Structural Biology
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Lecture – 35
Raman Spectroscopy, Raman Microscopy and Raman Crystallography for Studying Protein

Hi everyone welcome again to the course of structural biology. We are going through structural biology techniques. We have already covered high-resolution techniques like X-ray crystallography NMR. Now we are going through different low-resolution techniques in spectroscopy. We already discussed the principle of Raman spectroscopy and how it is correlated with infrared, they both are operating in the vibrational mode.

So, we have discussed their mutual exclusiveness and their differences, similarities, advantages, and disadvantages. Today we will discuss how Raman spectroscopy uses their fingerprints to get information about protein structure. And more importantly, we will discuss a very recent technique which is Raman microscopy and a modified version of Raman microscopy called Raman spectroscopy.

Very few people have the opportunity to work using this technique. I have worked with that, and Raman crystallography is an amazing technique, which is very novel because you could trap a live reaction by using this technology. I repeat, you could trap a live enzymatic reaction. So, let us go to and start Raman spectroscopy, Raman microscopy, Raman crystallography for studying protein.

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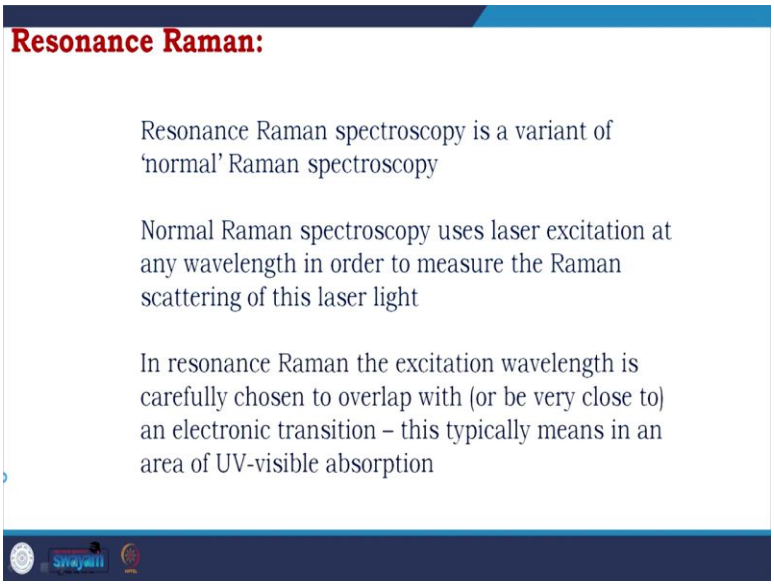

We will start with resonance Raman: Resonance Raman spectroscopy is a variant of normal Raman spectroscopy. You probably remember that in the last class, I talked about Raman signal is very weak. So, normal Raman was not very fruitful in experimental purposes in data collection it needs very specific instrumentation. Normal Raman spectroscopy uses lesser excitation at any wavelength to measure this laser ray's Raman scattering.

In resonance Raman the excitation wavelength is carefully chosen to overlap with or be very close to an electronic transition, this typically means in an area of UV visible absorption.

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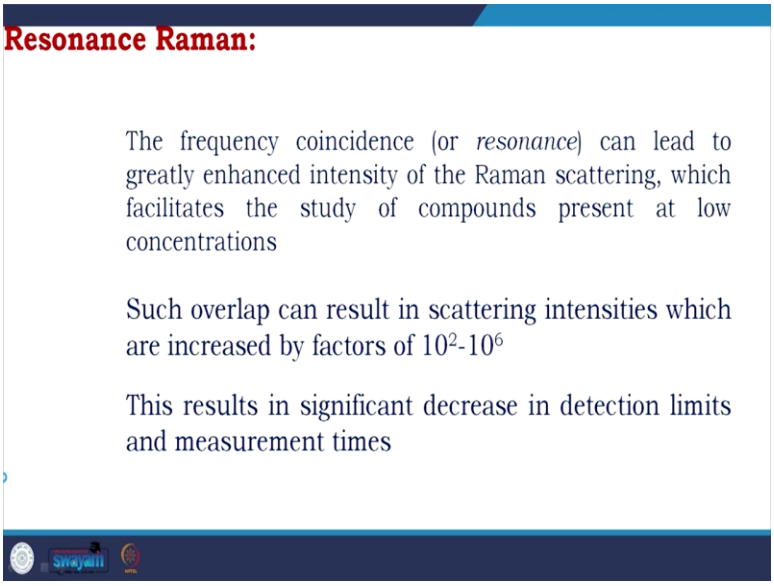

The frequency coincidence or resonance can lead to greatly enhanced intensity of Raman scattering, which facilitated the study of compounds present at a low concentration. Such overlap can result in scattering intensities increased by factor 10^2 to 10^6. This results in a significant decrease in detection limit, increase the signal intensity, and decrease the measurement time.

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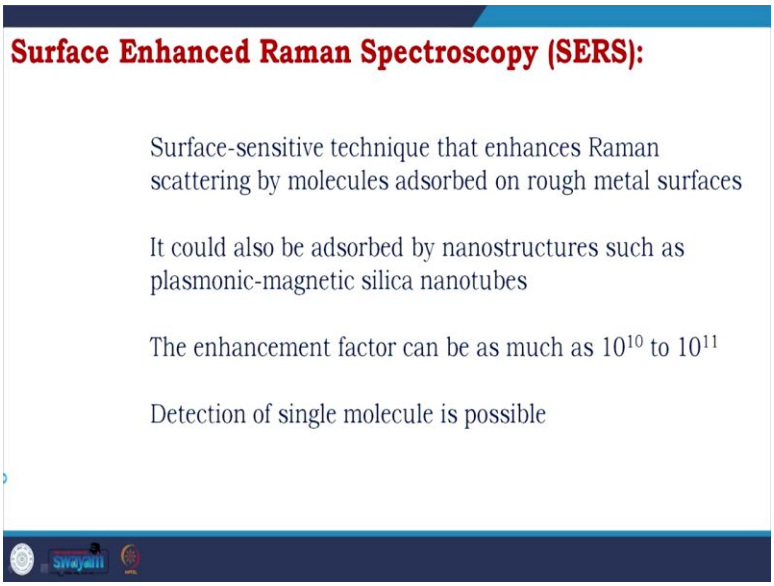

