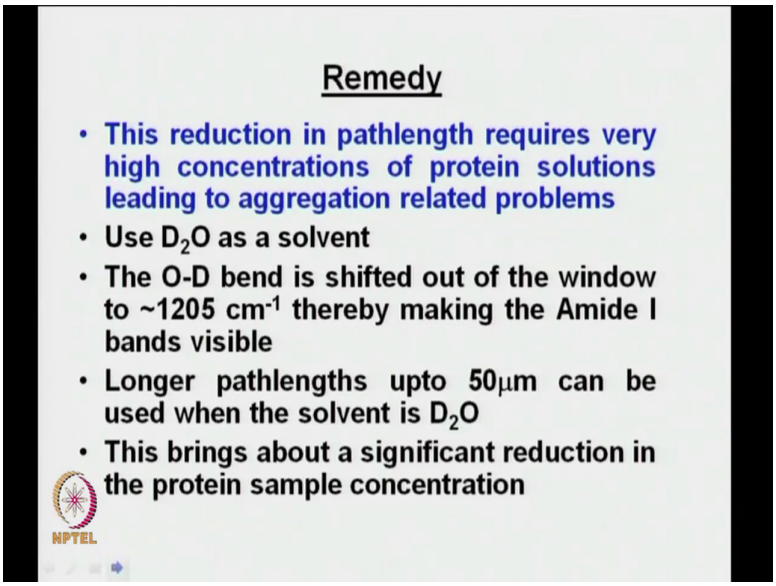

Now, let us look at it a little more deeply now; obviously, what is the remedy, the reduction in this reduction in path length requires very high concentrations of protein solutions because you see, if you would remember your Lambert Beers law. What is Lambert Beers law

absorbance is equal to epsilon the molar absorptivity molar extinction coefficient and C into L, C is the concentration, L is the path length the longer is the path length right the more is absorbance the same thing will happen here.

However what is the problem, the problem is that you can increase the path length go for a small protein concentration, but if you are doing it in water, the water absorbance would increase proportionately right again you will be running into a problem. So, you go for a smaller path length as we just looked at in the previous slide; now even if you go for a smaller path length; that means, to get a workable or what should I say decent or sizeable contribution or significant contribution of the protein amide 1 mode you have to make sure that you have taken a huge amount of protein concentration depending upon the size of the protein and the nature of the protein.

Now, this is not always so easily done, one proteins are sometimes not available to their extent because you know proteins can be very expensive to work with, number 2 is some proteins are very prompt to aggregation. So, if you increase the concentration, then they would easily aggregate. So, you would not be able to do the measurements you wanted to do with the protein.

So, then; obviously, the remedies again if you are doing transmission measurements all these i talking about transmission measurements I am not talking about any of these reflection measurements. That means, that on the solid sample or pastes all these are transmission IR measurement safety IR measurements I am talking about.

So, use D 2 O as a solvent as I just said The O-D bend is shifted out of the window to about 1205 centimeters inverse you know this is the bending I am talking about the bend in this case. So, that is what I am saying not band bend there by making the amide 1 bands visible and masked, longer path lengths up to 50 micron can be used when the solvent is D 2 O good.

The good thing is you can have a smaller protein concentration increase the path length without having too much of background contribution from D 2 O and still do a proper background subtraction because you are well within the linear range of the detector and the D

2 O bending mode is not even there. Now, this brings about as it says this brings about a significant reduction in the protein sample concentration.

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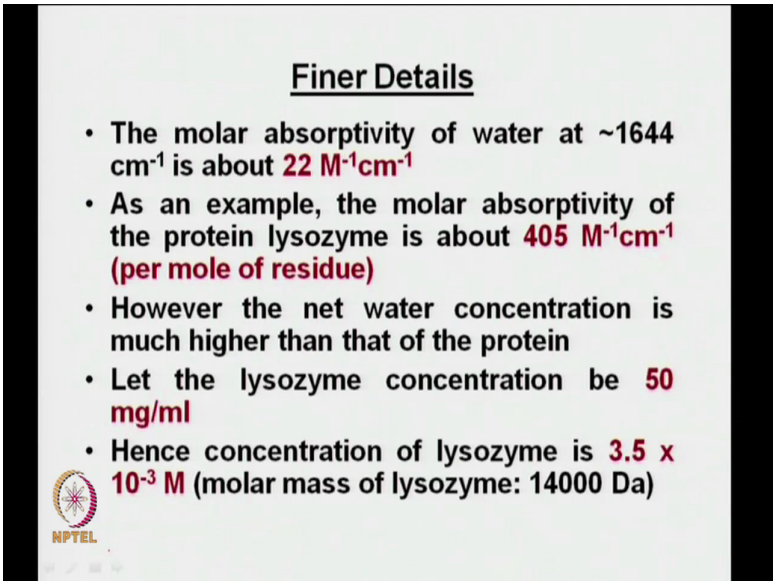

Now, what are the finer details? Let us look at this real quick and this is how we will end the class. The molar absorptivity of water at 1644 44 centimeter inverse is about 22 mole inverse centimeter; this is a molar absorptivity right ok.

Now, as an example let us taken an example the molar absorptivity of the protein lysozyme is 405 mole inverse centimeter inverse. This is per mole of residue means amino acid per mole of amino acid in lysozyme.

