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

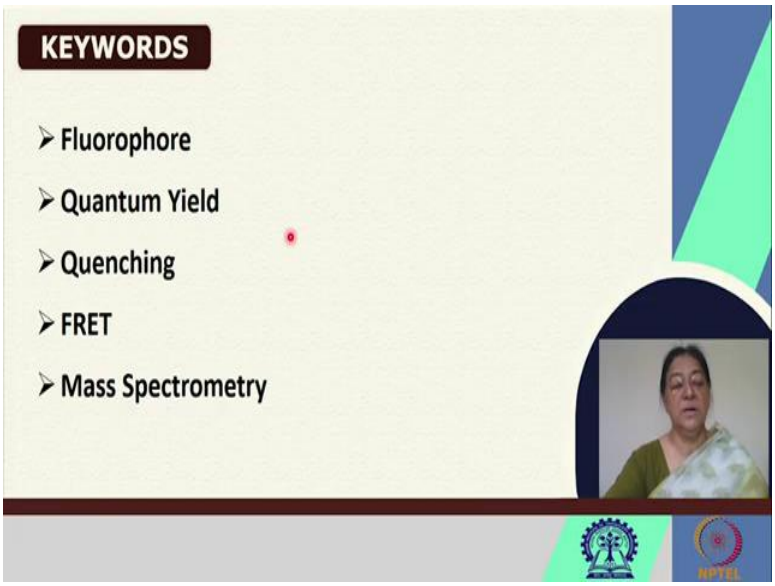
Module - 04
Protein isolation and characterization
Lecture - 19
Biophysical Methods - II

We will continue our discussion on biophysical methods. In the last class we learnt about UV spectroscopy, IR and a bit about circular dichroism.

(Refer Slide Time: 00:26)

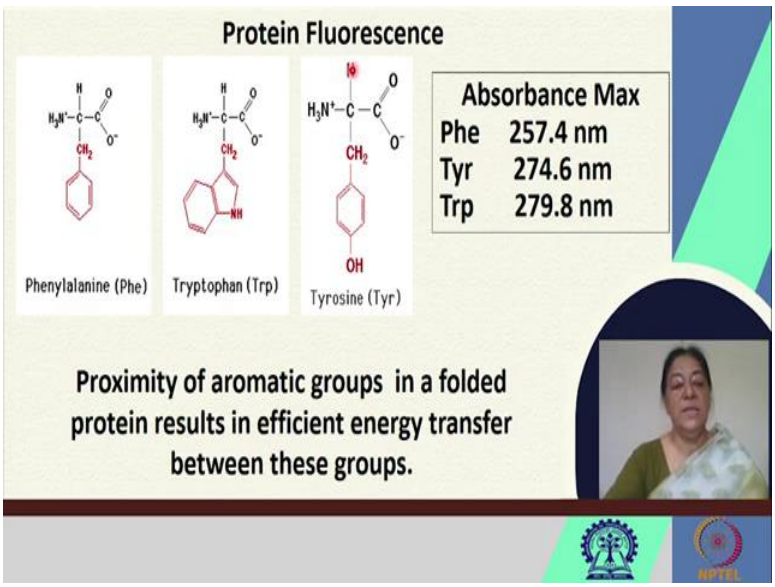
What we will do in this class, is we will look at protein fluorescence, protein resonance energy, fluorescence resonance energy transfer and a bit about mass spectrometry.

(Refer Slide Time: 00:40)



The specific idea that we are going to look at here is what the fluorophores are, what we mean by a quenching and what we mean by fluorescence resonance energy transfer and how that can help us in estimating our protein in its folded content, in its unfolded condition and also protein-ligand binding which we will look at later on, when we consider the specific topics.

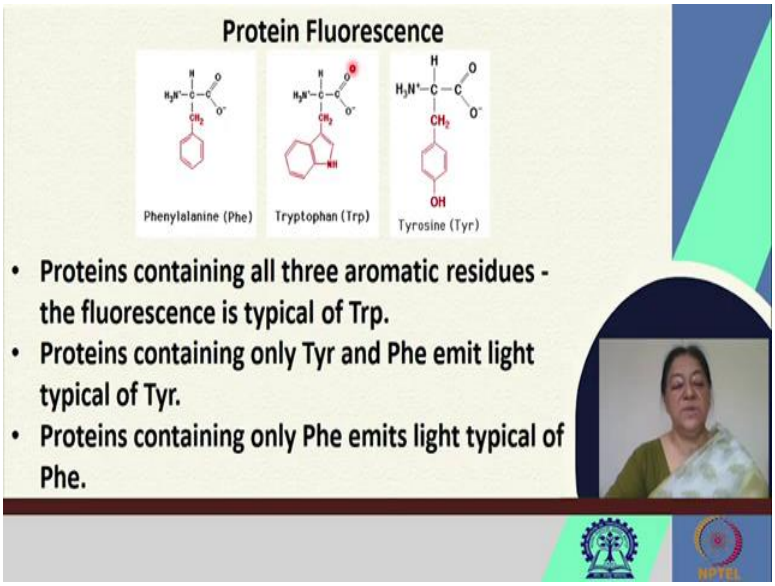
(Refer Slide Time: 01:05)



When we look at protein fluorescence as we have seen before, we look at these [refer to slide] three aromatic amino acid residues that are responsible for protein

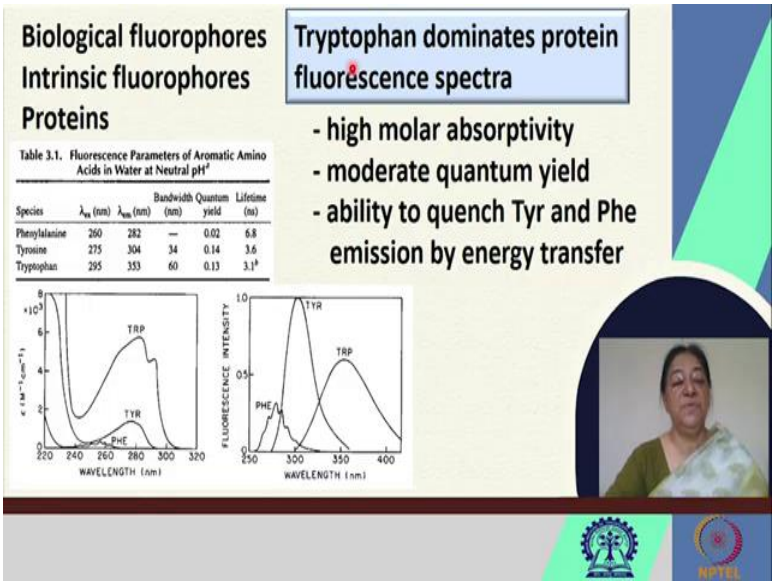
fluorescence. In a knowledge of the proximity of the aromatic groups in a folded protein, there is efficient energy transfer between these groups.