Another technique that help Raman spectroscopy to grow is surface-enhanced Raman spectroscopy or SERS. Surface sensitive technique that enables Raman scattering by molecules absorbed on rough metal surfaces. It could also be absorbed by nanostructures such as plasmonic magnetic silicon nanotubes. The enhancement factor can be as much as 10^{10} to 10^{11}. Detection of a single molecule that is critical using surface enhanced Raman spectroscopy is now enabled to identify a single molecule.

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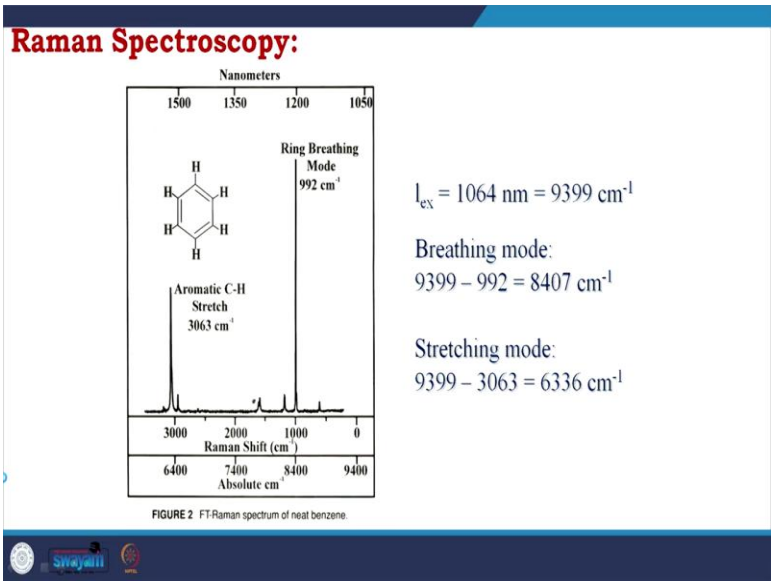

So, in; Raman spectroscopy, if you see the breathing mode and stretching mode in the FT Raman spectrum of benzene. So, you could get the aromatic CH stretch at 3063 cm^{-1} and ring breathing mode at 992 cm^{-1}. So, you could calculate characteristic spectra from there.

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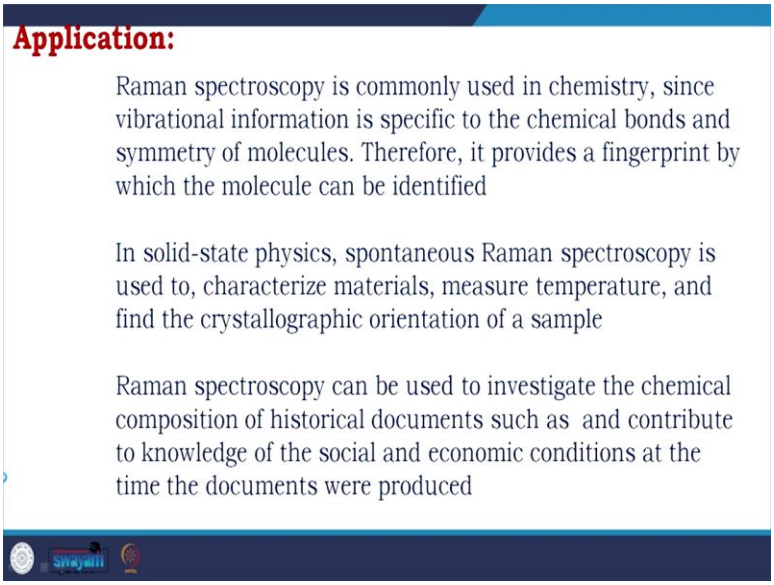

Application Raman spectroscopy is commonly used in chemistry since vibrational information is specific to molecules' chemical bonds and symmetry. Therefore it provides a fingerprint by which molecule can be identified. In solid-state physics, spontaneous Raman spectroscopy is used to characterize the material, measure temperature and find the crystallographic orientation of the sample. Raman spectroscopy can be used to investigate the chemical composition of

historical documents and contribute to knowledge of the social and economic condition when the documents were produced.

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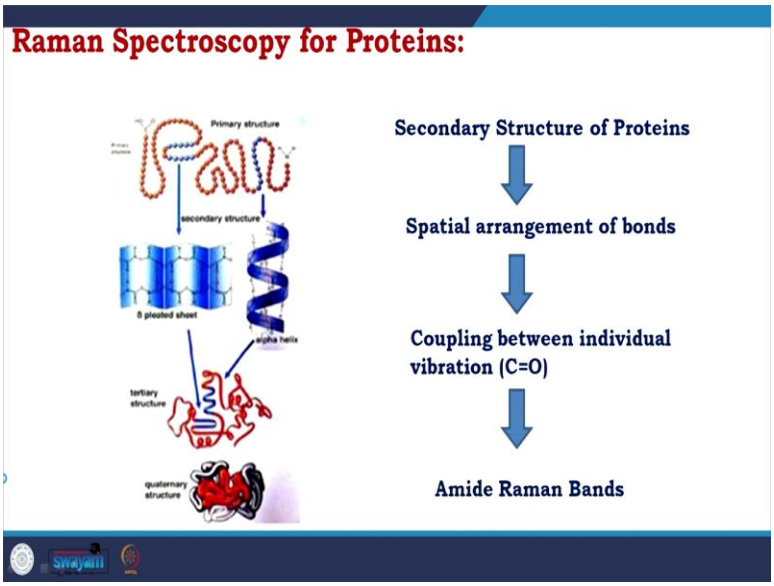

Coming to what we are for, Raman spectroscopy for protein is very relevant for this course. Secondary structure of a protein, you have a special arrangement of bonds the amide bonds coupling between individual vibration of CO and amide Raman bands.

