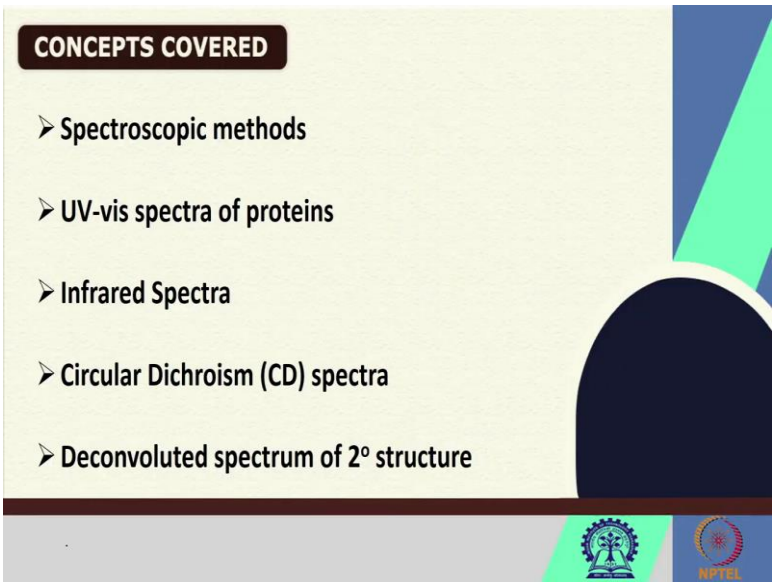
Fundamentals of Protein Chemistry
Prof. Swagata Dasgupta
Department of Chemistry
Indian Institute of Technology, Kharagpur

Module - 04
Protein isolation and characterization
Lecture - 18
Biophysical Methods - I

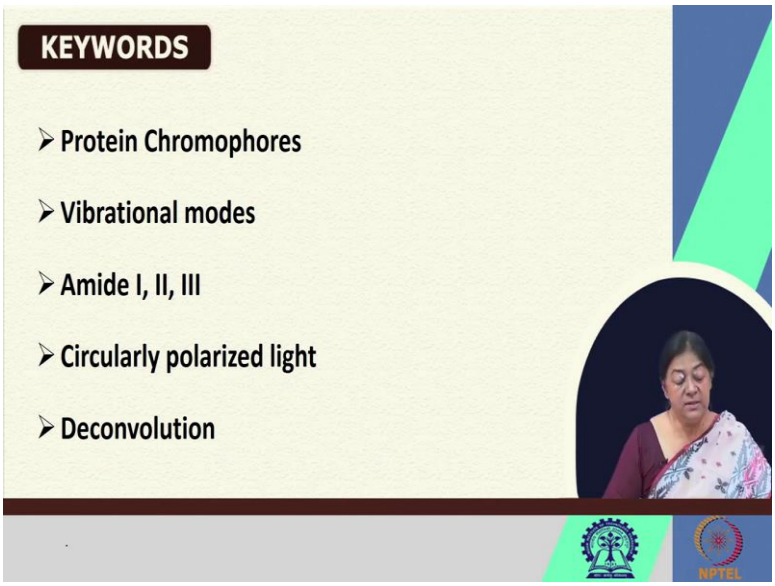
In module 4, we have been looking at protein isolation and characterization methods. What we will do in this lecture is look at some of the biophysical methods that are involved in the characterization as well as in the isolation procedures, to understand whether we have our protein of interest.

(Refer Slide Time: 00:33)



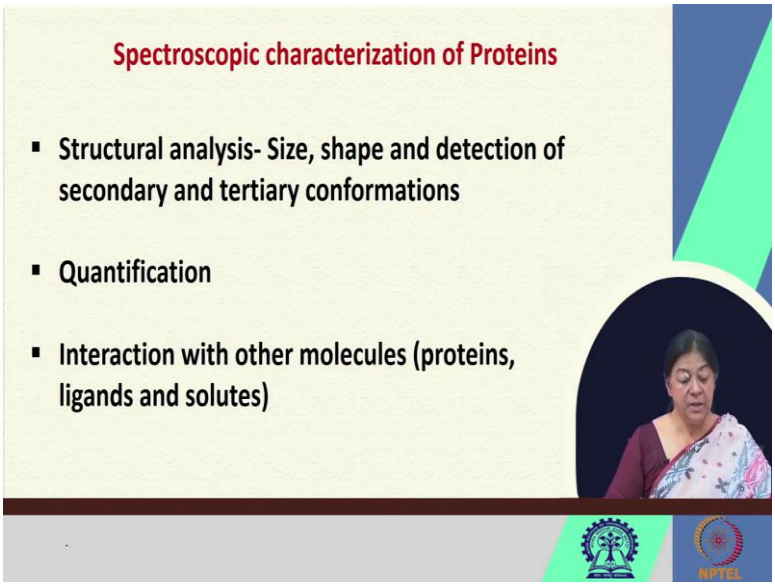
The concepts that are going to be covered are the specific spectroscopic methods associated with these types of characterizations in terms of UV visible spectra, infrared spectra and circular dichroism spectra.

(Refer Slide Time: 00:47)



And what we understand is the protein chromophores, how we would know that we would have a spectroscopic signal associated with the proteins and specific vibrational modes in terms of the IR spectroscopy techniques and what we look at for circularly polarized light.