(Refer Slide Time: 01:26)



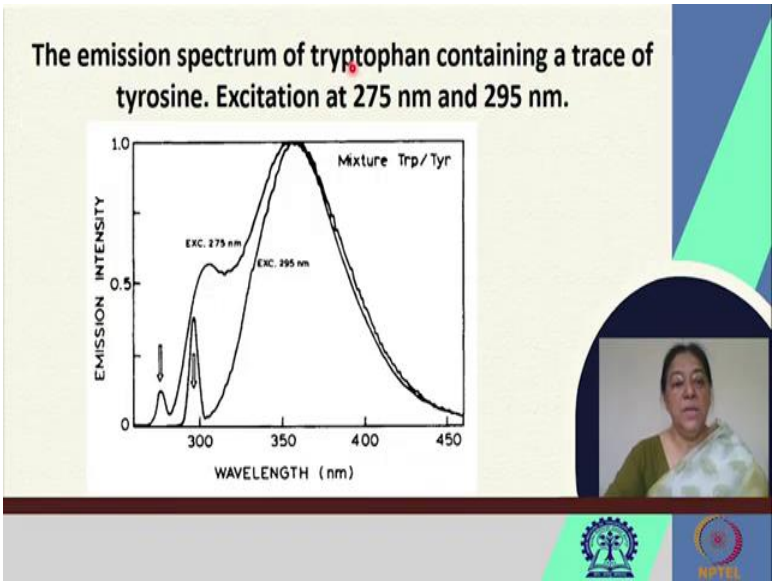
In protein fluorescence when we have the three aromatic residues and we have tryptophan, the fluorescence emission is typically controlled by tryptophan and the emission that we see is due to this. With a combination of the aromatic amino acids we either see that typical of tyrosine, depending upon the presence of the aromatic residues in the protein sequence.

(Refer Slide Time: 01:56)



The biological fluorophores that we look at in the intrinsic fluorophores, we understand now that tryptophan dominates protein fluorescence spectra. This is because it has a high molar absorptivity even though it has moderate quantum yield and it has the ability to quench tyrosine and phenylalanine emission by energy transfer.

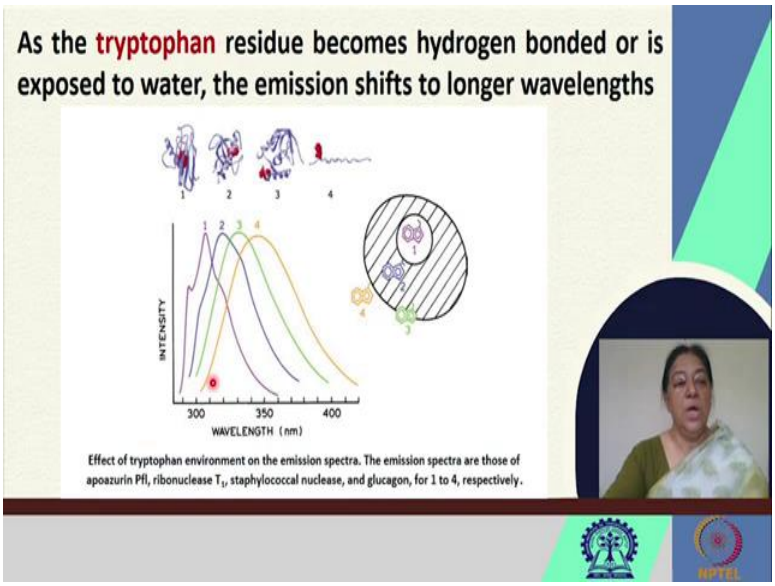
(Refer Slide Time: 02:17)



The emission spectrum of tryptophan, with a small content of tyrosine, can be seen here [refer to slide] at the specific excitation wavelengths. At an excitation wavelength of 275 nm there is a possibility that we are exciting both tyrosine and tryptophan.

The emission intensity that we observe is going to be a mixture of tryptophan/ tyrosine. So the common method is to excite the protein at 295 nm. So, that we do not see any interference from the tyrosine residue.

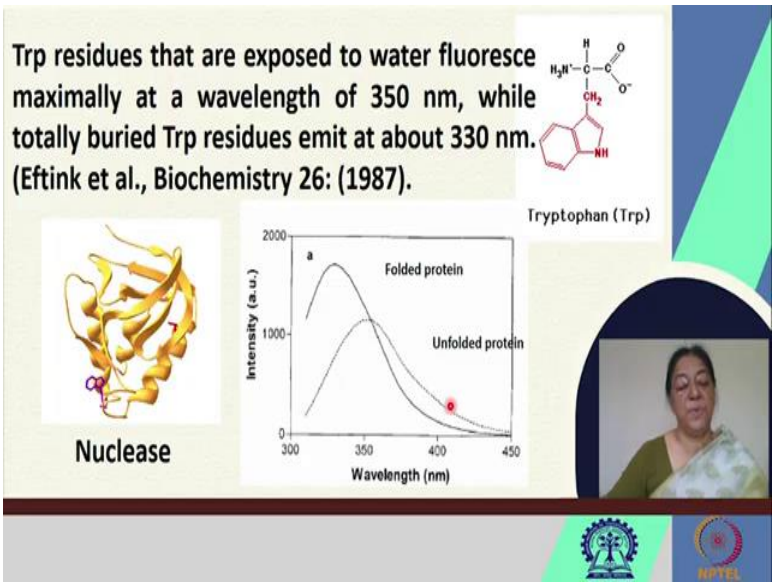
(Refer Slide Time: 03:04)



As the tryptophan residue now becomes hydrogen bonding, there is an exposure of this tryptophan to the solvent. This exposure results in what is called a red shift as is evident from the spectra that we see here [refer to slide], where we have the fluorescence emission intensity on the y axis and the wavelength on the x axis.

As the tryptophan becomes hydrogen bonded or is exposed to water, the emission shifts to longer wavelength that gives us an indication not only of the exposure of the tryptophan, but if we have ligand binding we can also observe quenching of the tryptophan fluorescence.