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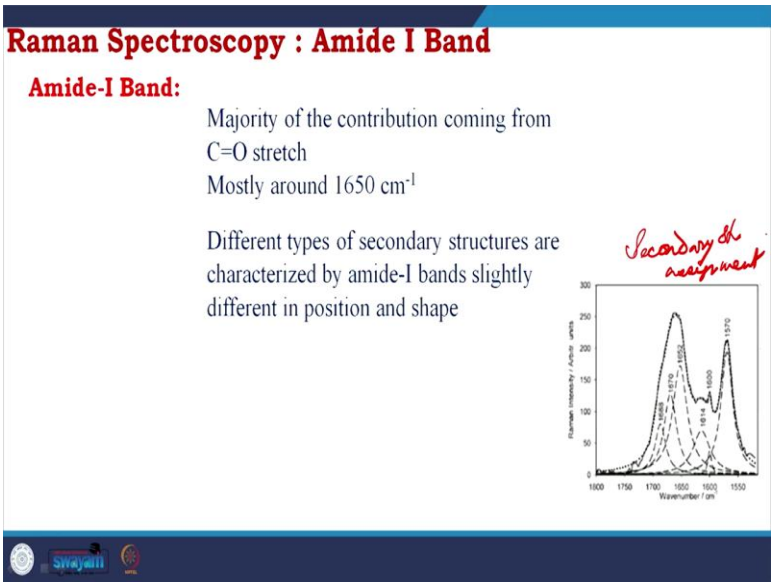

There are fingerprints of amide Raman bands. In secondary structure analysis, nine normal modes are allowed for the amide band for the protein. These are called 1 to 7 and A, B. The nine amide bonds, each band shows different frequency and shift because of stretching and bending.

So, amide one band is the majority of the contribution coming from C double bond O stretch mostly around 1650 cm^{-1}. Different types of secondary structures are characterized by amide one band slightly different in position and shape. So, you could do the assignment of secondary structure from amide one band.

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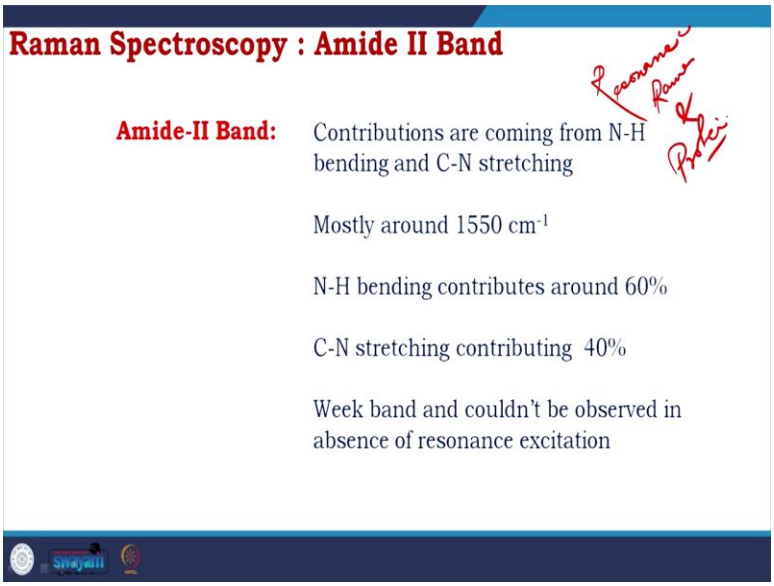

Amide 2 band contributions come from N-H bending and C-N stretching mostly around 1550 cm^{-1}. N-H bending contributes around 60%, C-N stretching contributes 40% weak band and could not be observed without resonance excitation.

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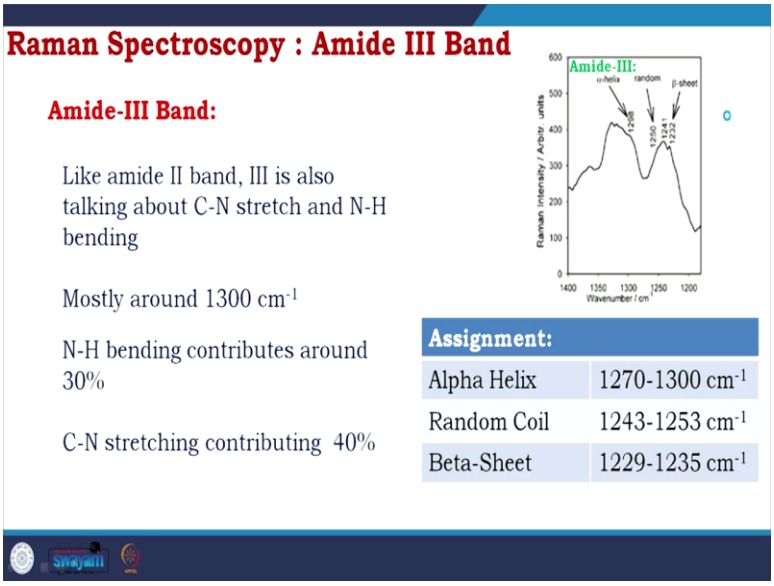

Like Amide 2 band, Amide 3 band also talks about C-N stretch and N-H bend mostly around 1300 cm^{-1}, N-H bending contributes around 30%, and C-N stretching contributes 40%. So, if you see alpha-helix and beta-sheet. Alpha helix mostly as 1270 to 1300 cm^{-1} random coil 1243 to 1253 cm^{-1} beta-sheets 1229 to 1235 cm^{-1}. So, that is why it is also helping in the assignment.