However the net water concentration is much higher than that of the protein right, why is it so, you will see in i mean you will see it very soon. Let the lysozyme concentration be 50 grams per ml now this is a pretty high concentration.

Hence based on the molar mass of lysozyme which I have taken to be approximately 14 kilo Dalton or 14000 Daltons, the concentration of lysozyme at this 50 grams milli grams per ml is 3.5 milli molar that is that is by no means a small concentration that is pretty high.

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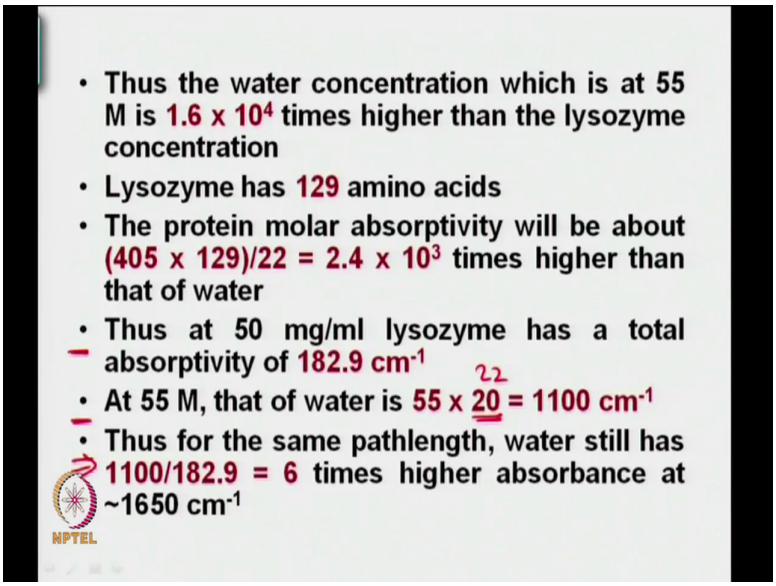

So, thus the water concentration which is at 55 molar because that is what the water concentration is, is still about 1.6 times 10 to the power 4 times higher than lysozyme concentration, because water is 55 molar and lysozyme we just saw was 3.5 10 to the power minus 3 molar right.

Now, lysozyme has 129 amino acids. So, as I said the molar absorptivity per residue was 405 mole inverse centimeter inverse. So, if you take this if you what you do is, if you take 405 times it by the number of amino acids which is 129 this is the total molar absorptivity of the protein, then what you do is you divide it by 22. So, 22 is what? 22 is a molar absorptivity of water.

So, what you see is now this is encouragingright what you see is that, the protein molar absorptivity is about 2.4 times 10 to the power 3 times higher than that of water good why should be going this is why. Thus at 50 milligrams per ml you look at the next point thus at 50 milligrams per ml lysozyme has a total absorptivity of 182. 9 per centimeter of path length.

At 55 molar now this is a key point. So, these two are the key points at 55 molar that of water is 55 it should be actually 22 anyway it should be 22 by mistake I have written 20 it is close to this.

Now, though in the third point on the slide you have to seen that the protein molar absorptivity is 2.4 times 10 to the power 3 times higher; however, the concentration of lysozyme is small and the net water concentration is very high. So, that actually over comes whatever difference you have.

So, here if you look at the last point, the last point says thus for the same path length; that means, if you are using the same path length water still has still has 6 times higher absorbance at about 1650 centimeter inverse that is what the amide 1 band absorbs. Now, no matter what you did no matter what you did, you increase the concentration to about you know 3 or 4 or 5 milli molar that is a very high concentration, but you look at the concentration of water it is 55 milli molar you see you can see how much of you know contribution from the O-H bend you would be having in this amide 1 mode.

So, the amide 1 mode when you see would be the actual contribution for the amide 1 mode of the protein plus that of water and that increase the total intensity ok.

So, what we will do is, as you can understand we will stop here in this class, but we will what we will what you can take home from here is that just taking an IR spectrum is not so easy there are some things you have just keep in mind. First of all you go to make sure whether you going to take the protein in water or D 2 O, if you have to do the protein in water if you have to do the protein in water because sometimes people say that if you exchange with D 2 O then what can happen is the protein conformation can change or some other effects can happen.

So, if you want to do it in H 2 O then you have to go for a very small path length less than 10 micron you know companies like Brooker, Nicolette they suggest 7 or 8 micron path lengths or even 6 micron path length cells right, but then you can understand these are less than 10 micron, but the what is the problem. The problem is yes, the water contribution would decrease, but think about the protein concentration how much you have to ramp it up by and if you are going to ramp it up, you can have many other RD facts like protein aggregation many other things coming in right which you do not want ok.

And the other thing is though the protein molar absorbance; that means, the whole absorbance of the protein is higher than that that of water it is just the concentration of water which is so high which is on the order of 55 molar which makes it so difficult for us to do a proper background subtraction if we have a protein dissolved in aqueous buffer.

So, that is why if you are doing a transmission measurement if you are doing a transmission measurement then in almost all cases if you would know that the protein is not going to get effected which in most cases they are like that then you would do it in D 2 O right. However if you do have to do if you do have to do a measurement with aqueous solution then what you will have to do is, you will have to resort to ATR- FTIR this is something we will take up in the next class and that is how we will end our discussion on IR spectroscopy.