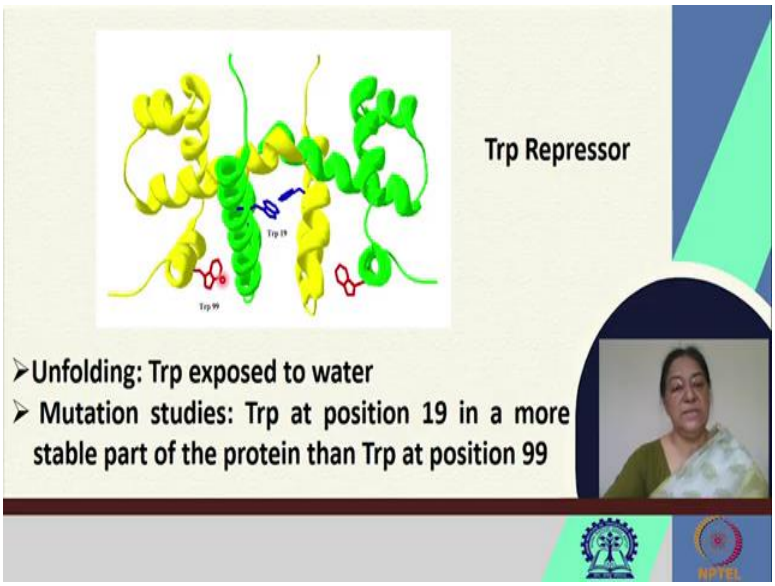
(Refer Slide Time: 03:51)



So the tryptophan fluoresce residues that are exposed to water fluoresce maximally at a wavelength of 350 nm, while those that are totally buried emit at about 330 nm. Given this range we can understand where our tryptophan residue lies. For example, if we look at the new tryptophan in the nucleus protein here [refer to slide], we see that this is where it exists in the folded protein, where it is situated in the folded protein.

If we do a particular experiment where we unfold the protein what we can observe is we can see that the tryptophan emission intensity has red shifted for the unfolded protein which is this spectrum here.

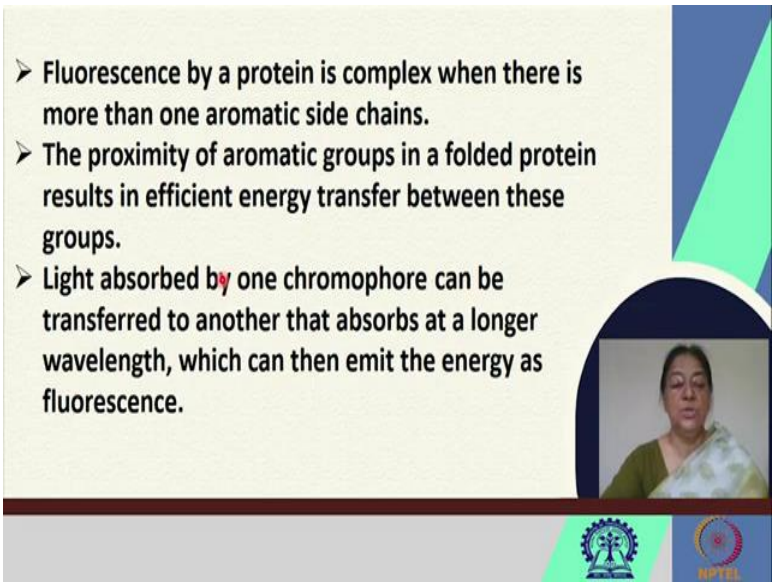
(Refer Slide Time: 04:37)



This can also lead us to an identification of locations of important tryptophan residues. A knowledge of the tryptophan repressor tells us that there are two tryptophans per monomeric unit, one of these tryptophans as we can see marked in blue here [refer to slide] is located buried in this structure and this is located relatively on the surface. So what can be done is a look at the tryptophan exposure to water can also be monitored if we do a mutation study.

Whereby, a single mutation will change the tryptophan residue present, say at position 19 and tell us which tryptophan is actually in the more stable part of the protein in comparison to one another, we see that the tryptophan at position 19 is in a more stable part of the protein than that at 99.

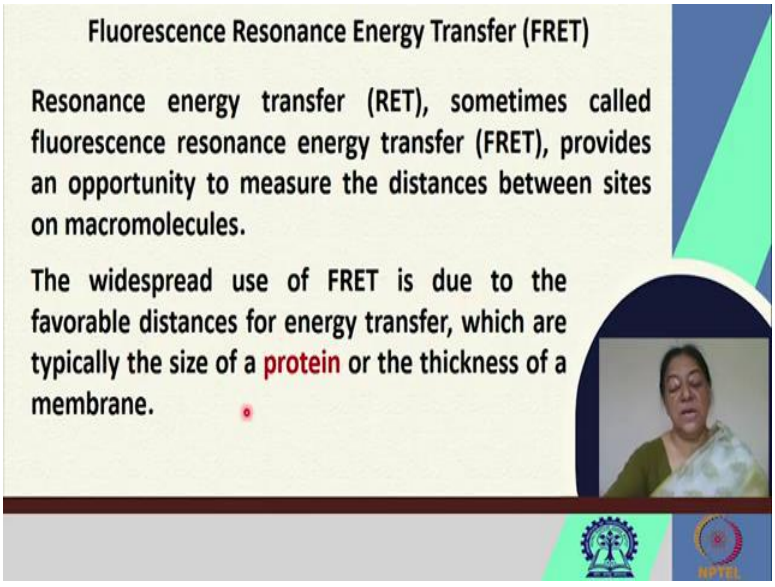
(Refer Slide Time: 05:36)



So this fluorescence by a protein is complex when there are more than one aromatic side chains, because we have the contributions from the others as well and when we have multiple tryptophan residues also. The proximity of the aromatic groups in a folded protein results in efficient energy transfer between these groups.

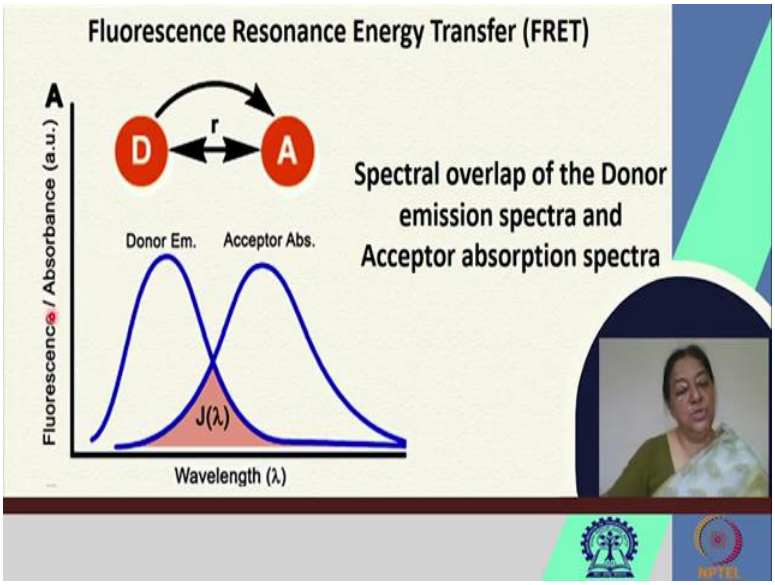
And the light absorbed by one chromophore can also be transferred to another that absorbs at a longer wavelength, because we know that the fluorescence emission spectra is red shifted from the absorption and can also emit the energy as fluorescence.