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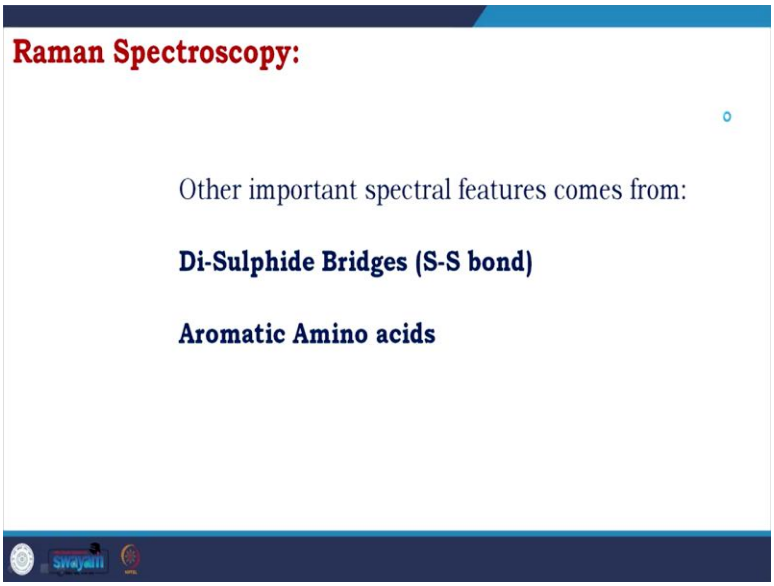

Other important spectral features come from disulfide bridges the (S-S bond), the aromatic amino acids.

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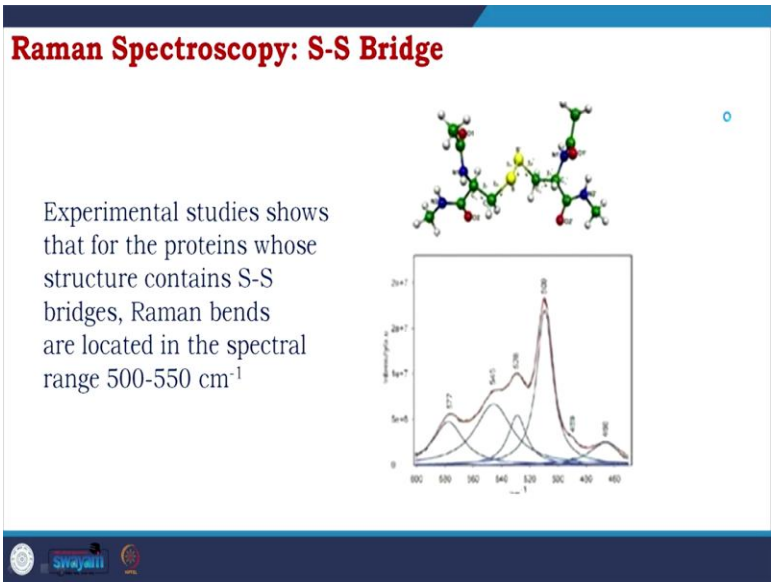

Disulfide experimental studies show that the proteins whose structure contains S-S bridges Raman bends are located in the spectral range of 500 to 550 cm^{-1}.

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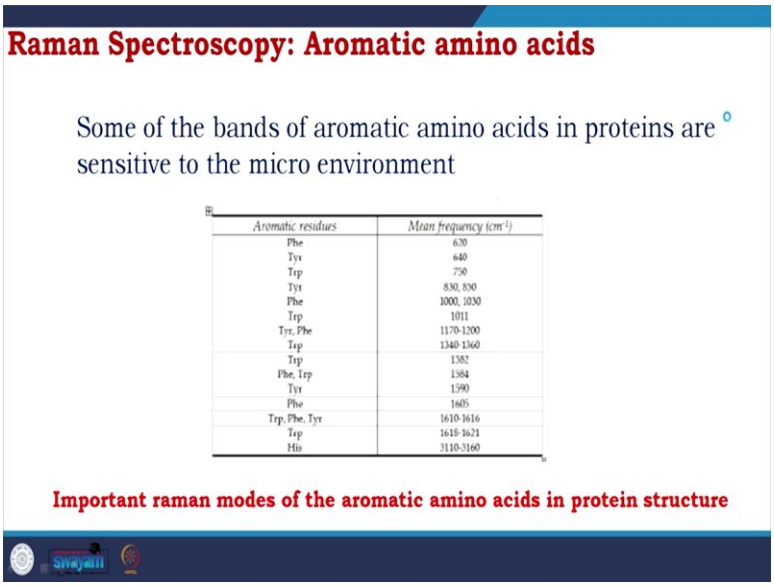

Some of the bands of aromatic amino acids in proteins are sensitive to the microenvironment. Important Raman modes of aromatic amino acids in protein structure could be found by looking at those frequencies, which is possible to identify.