(Refer Slide Time: 01:09)



In the spectroscopic characterisation of proteins, there are methodologies that are going to tell us about the size, shape and the detection of the secondary and the tertiary

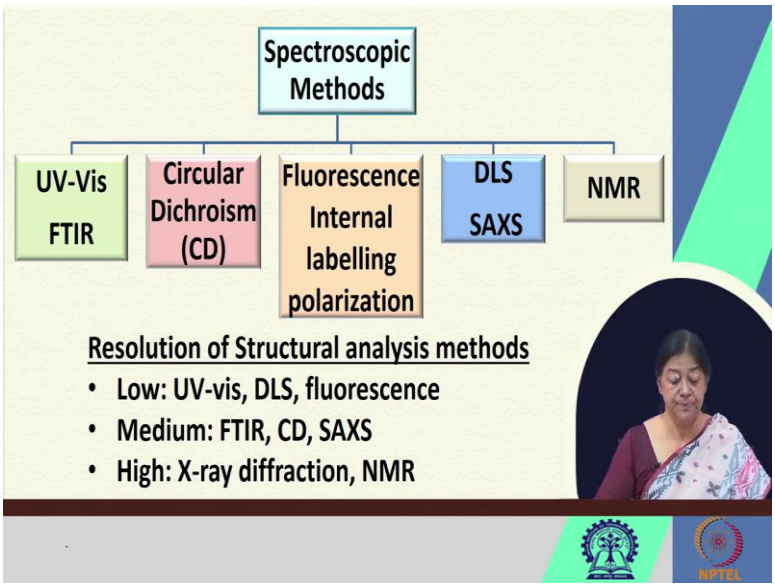
confirmations. It is important we know for the proper functioning of the protein, to have the proper structure of the protein.

To know that the secondary structure elements or the tertiary confirmation of the protein are in their correct three-dimensional confirmation and some of these aspects can be identified or can be determined by the specific spectroscopic techniques.

The quantification of proteins in terms of finding out their concentrations, is something that we have looked at in a previous lecture related to the amino acids, where we identified the specific amino acids that can comprise the protein chromophoric units.

In addition, when we will be looking at protein ligand interactions and protein-protein interactions, these spectroscopic aspects are extremely important to understand the binding characteristics and to give us some idea of the affinity of the ligand or the protein to other molecules.

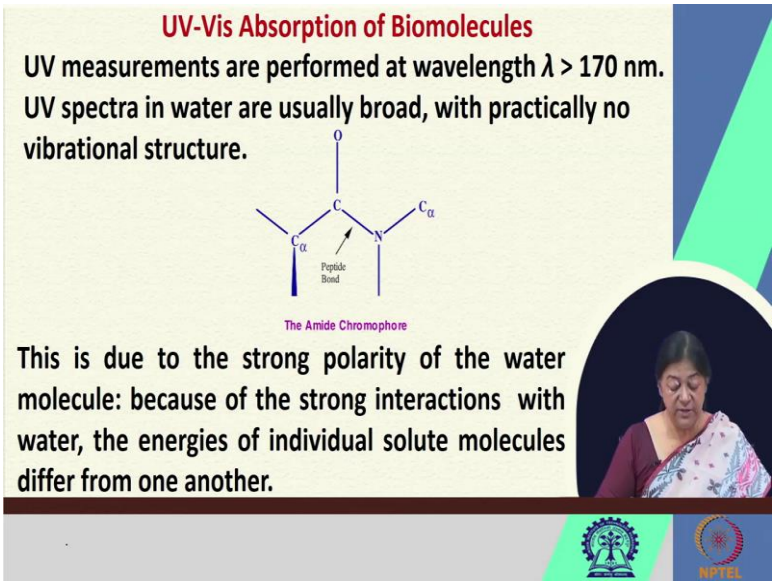
(Refer Slide Time: 02:27)



When we look at the spectroscopic methods that are important or are utilized in an understanding, we have the UV visible the FTIR, circular dichroism, fluorescence, DLS SAXS and NMR. Now, each of these have their own particular spectroscopic characteristics and they can be used for specific identification, characterization and determination of specific information or provide us with specific information and the resolutions are such that if we are looking for low resolution, we can look for UV visible

DLS and fluorescence. We are not talking about sensitivity. Sensitivity is very high for these methodologies; but the resolution of the structure analysis that we can use is low and then medium for FTIR and CD and very high for X-ray diffraction and NMR because we are looking at the specific atoms and the specific local environments for the structures.

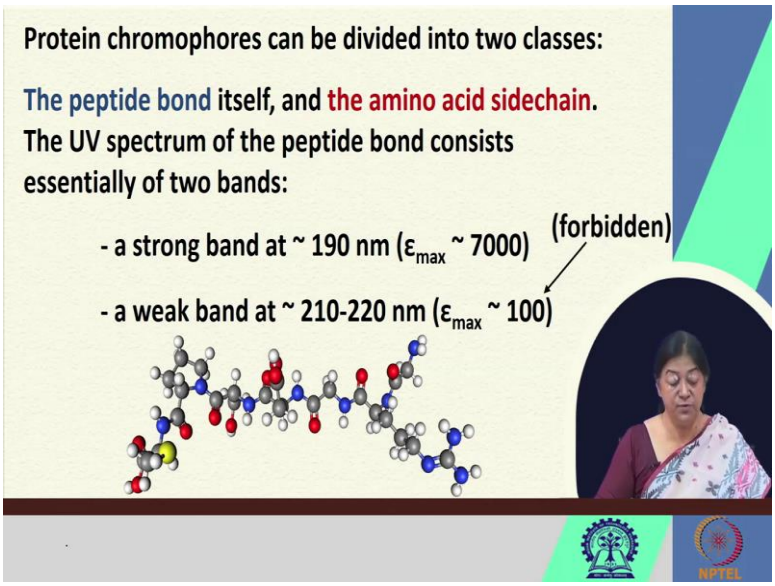
(Refer Slide Time: 03:33)



If we look at the UV visible absorption of biomolecules as we looked at, we had the UV spectra in water. Now considering that most of the protein solutions that we make are in a buffer solution, we have no vibrational structure as then, but we have the peptide bond that is also a chromophoric moiety information of the amide chromophore.