(Refer Slide Time: 06:16)



This is what accounts for fluorescence resonance energy transfer. In this resonance energy transfer it gives us a method to measure the distances between sites on macromolecules. The important part about this is, FRET is used due to favorable distance for energy transfer, which are typical of the size of the protein and also of the thickness of the membrane.

(Refer Slide Time: 06:42)

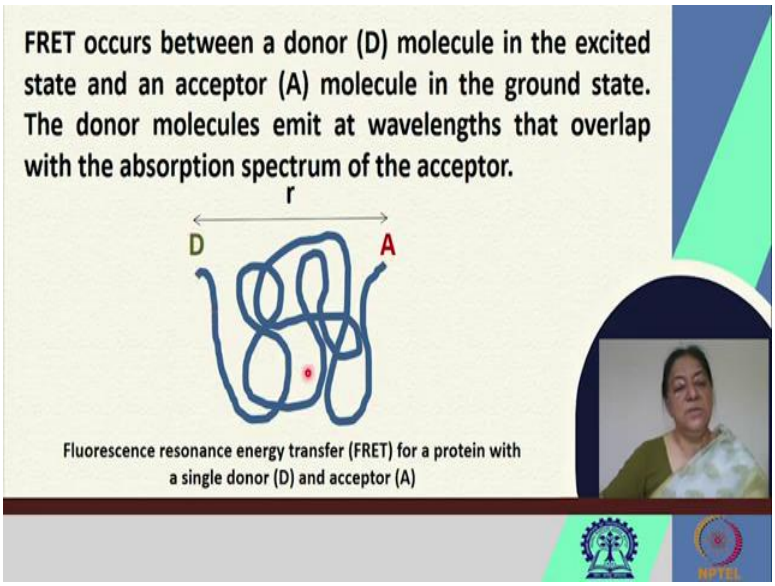


If we look at the methodology of FRET, what we see is the spectral overlap of the donor emission spectra and the acceptor absorption spectra, which is evident from this diagram that we see here [refer to slide]. The donor absorption is somewhere more blue shifted to a lower wavelength here and this is the donor emission.

Now this emission can result in the excitation of an acceptor within a certain distance from it. So if it is in close proximity, this donor emission can be able to excite the acceptor absorbance, where there is an overlap of the donor emission spectra and the acceptor absorption spectra and this is called the overlap integral.

The acceptor in turn can also fluoresce which will be further red shifted in this direction, so we have the fluorescence resonance energy transfer, that is a measure of the distance from the donor to the acceptor.

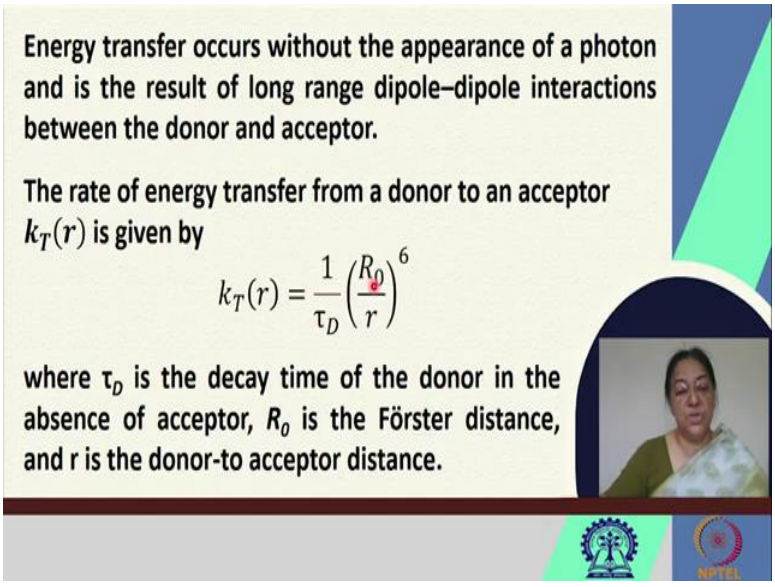
(Refer Slide Time: 07:55)



FRET occurs between a donor molecule in the excited state and an acceptor molecule in the ground state and the donor molecules emit at wavelengths that overlap with the absorption spectrum of the acceptor.

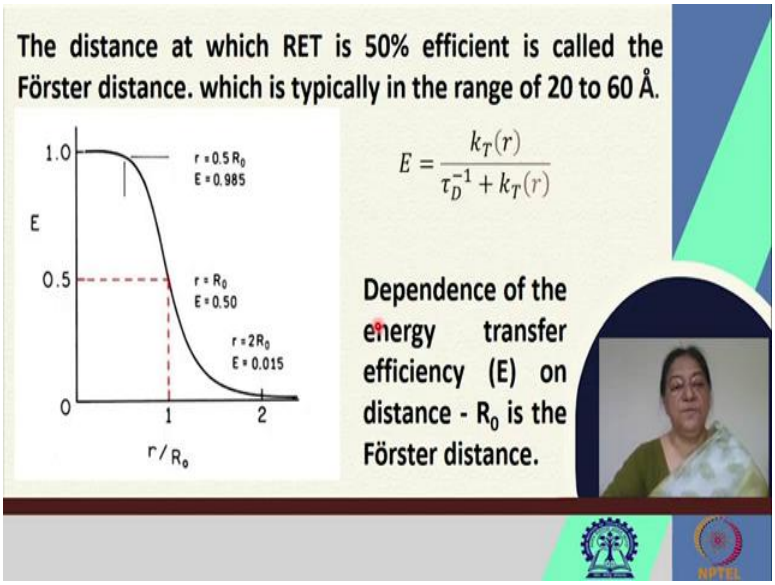
So, we can actually then mark our proteins or tag our proteins with an acceptor and a donor and then monitor the folding or we can monitor the distance at which they gradually approach each other while the protein is folding because it has to be within a particular distance r for the resonance energy transfer to occur.