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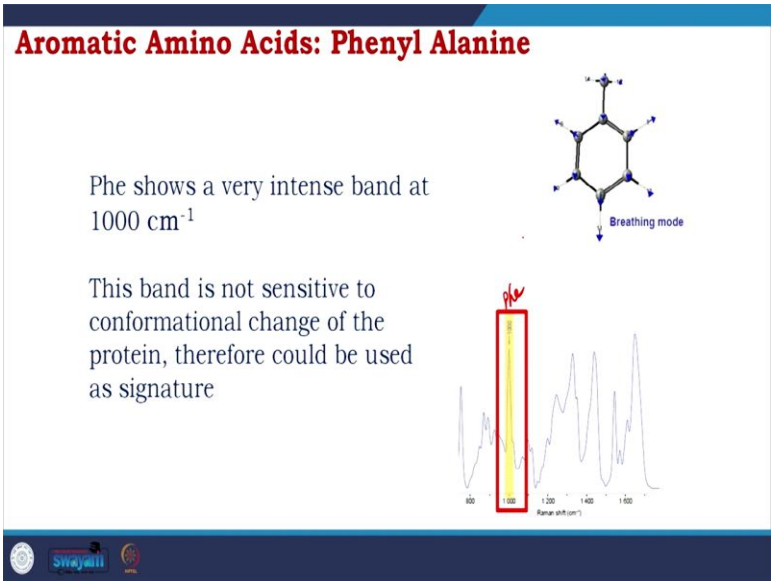

Phenyl Alanine shows a very intense band at 1000 cm^{-1}. So, this is the band for Phenyl Alanine. This band is not sensitive to the conformational change of the protein; therefore could be used as a sign you should get it with the presence of Phenyl Alanine.

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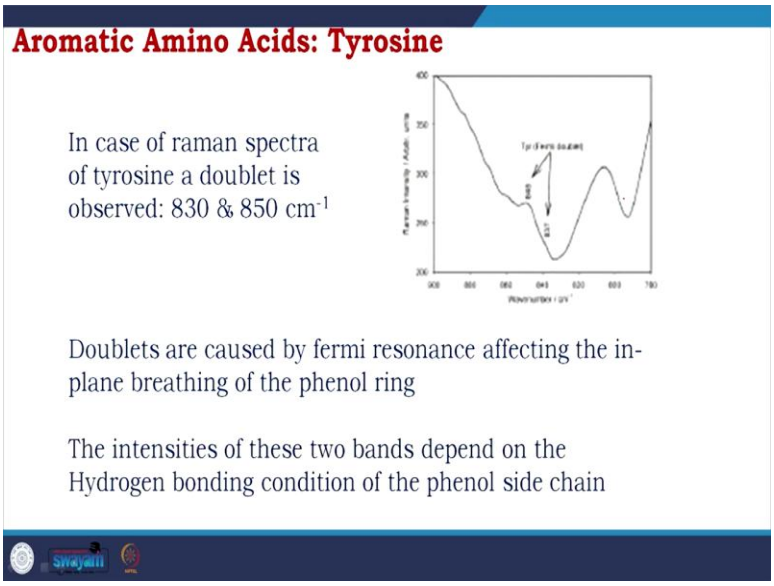

In the case of Raman spectra of tyrosine, a doublet is observed at 830 and 850 cm^{-1}. The doublets are caused by Fermi resonance affecting the in-plane breathing of phenol rings. The intensities of these two bands depend on the hydrogen bonding condition of the phenol side chain. So, you could detect the state in which the phenolic proton is present.

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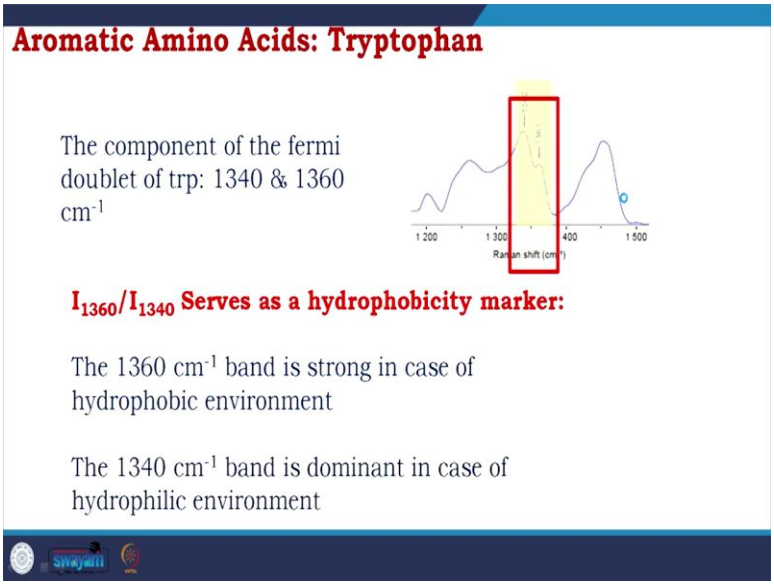

The component of the Fermi doublet at tryptophan 1340 and 1360 cm^{-1}, I_{1360}/I_{1340} serve as a hydrophobicity marker. The I_{1360} cm^{-1}band is strong in the hydrophobic and the other one in (I_{1314}) hydrophilic environments.

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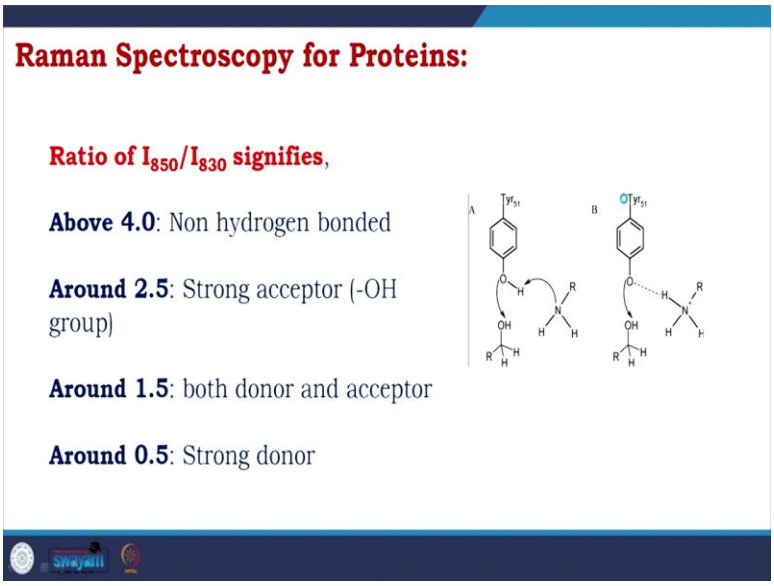

So, I_{855} / I_{830} signifies above 4.0 non-hydrogen bonding or no hydrogen bonding, around 2.5 strong acceptors for the hydroxyl group, around 1.5 both donor and acceptor, around 0.5 strong donors. So, it is a very good signature.