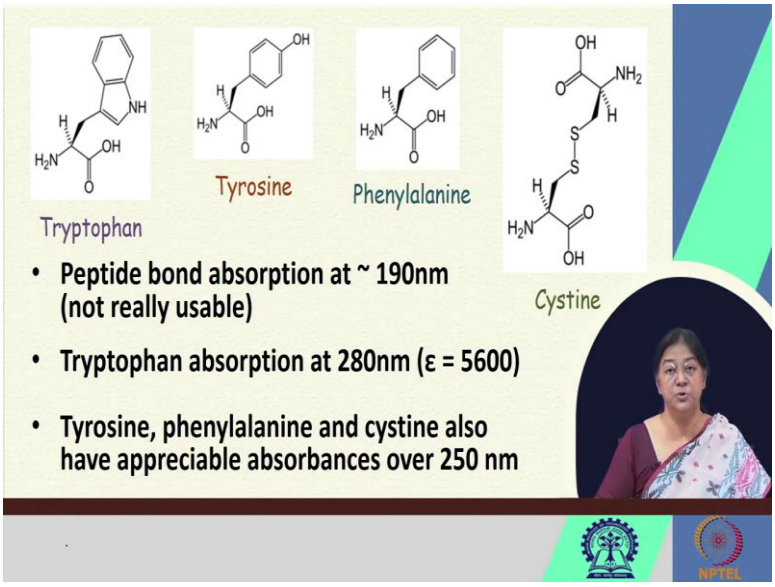
So the strong polarity of the water molecule, sometimes gives us a broad spectra with no vibrational structure per se, because with the water the energies of the individual solute molecules they will differ from one another.

(Refer Slide Time: 04:13)



But nevertheless, we do get specific information. We know that the protein chromophores themselves can be divided into the amide chromophore, that is the peptide bond itself and the amino acid sidechain and we have two strong bands here; one at 190 nm and the other at 210 to 220 nm that is essentially a forbidden transition.

(Refer Slide Time: 04:37)

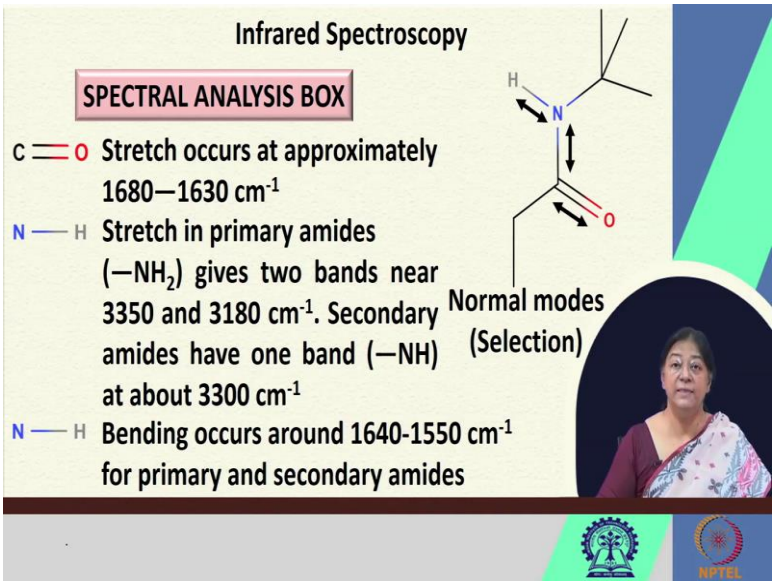


But nevertheless we have information from these aromatic sidechains and cystine, the disulphide bond formation that is formed between two cysteine amino acid residues.

These are the specific chromophoric moieties, for knowledge based information about our proteins.

The peptide bond absorption is not really usable, but the tryptophan absorption having a very high extinction which we learned before, is very important in addition to tyrosine, phenylalanine and cystine that also show appreciable UV absorption above 250 nm.

(Refer Slide Time: 05:17)

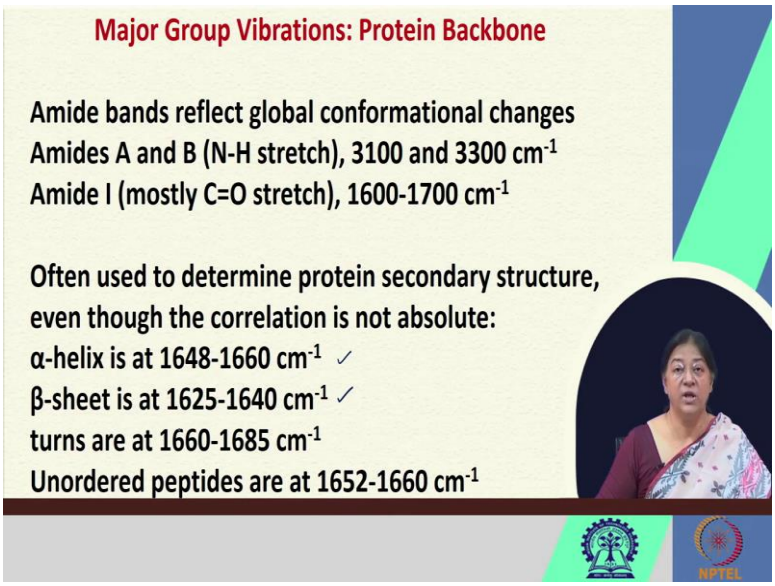


Each of the spectroscopic methods that we're briefly touching upon here, will be shown in their specific aspects in an understanding as and when we study specific techniques and specific ideas related to the subject or the topic at hand. When we are looking at infrared spectroscopy, we have normal modes and we have what are called stretching and bending of these bonds.

Now, this stretching and bending is going to give rise to the vibrational spectroscopy. In the stretching of the primary amides, there are variations when we have say hydrogen bond formation.

So whether we have a free C = O, N-H or whether it is involved in hydrogen bonding, where we are going to have variations in their frequencies, depend upon what type of interaction they are involved in. That is what we can determine from IR spectroscopy.

(Refer Slide Time: 06:39)



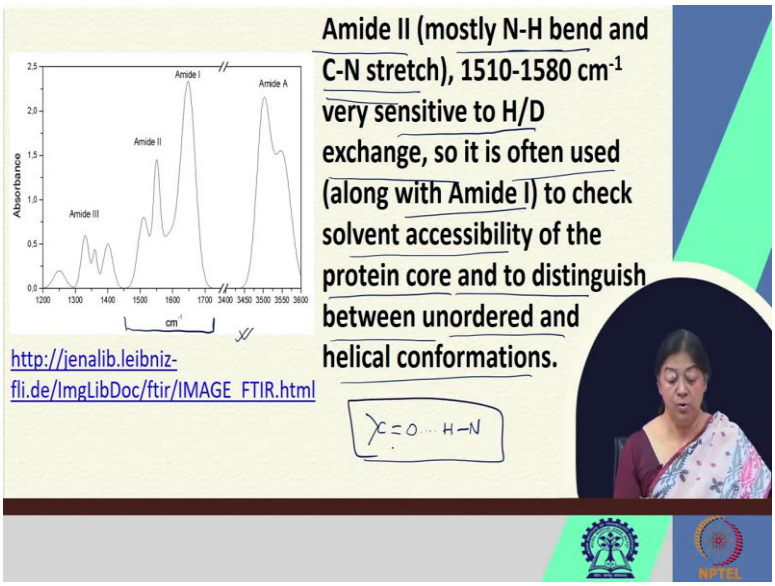
So we have the stretching and the bending modes that are important. In case of proteins, we have the major group vibrations because of the protein backbone. Now what information do we get from the protein backbone? We have the C = O and the N-H; a hydrogen bond formation that is possible and nevertheless, they can go through specific amide interactions in addition to the hydrogen bonding between them.