(Refer Slide Time: 08:37)



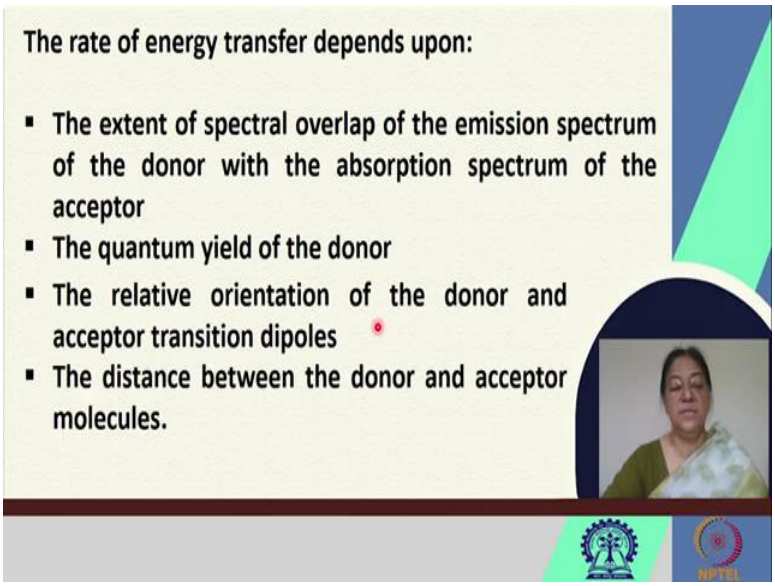
This energy transfer occurs without the appearance of a photon and is the result of the long range dipole-dipole interactions between the donor and the acceptor. The rate of energy transfer is given by k_T and the expression is $k_T(r)$, which is the distance $1/\tau$, where τ is the decay time of the donor in the absence of the acceptor, R_0 is the Forster distance, and r is the donor-to-acceptor distance.

(Refer Slide Time: 09:08)



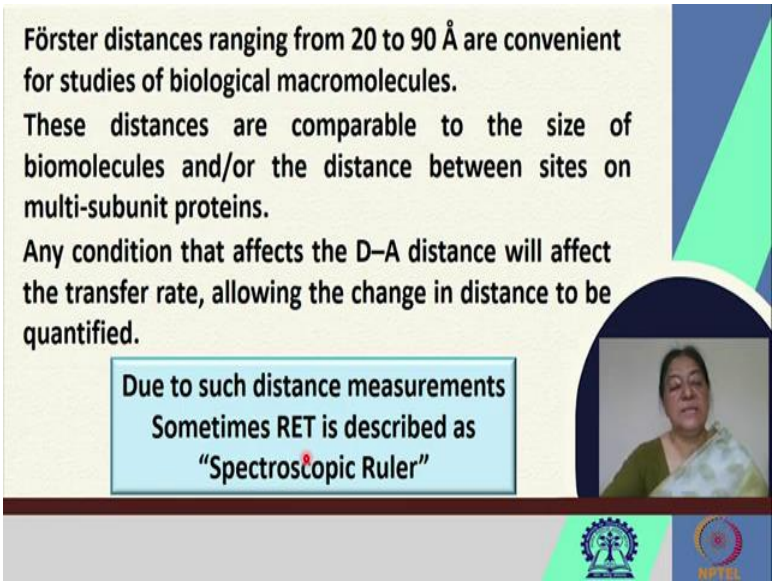
If we look at the specific diagram of the energy versus the r/R_0 ratio, when we have this $r = R_0$ with 50% energy transfer efficiency then we call this R_0 as the Forster distance which is typically in the range of 20 to 60 Å. And this dependence of the energy transfer on the distance, is important in understanding how we can measure the proximity of the donor and the acceptor molecules.

(Refer Slide Time: 09:38)



This rate of energy transfer would therefore depend upon the extent of the spectral overlap of the emission spectrum of the donor, with the absorption spectrum of the acceptor; the quantum yield of the donor; the relative orientation of the donor and acceptor transition dipoles and the distance between the donor and the acceptor molecules.

(Refer Slide Time: 10:02)

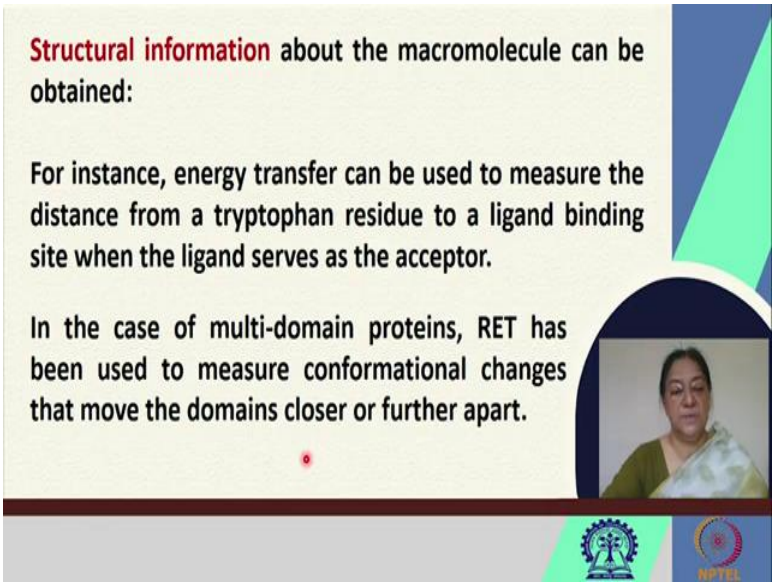


Forster distances ranging from 20 to 90 Å are convenient for studies of biological macromolecules and these distances are actually comparable to the size of the

biomolecules and the distances between multi-subunit proteins and this D-A distance will be affected by the transfer rate and it can allow the change in the distance to be quantified. So it is sometimes referred to as a "Spectroscopic Ruler."

What we did look at is the possibility of locating specific residues that can be tagged with fluorophores as a donor and an acceptor, and based on this distance we can look at protein folding/unfolding techniques or even protein-ligand transfers, protein-ligand interactions, which we will see later as we go along in the course.