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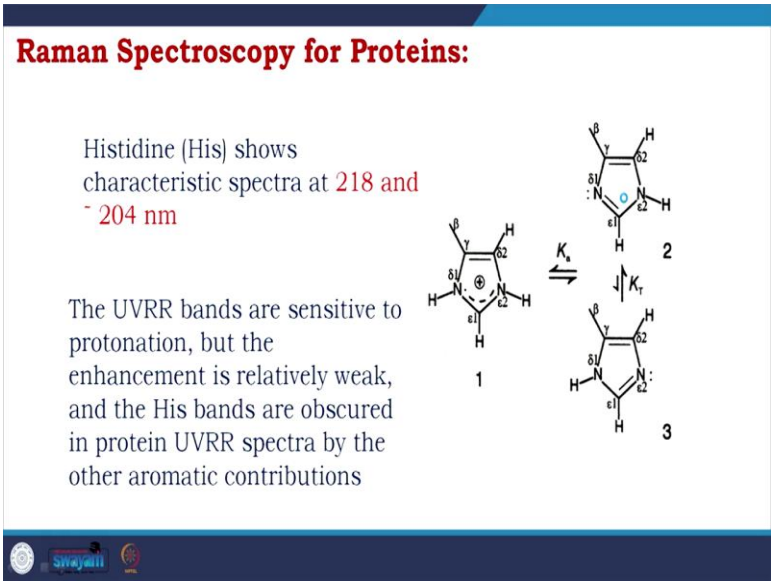

Histidine shows characteristic spectra 218 and 204 nm. The ultraviolet resonance Raman brands are sensitive to protonation. Still, the enhancement is relatively weak, and the Histidine bands are obscured in protein due to resonance damage spectra by the other aromatic contributions.

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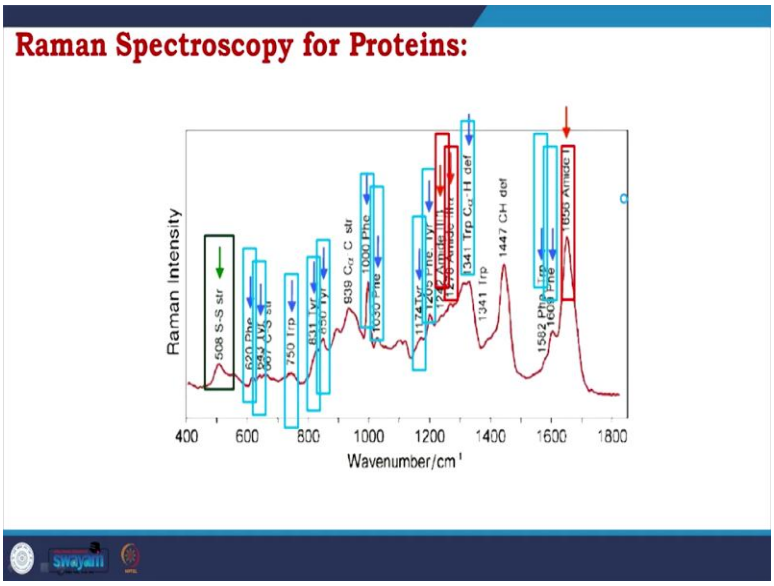

This is the spectra of a protein where you can see the effect of disulfide bond around 500 cm^{-1}. The amino acids are shown in sky blue; you see individual amino acids are coming here. So by putting Raman intensity versus wavenumber, you could identify the different modes like the presence of disulfide bond, specific amino acids specially the aromatic ones, and the change of conformation of the peptide bond.

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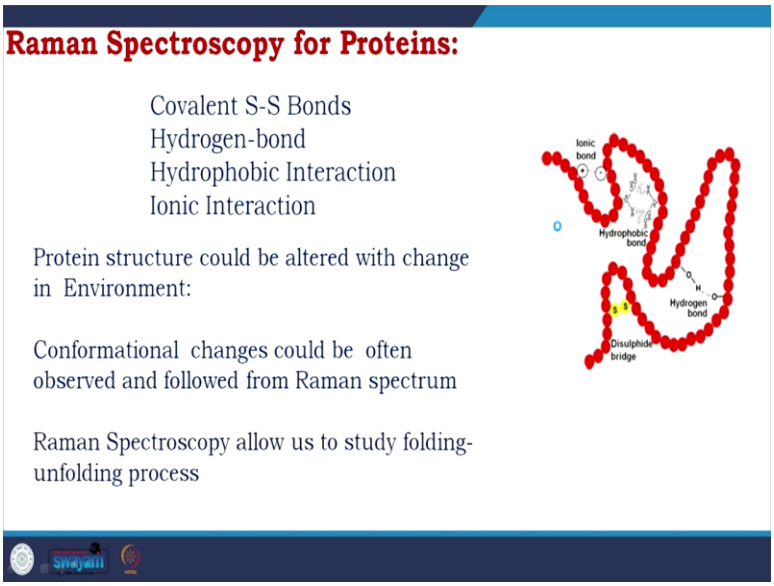

So, covalent disulfide bond could detect hydrogen bond, hydrophobic interaction, ionic interaction, protein structure, altered with change in environment, the conformational change

could be often observed and followed from Raman spectrum. Raman spectroscopy allows us to study the folding unfolding process with more sensitivity.