The amide bonds or the amide bands here actually reflect the global conformational change. We have amides A and B that comprise the N-H stretch, that is seen at 3100 and 3300 wave number that is given in cm^{-1} and amide I that corresponds to the C = O stretch.

Variations in the N-H and the C-O stretch, give us indication that there are some interactions involved. So what we have is the interactions we know in terms of C-O and N-H, are possibly hydrogen bonding interactions and where do we see these primarily? We see them in protein secondary structures.

Though the correlation is not absolute, we do have α-helix interactions where we can see them ranging from 1660 to 1648 cm^{-1} and for the β-sheet from 1640 to 1625 cm^{-1}. Now we have turns and we have other unordered peptides. So depending upon where their appearance is in our specific spectra, we can actually identify what kind of secondary structure we do have.

(Refer Slide Time: 08:19)



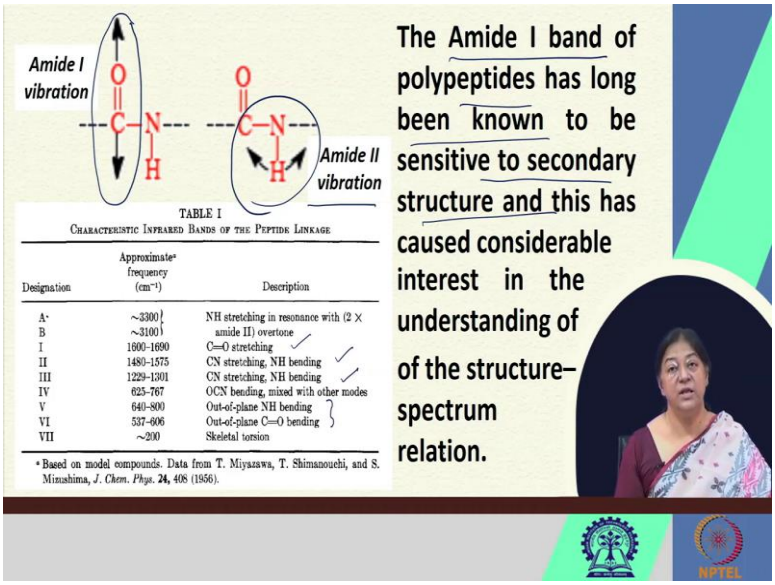
We have the amide I and the amide II that are mostly important for our protein determinations. This region here [refer to slide] of the amide I and the amide II spanning from 1700 to around 1500 cm^{-1}, is what we are interested in.

This information tells us that when we have the amide II, we have mostly an N-H bend and a C-N stretch. We are looking at the backbone information that is provided through the IR spectra. So, we have a very sensitive to the H/D exchange. We use this along with the amide I to check solvent accessibility, where we look at H_2O, D_2O, H/D exchange because we have our solution, our protein in D_2O.

We will look at these when we go to specific interactions; when we look at say protein legal interactions or determinations of other types of interactions. So, we can actually look at the protein core information to give us the unordered and the helical confirmations.

This information is important because we are now looking at a C = O, we have the stretching that could be important here and for the amide II, we have N-H bending and C-N stretching and the amide I will give the stretching.

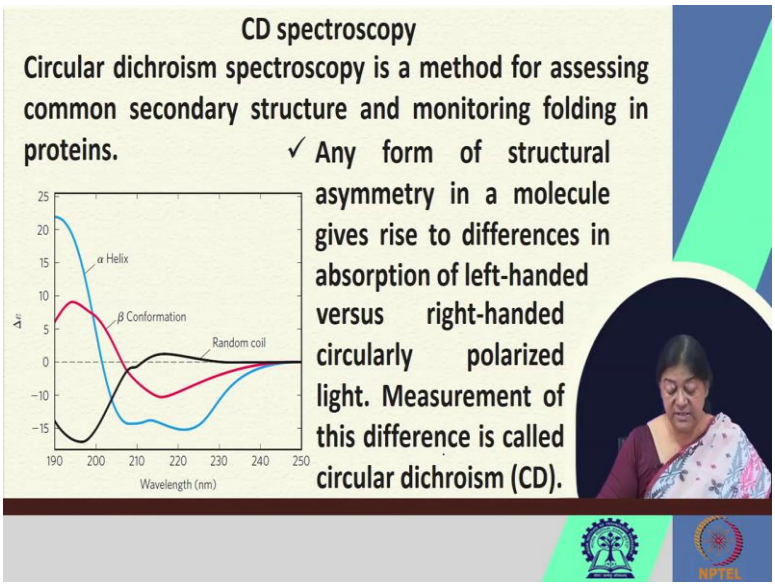
(Refer Slide Time: 10:13)



This provides us with information about the specific type of secondary structures that may be present. When we look at the amide bond, we look at the amide I that is the C-O stretching, we look at the amide II that corresponds to the N-H bending.

When we see the C-O stretching, we have the C-N stretching, the N-H bending; the C-N stretching and the N-H bending, then out-of-plane bending that is also possible, that gives us information about the specific interactions that are involved. For proteins, we look primarily at the amide I and the amide II. The amide I band for the polypeptides is sensitive to the secondary structure and this gives us a relation between the structure and the spectra that we can obtain for the specific protein.