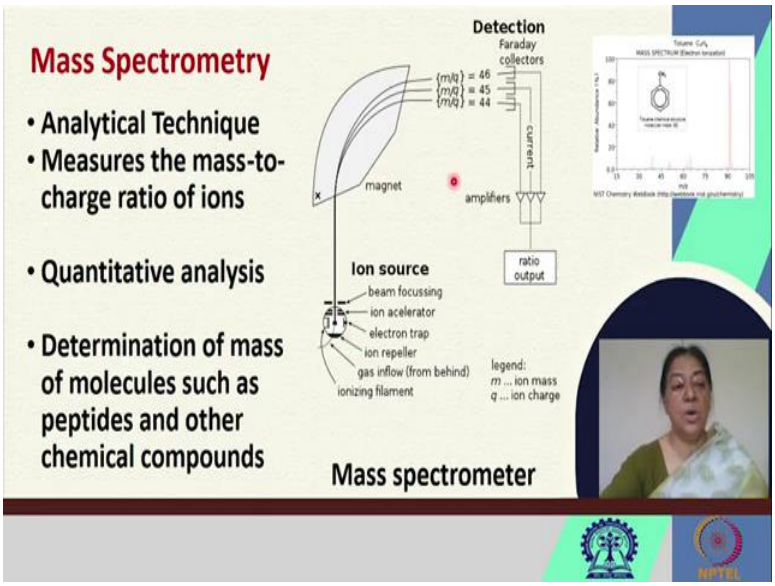
(Refer Slide Time: 11:05)



The structural information about the macromolecule therefore that can be obtained; for instance, if we look at the energy transfer we can measure the distance from a tryptophan residue to a ligand binding, where we can look at not only at the fluorescence emission quenching, but also to a resonance energy transfer.

And in the case of multi-domain proteins, this resonance energy transfer can be used to measure conformational changes that move the domains closer or further apart depending upon the donor acceptor distances.

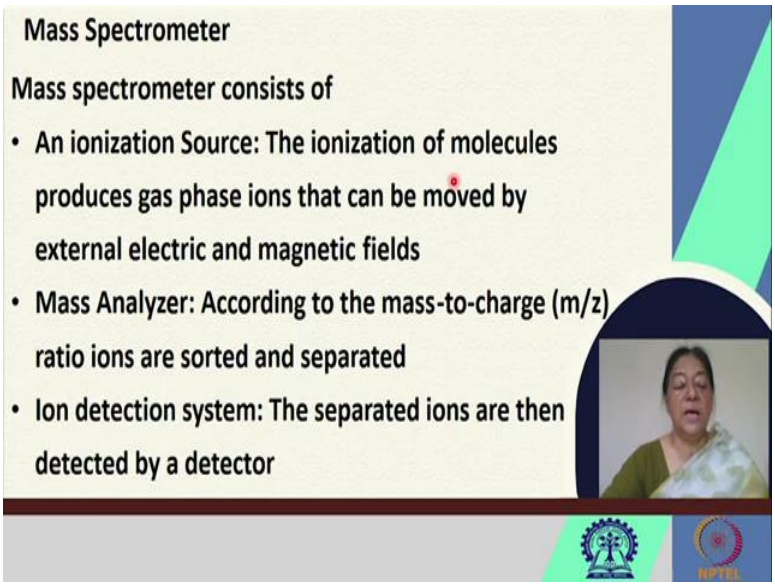
(Refer Slide Time: 11:39)



In our next understanding of what we can do with our protein molecules that we have isolated, there is a methodology that measures the mass of the molecule. Mass spectrometry is an analytical technique that measures the mass-to-charge ratio of ions. It is a quantitative analysis and what it does, it can determine the mass of the molecules such as peptides and other chemical compounds.

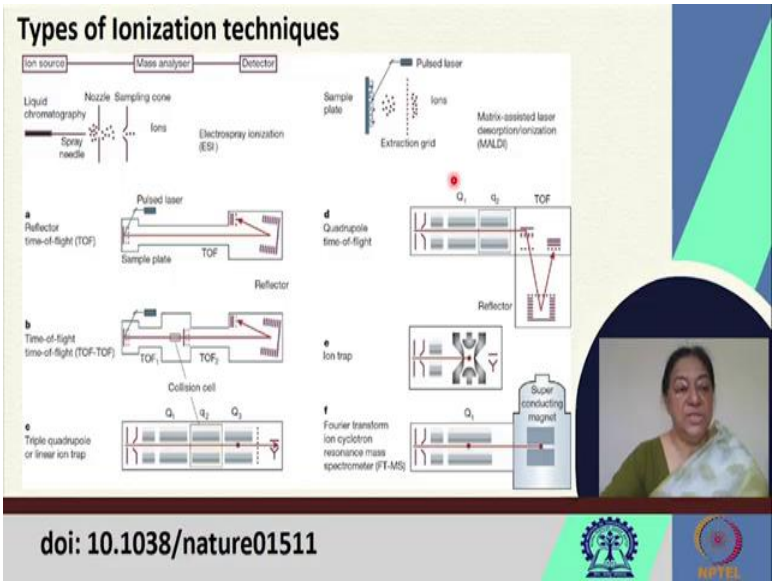
It typically has an ion source, an electromagnetic system here [refer to slide] and then a detector, where we can actually look at and monitor the specific mass-to-charge ratio.

(Refer Slide Time: 12:20)



The spectrometer contains of an ionization source, where we have the ionization of the molecules that produce gas phase ions, that can be moved by the external electrical and magnetic fields depending upon the mass-to charge-ratio. This mass-to-charge ratio will then sort and separate the ions where we can have a mass analyzer and finally, there is an ion detection system where the separated ions are then detected by a specific detector.

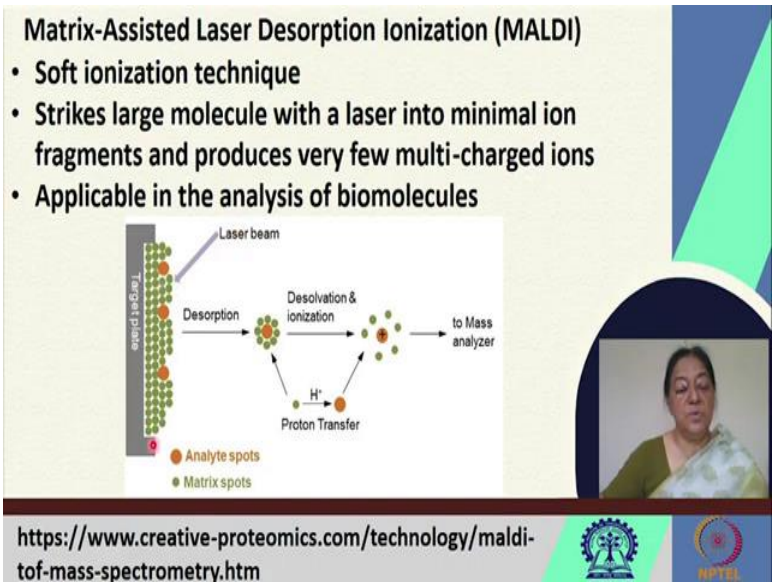
(Refer Slide Time: 13:01)



There are different types of ionization techniques. All the methodologies use an ion source, a mass analyzer, followed by a detector. When we are looking at liquid chromatography we have a spray needle, a nozzle and we have electrospray ionization and because of the presence of the electromagnetic field here [refer to slide], the ions move to the detector based on their mass-by-charge ratio.