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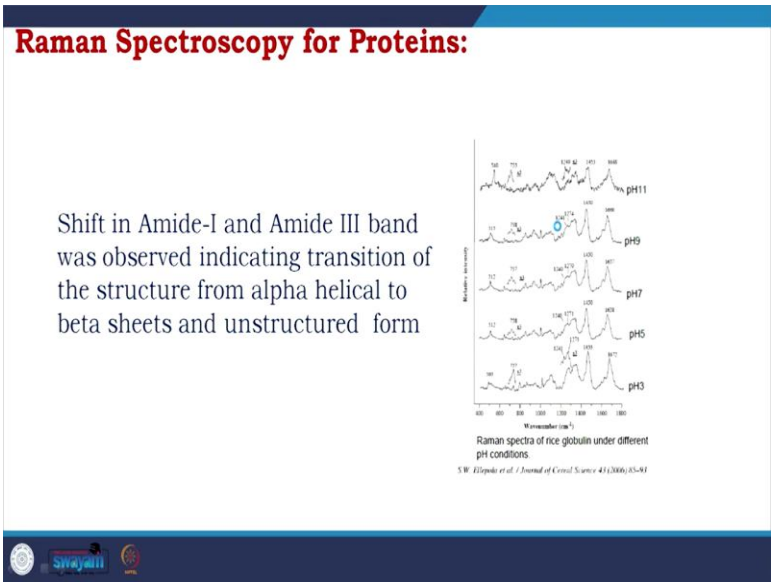

Shift in amide 1 and amide 2 bands was observed, indicating the structure's transition from alpha-helical to beta sheets and unstructured form, here. You could see Raman spectra of rice globulin under different pH condition. How the characteristic spectra's are shifting looking at that we could talk about the content of the secondary structure.

Still, resonance Raman or surface enhanced Raman, which gives good intensity for small molecule or other polymers and all for protein. There is a problem in designing those experiments because there is a possibility of protein being denatured in those set up. To overcome this today, I will talk about a extremely new technology that started with Raman microscopy's concept.

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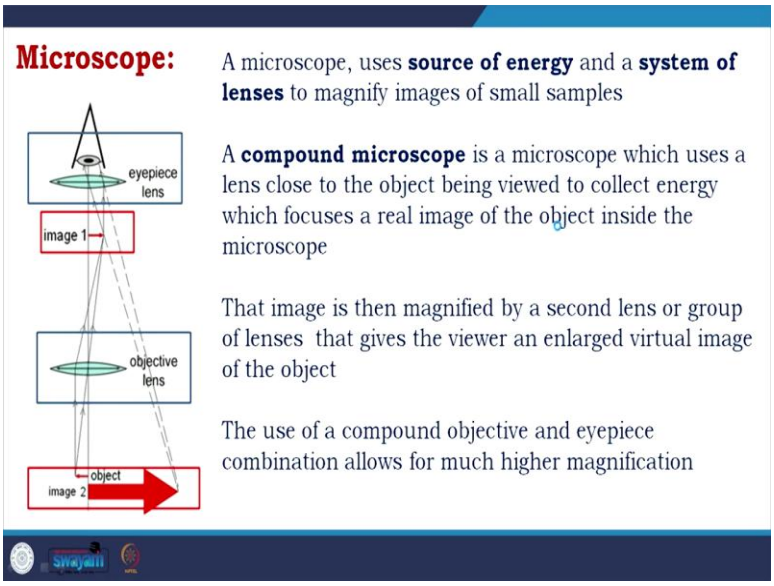

A microscope use a source of energy and a system of lenses to magnify images of small samples. So, there are set of systems you have objective lens and eyepiece lens and many of them. A compound microscope is a microscope which uses a lens close to the object being viewed to collect energy which focuses a real image of the object inside the microscope that image is then magnified by a second lens or more importantly group of lenses which is now used in modern microscope that gives the viewer an enlarged virtual image of the object.

The use of a compound objective and eyepiece combination allows for much higher magnification.

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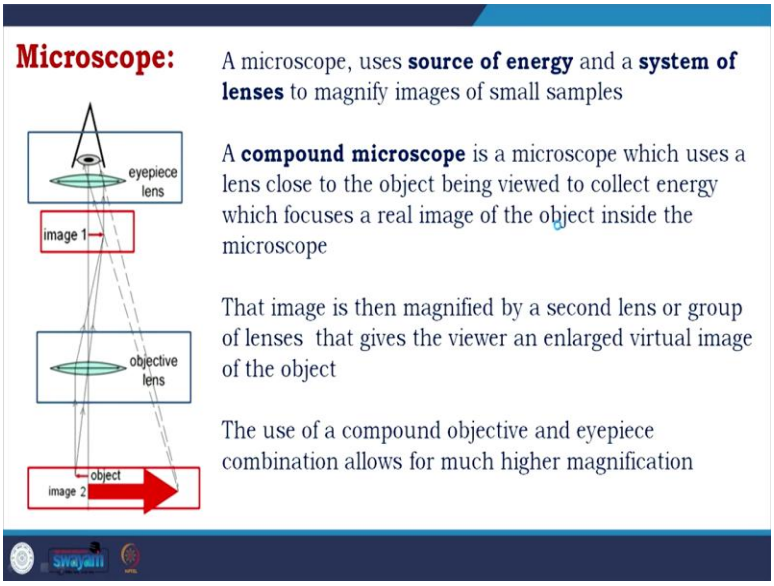

Now I will talk about Confocal Microscopy. If you look at confocal microscopy picture, you see the pinhole. Pin hole is the key to confocal microscooopy. Only the light from the focal plane passes through the pin hole to the detector, and confocals have good z-axis resolution.