(Refer Slide Time: 11:07)

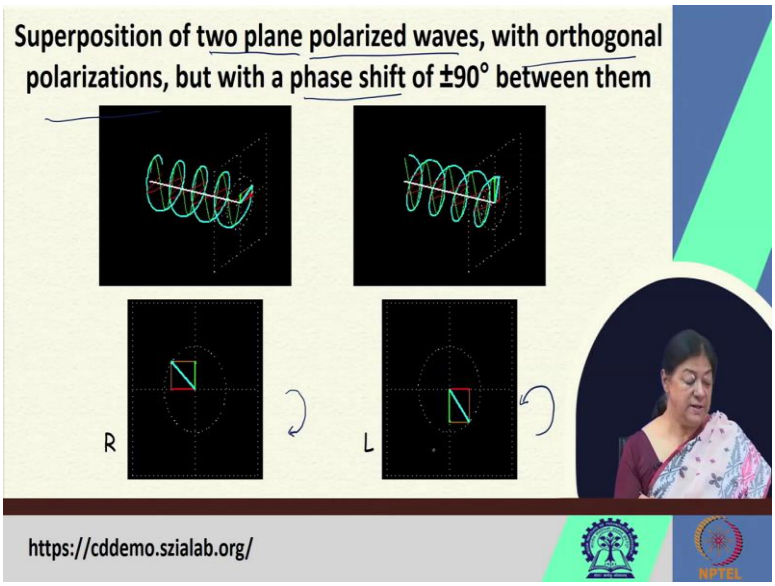


Another type of spectroscopy that is used very often is circular dichroism. Now circular dichroisms spectroscopy is again usually a method for accessing the common secondary structure and monitoring the folding in proteins. If we know that our protein is correctly folded, the content in terms of secondary structure, the α-helical content, the β-sheet content is going to give us a specific value.

We can actually quantitatively get the percentage say of the α-helix in the protein. An understanding of the structure or knowledge of the structure is essential. Therefore, from a structure stand point in terms of its function because we know that if the protein has to function correctly, it has to be folded correctly.

If it is folded correctly, then its secondary structural elements are in their correct confirmations and they have a specific secondary structural content in their structure. So, any form of structural asymmetry in a molecule, gives rise to differences in left handed versus right handed circularly polarized light. We will see what we mean by these terms in the couple of slides later on and the difference is called circular dichroism.

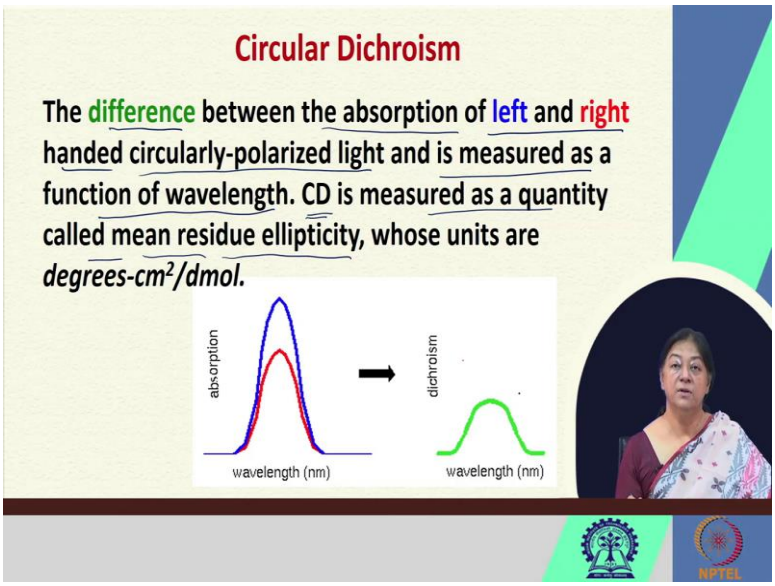
(Refer Slide Time: 12:35)



So, if we look at the superposition of two plane polarized waves, with orthogonal polarizations, but with a phase shift, we have this [refer to slide] as the right circularly polarized right and this as the left circularly polarized light. Now, when we are looking at any polarized light, we see that the left and the right when superimposed, it is going to give us linear circulizer. So, the important aspect here is we have two plane polarized waves that have orthogonal polarizations.

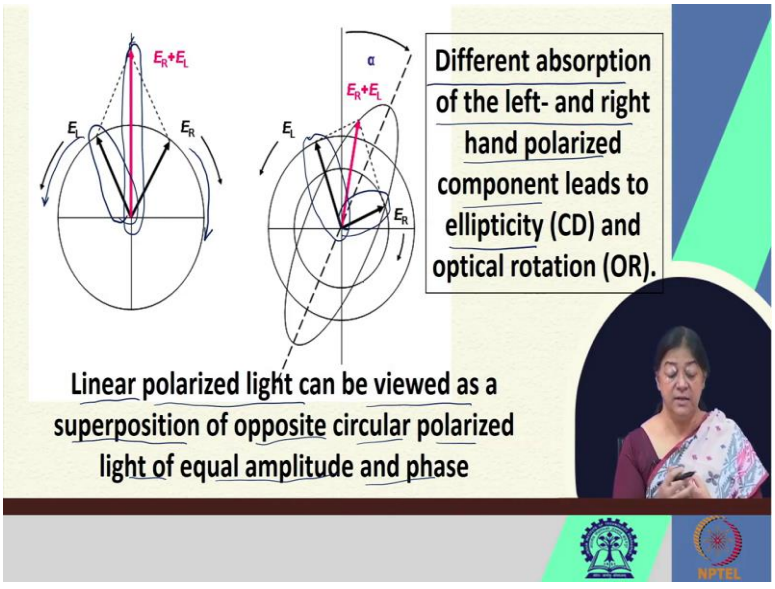
If we look at the animations here and we look at the rotation, we have the red and the green and the red and the green here [refer to slide]. The red and the green when combined give us the right circularly polarized light for the one on the left and what we see here is we see again the superposition with a phase shift that is going to give us left circularly polarized light.

(Refer Slide Time: 13:51)



Now, what happens is we are looking at the difference between the absorption of left and right handed circularly polarized light and this is measured as a function of the wavelength. This circular dichroism is measured as a quantity called the mean residue ellipticity.