There is also a reflector time-of-flight, a TOF as it is called. A TOF or a time-of-flight time-of-flight, that just increases the distance here and the detector is located in a position where the mass-by-charge ratio of the different fragments can be collected and analyzed. A common methodology for the mass of proteins is Matrix-Assisted Laser Desorption Ionization MALDI. MALDI-TOF which we will see in a bit detail in the next slide.

(Refer Slide Time: 14:07)

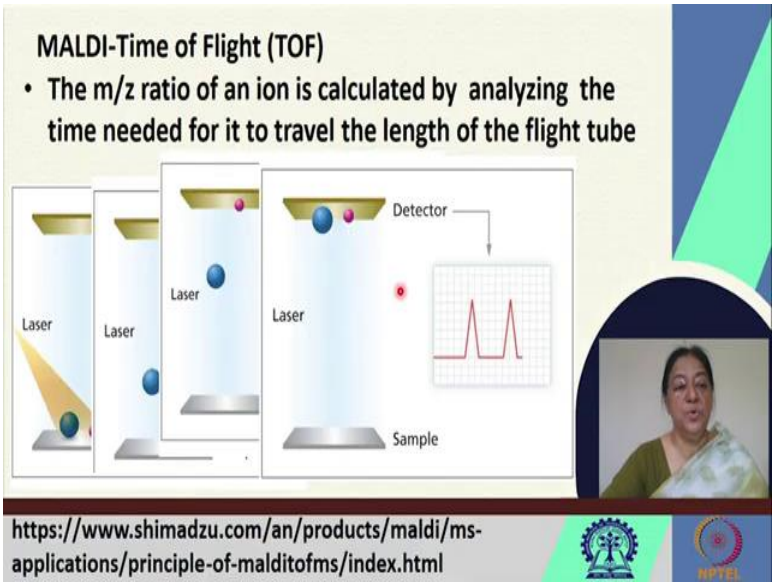


In matrix-assisted laser desorption ionization, the protein molecule is mixed with a matrix. This is a soft ionization technique. It strikes the large molecule with a laser, into ion fragments and produces few multi-charged ions. It is applicable in the analysis of the biomolecules and this is a typical methodology.

We have a target plate which has the analyte spots and the matrix, where we have the protein mixed with this specific matrix. The laser beam is shown [refer to slide] on this target plate and there is desorption due to the laser beam that is shown on the plate. Then there is desolvation and ionization, proton transfer leading to a mass-by-

charge ratio that then moves on in the TOF technique, in the time-of-flight to the mass analyzer in the detector.

(Refer Slide Time: 15:14)

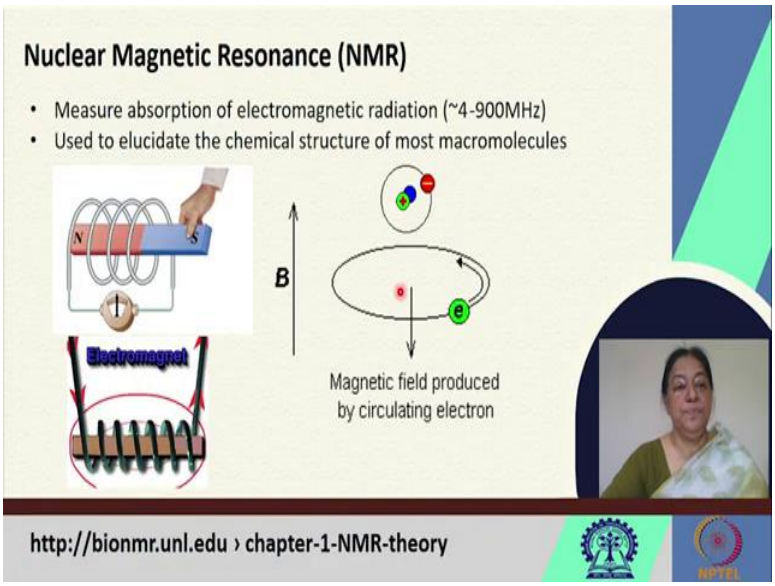


In the MALDI-time of flight, the m/z ratio of an ion is calculated by analyzing the time needed for it to travel the length of the flight tube. This is a typical example of how it takes place. Here [refer to slide] we have an analysis in this inset, where we have the sample on this plate, mixed with the matrix.

We have the laser on the matrix plate, then with the production of the laser it ionizes and gradually moves to the detector and depending upon the m/z ratio, it would reach the detector and we would get a peak depending upon which molecule has reached earlier; which would depend upon the mass-by-charge ratio in the time-of-flight.

So the finally, we would get these two peaks depending upon the mass-by-charge ratio, giving us an indication of the mass of the molecules that we had present in our mixture of proteins.

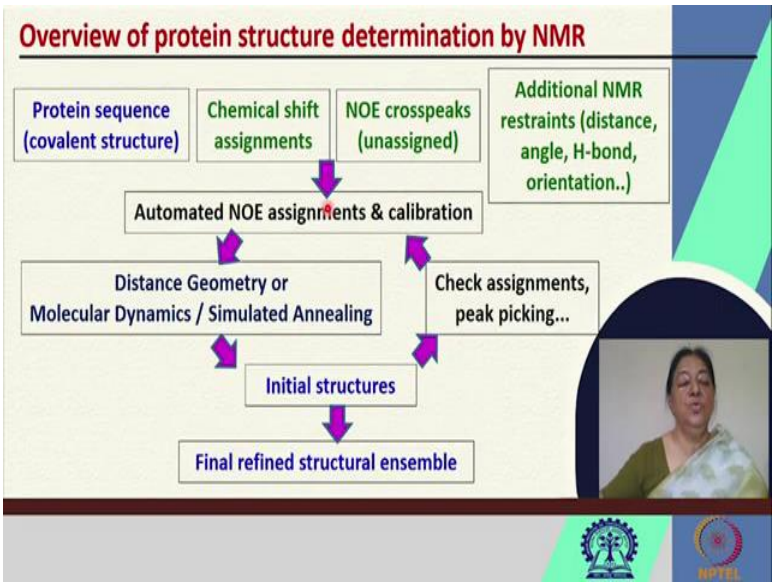
(Refer Slide Time: 16:29)



We now look at a different methodology, where we can also understand the chemical structure of many macromolecules in nuclear magnetic resonance. In our overview of the protein structure prediction, we had briefly looked at NMR in the understanding of a protein structure and how it may be predicted.