(Refer Slide Time: 14:21)



So what we have is we have absorption giving us some different spectra, that is a manifestation of the sample through which this light has been passed through. So, when we are looking at right polarized light, that is going to be travelling in this direction

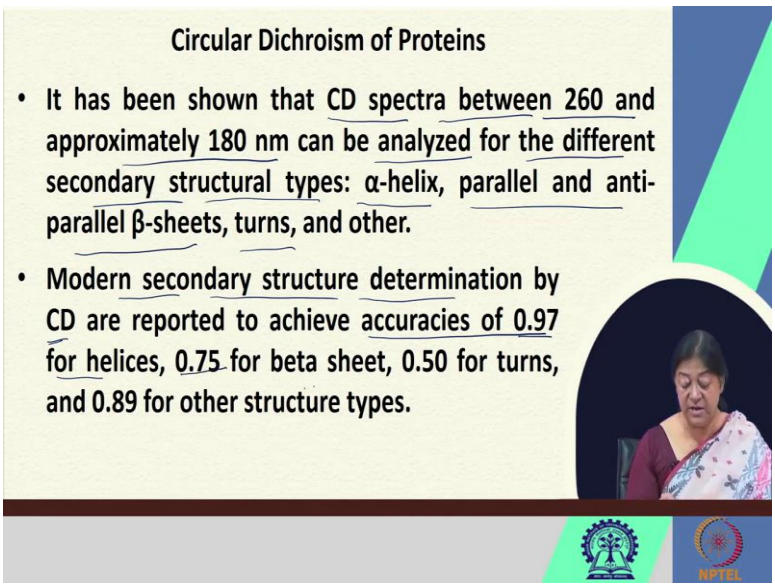
[refer to slide] shown by the arrow here or left polarized light that is going to be following the arrow on the left hand side, the summation of this is going to give us this linear light.

Now, what happens is if our sample absorbs the light differently. Essentially what we have is we have linear polarized light. This is viewed as a super position of the opposite circularly polarized lights or which are of equal amplitude and phase. So, if we have the right circularly polarized light and the left circularly polarized light and they are super posed, what we get is we get linear polarized light.

Now, if our sample is such that it absorbs preferentially one particular type of light, resulting in different absorption of the left and the right handed polarized light; what happens then is, we can see here [refer to slide] the left component is the same as it was originally; but part of the right component has been absorbed.

As a result of what has happened, we now have ellipticity because of a specific component in our material, it is called a rotational absorption. In this case our protein that has preferentially absorbed the right circularly polarized light and not the left, as a result of which we have got ellipticity.

(Refer Slide Time: 16:29)

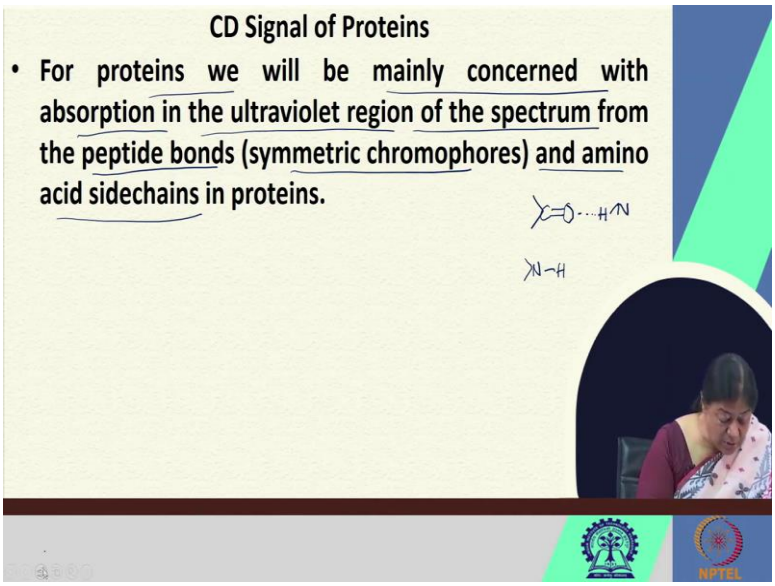


If you look at the circular dichroism of proteins, the CD spectra that is found between 260 and approximately 180 nm, can be analyzed for the different secondary structural

types. So we can look at the characteristic spectra, CD spectra of a protein and distinguish α-helix, parallel and anti parallel β-sheets, turns and other random structures.

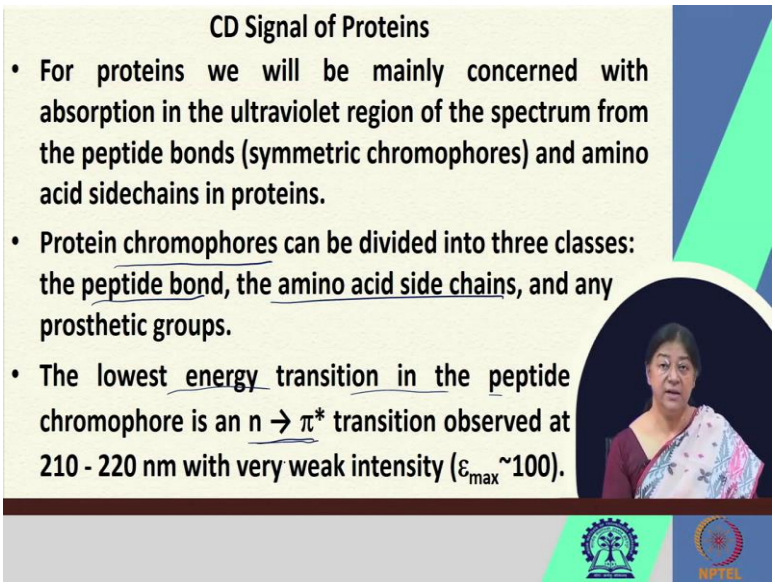
The identification of an α-helix tells us a lot of information about the proteins in terms of its secondary structural units. In fact, the modern secondary structure determination by circular dichroism actually can achieve accuracies of up to 97% for helices and 75% for β-sheets.