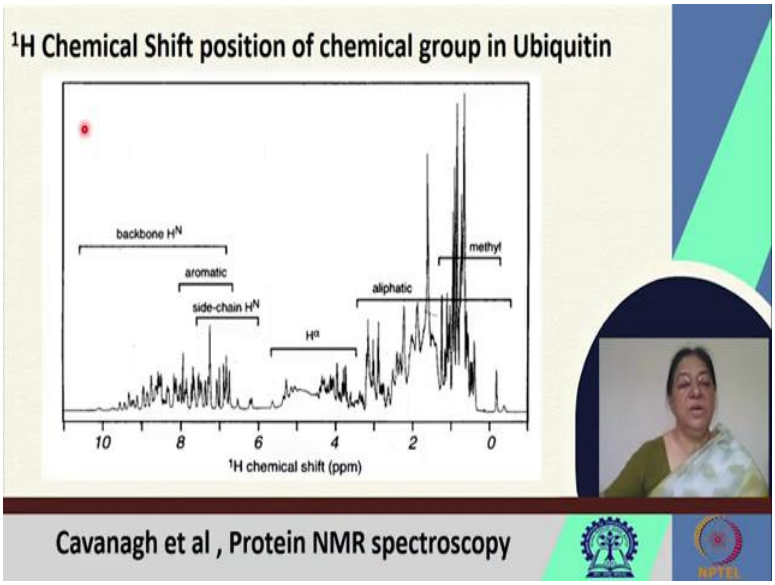
When we look at NMR in this context we are looking at a characterization of the protein to know whether the protein is in its folded form, to know whether the protein will be able to perform its function and later on we will also look at the variations in the spectrum, due to ligand interactions with the protein.

(Refer Slide Time: 17:33)



In the methodology of NMR we know that there is a magnetic field that is produced by the circulating electron, further analysis done to look at the specific chemical shift assignments that will give us additional NMR restraints that can be added in terms of distance, angle, hydrogen bond and the specific orientations. And this will lead to specific chemical shift assignments followed by calibration, giving us a specific distance geometry that would give us some basic structure of the protein that we are interested in.

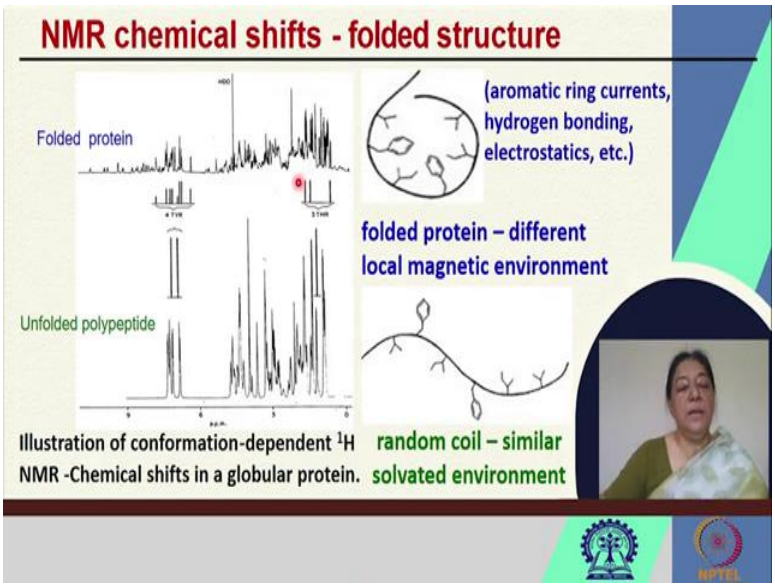
(Refer Slide Time: 18:12)



When we look at the protein chemical shifts, we can see the proton chemical shift position of a chemical group say in ubiquitin. Now, if we look at the specific types of protons that are present in proteins, these are the different types of protons that we see. Knowing that the amino acids linked together have their hydrogen atoms in the backbone, in aromatic amino acids, in the specific side chains, also in α-hydrogen atoms that we see and the aliphatic and the methyl groups.

Depending upon this, there is going to be a specific chemical shift associated with the proton that is dependent on the chemical group, particularly present in the proteins.

(Refer Slide Time: 19:06)



For example, if we look at NMR chemical shifts in a folded structure, we are looking at say the tyrosine or the threonine or different types of residues that have hydrogen in them. If we are looking at a proton NMR, we are interested in the chemical shifts in the globular protein. So when we look at the folded protein we realize that the local magnetic environment is going to be different because of its folded structure as can be observed here [refer to slide].

What is different here is the aromatic ring currents, the hydrogen bonding is different, the overall electrostatics is different. Which results in a different local environment, a different magnetic environment, giving us a completely different structure overall, a different NMR spectrum for the folded protein.

However when the protein is unfolded, what happens in this case is we are looking at a random coil where we are going to have a solvated environment. But the overall local environment is going to change, resulting in chemical shifts that are indicative of the difference of the folded protein from the unfolded polypeptide.

This indication is important in an understanding where look at specific chemical shifts to see whether we have our ligands bound to the specific, say amino acids in the active site of enzymes. If we are interested to develop inhibitors to understand whether we have the specific chemical shifts or variations because of these specific interactions.

So, what we looked at in this lecture is an understanding of protein fluorescence, telling us that we have the aromatic amino acid residues that contribute to our overall structure, our overall protein fluorescence with tryptophan dominating the fluorescence.

Then this tryptophan can have its emission intensity shifted to the right, red shifted to a higher wavelength, that is indicative of its exposure to solvent or we could have fluorescence quenching, that is indicative for protein-ligand binding, which we will see in subsequent lectures.

Another concept that we looked at is fluorescence resonance energy transfer, where the fluorescence donor emission can excite an acceptor molecule if there is an overlap between the donor emission and the acceptor absorption. This can lead us to identifying donor absorption or donor acceptor distances, that are important in understanding the folding of proteins or even multi-subunit proteins, to see the proximity of specific donors and acceptors that may also be tagged along into the proteins.

Another aspect is looking at protein-ligand binding, where we could have donor to acceptor energy transfer. We looked at a brief introduction or a brief discussion on mass spectrometry and how it may be used to determine the molecular weight from a mass-by-charge ratio, using MALDI-TOF most commonly used for proteins.

Another aspect of protein structure prediction, but also protein-ligand binding, is a looking at the chemical shifts of protons or specific atoms where we will be able to identify protein folding and unfolding in addition to protein-ligand interactions.

(Refer Slide Time: 23:16)

These are the references Biophysical Chemistry by Cantor and Schimmel, Lakowicz for Protein Fluorescence Spectroscopy and Principles of Physical Biochemistry in addition to the book by Cavanagh for Protein NMR Spectroscopy.

Thank you.