(Refer Slide Time: 17:25)



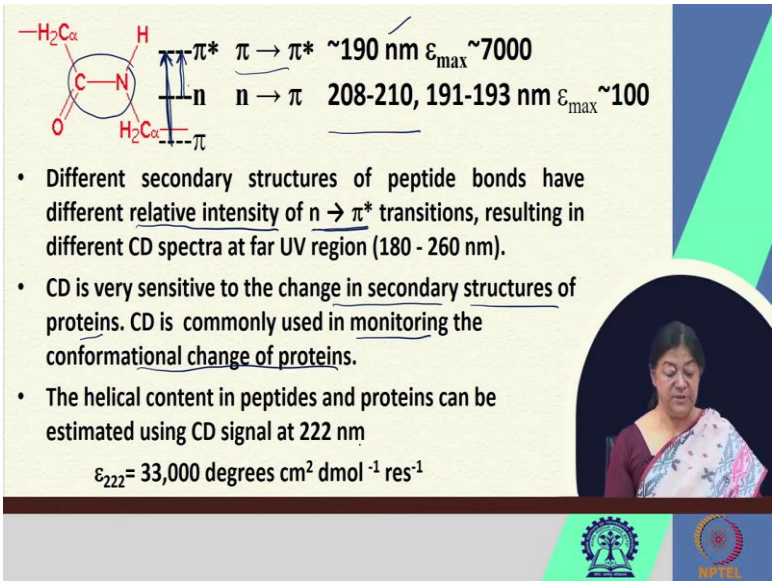
The CD signal is important here. So when we look at the CD signal, we look at the proteins. We are mainly concerned with the absorption in the ultraviolet region of the spectrum. So when we have a peptide bond formation between the C = O and the N-H, we find specific rotational strength because of the helix formation or hydrogen bond formation. We are going to have specific hydrogen bond formation because of the specific geometry that we can see for these molecules.

(Refer Slide Time: 18:25)



When we are looking at the specific geometry of the molecules, we know that the α-helix and the β-sheet have specific geometry. So, the protein chromophores in this case are the peptide bond and the amino acid sidechains. The lowest energy transition in the peptide chromophore is an n to π* transition, which we had observed or we had discussed earlier in our discussion for the peptide bond and its spectral characteristics.

(Refer Slide Time: 18:49)



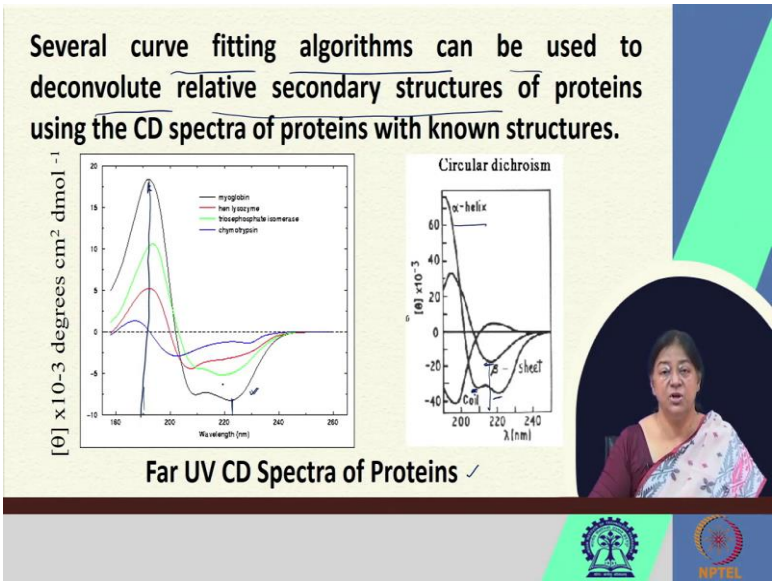
In our peptide bond here [refer to slide], we see a transition from an n to π*. So we are looking at a π to π* transition, means we are looking at this transition and n to π* means we are looking at this transition.

So, a π to π* transition is going to be one that is going to be of a lower wavelength and an n to π* transition, the energy gap being lower, is going to correspond to a larger wavelength. What we can get from this information is, different secondary structures of the peptide bonds have different relative intensity of their n to π* transitions.

This results in different CD spectra and the CD is extremely sensitive to the change in the secondary structure of the proteins and it is very commonly used to monitor the conformational change of the proteins. And the helical content of the peptides and proteins is estimated by a signal at 222 nm, as well as 208 nm.

So when we look at a CD spectra, it is very easy to identify the specific characteristics of the secondary structure because we have the relative intensity from the n to π* transitions that are being monitored in our specific spectra.

(Refer Slide Time: 20:19)



There are several curve fitting algorithms that are used to deconvolute the relative secondary structure of proteins and these are actually given by the specific CD spectroscope, the CD machines that we use and the far UV spectra. So we will have a band here [refer to slide] at 190.

This 190 corresponds to the π to π*. When we are looking at the other region here [refer to slide], we have a negative band at 222 and 208, this is characteristic of an α-helix. So here is the α-helix, where we have the 208 and the 222. If we look at the β-sheet there will be a combination around 217 nm.

Now, when we have a protein that has a combination of an α and a β structure, we will see some combinations. This is a typical α helical structure. Then we will look at the characteristic spectra that we observed, where we can see variations of the spectra because of the differential content of the secondary structural components that are present in the protein.

UV visible absorption spectroscopy is going to give us the concentration of the protein, it is going to give us relations related to the presence of a protein. So, when we have studied the protein chromatographic techniques, where we have looked at the isolation of proteins, we detected the presence of proteins using UV absorption spectroscopy because we know that the presence of the aromatic amino acids residues or any chromophoric moiety is going to give rise to the UV spectrum.

When we look at the specific vibrational spectroscopy, we are looking for specific characteristics in terms of the stretching and the bending modes of the bonds and when we term this as looking at secondary structural elements, we are looking at the C = O and the N-H.

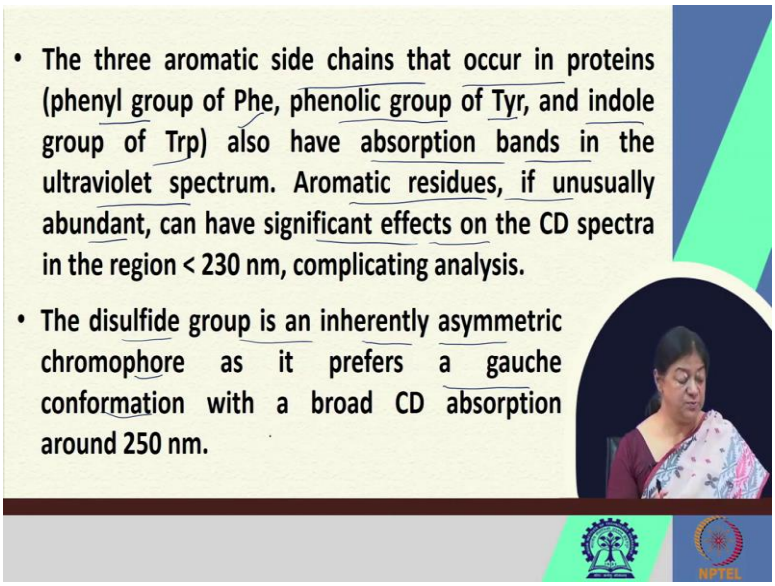
This information is important because any interaction associated with the backbone is going to have an effect on the overall confirmation of the protein, that is going to obviously affect the structure of the protein. And we know that since the structure and the function are related, any variations in the structure, is going to amount to a variation in the functional characteristics of the protein.

An idea of looking at say the secondary structure component which is an important part of a folded protein, is going to give us information that we would require to see whether we have the correctly folded structure. So when we are looking at the IR spectra, we are looking at the amide I and the amide II. The information from these C-O stretching, the N-H bending and the N-H stretching, gives us information about whether there are any variations.

When we are trying to understand whether we have the folded protein or the unfolded protein, there was specific spectroscopic characteristics that we could look at to monitor this effect.

We could look at IR variations, we could look at UV variations and now we see that we can look at also our circular dichroism variations, that will tell us that there is a disruption in the protein structure because of denaturating conditions.

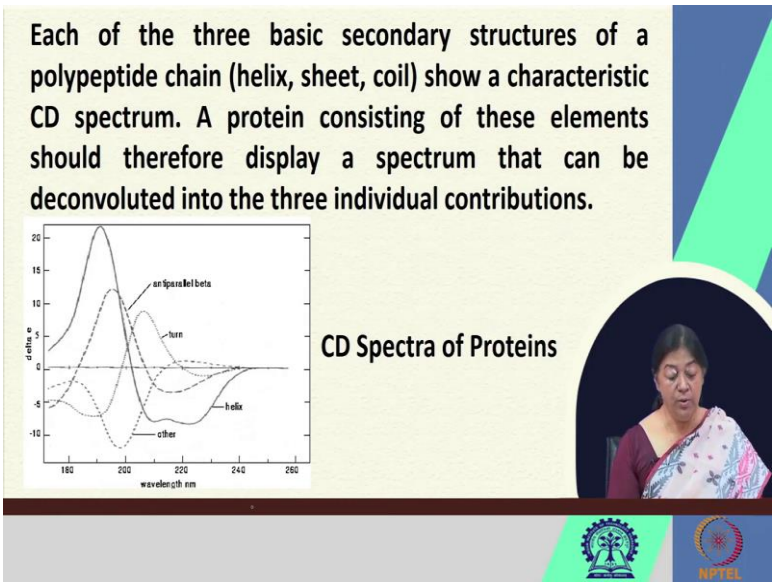
(Refer Slide Time: 24:43)



The three aromatic sidechains that occur in proteins the phenyl alanine that has the phenyl group, the phenolic group of tyroscene that is the OH and the indole group of tryptophan, they also have absorption bands in the UV spectrum as we were just discussing.

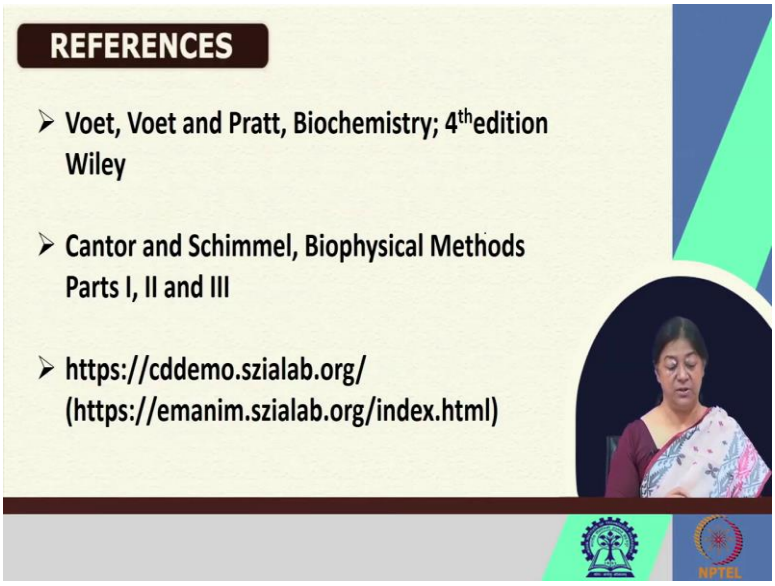
So, the aromatic residue which is unusually abundant, sometimes has effect on the CD spectra. But nevertheless this is more of a transition that we spoke about. In addition the disulfide group, which is inherently an asymmetric chromophore because it prefers a gauche conformation, with a broad CD absorption around 250 nm. But the range that we are looking at, is somewhere around this region [refer to slide].

(Refer Slide Time: 25:39)



Now each of the three basic secondary structures; the helix, the sheet and the coil, show a characteristic CD spectrum and this is the CD spectrum of the proteins and this can be deconvoluted into three individual contribution. So we can say, that this is the α-helical content, this is the β-sheet content and this is the content of random coil. And any variations related to protein ligand interactions, protein-protein interactions or even protein unfolding, can be detected from these methodologies.

(Refer Slide Time: 26:13)



There are many other animations that can be looked at; the books, Cantor and Schimmel and Voet, Voet and Pratt.

Thank you.