So, if you see you could have get the width, that is the reason this microscope is great for imaging thick samples because the hedge from the out-of-focus object is mostly eliminated.

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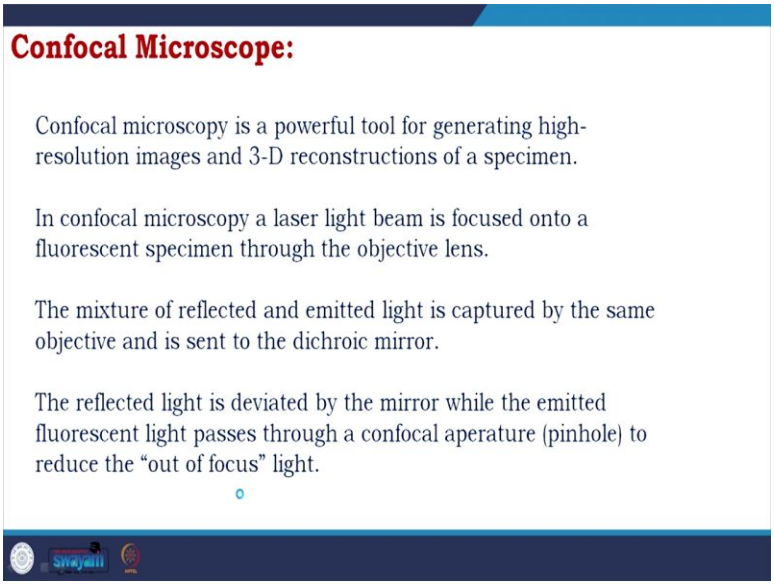

Confocal microscope is a powerful tool for generating high-resolution images and 3D reconstruction of a specimen. In confocal microscopy a laser light beam is focused onto a fluorescent specimen through the objective lens. The mixture of reflection and the emitted light is captured by the same objective and is sent to the dichroic mirror. The mirror deviates the reflected light while the emitted fluorescent light passes through a confocal aperture to reduce the out of focus light.

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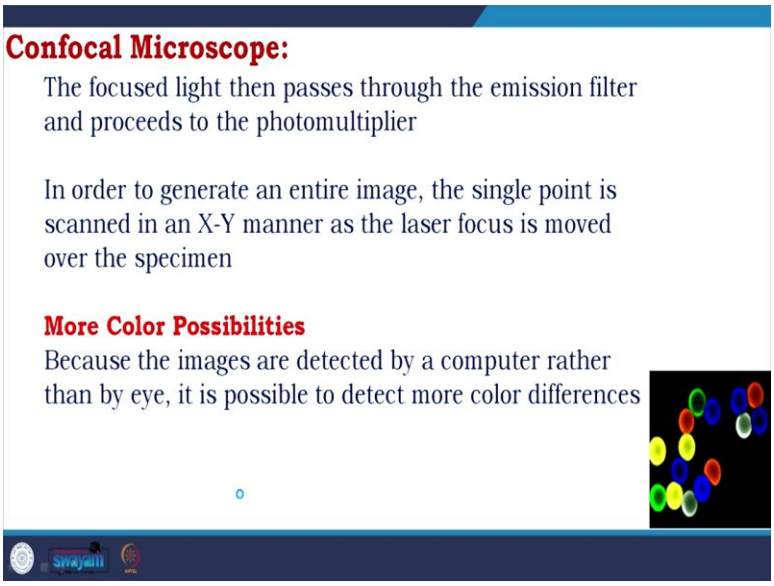

The focused light then passes through the emission filter and proceeds to the photo multiplier to generate an entire image. The single point is scanned in a xy manner as a laser focus is moved over the specimen. Also, there is more color possibilities because the images are detected by a computer rather than by eye it is possible to detect more color differences.

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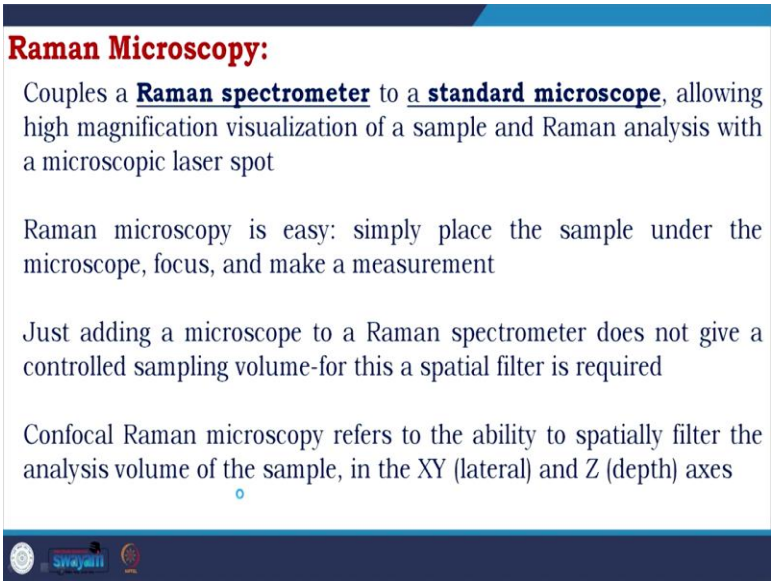

Now I talk about a Raman spectrometer and a confocal microscope. So, you couple a Raman spectrometer to a standard microscope allowing high magnification visualization of a sample and Raman analysis with a microscopic laser spot. Raman microscope is easy. Place the sample under the microscope focus and make a measurement. Just adding a microscope to a Raman spectrometer does not give a controlled sampling volume for this.

A spatial filter is required confocal Raman microscopy refers the ability to specially filter the analysis volume of the sample in the xy the lateral and the z the depth axis.

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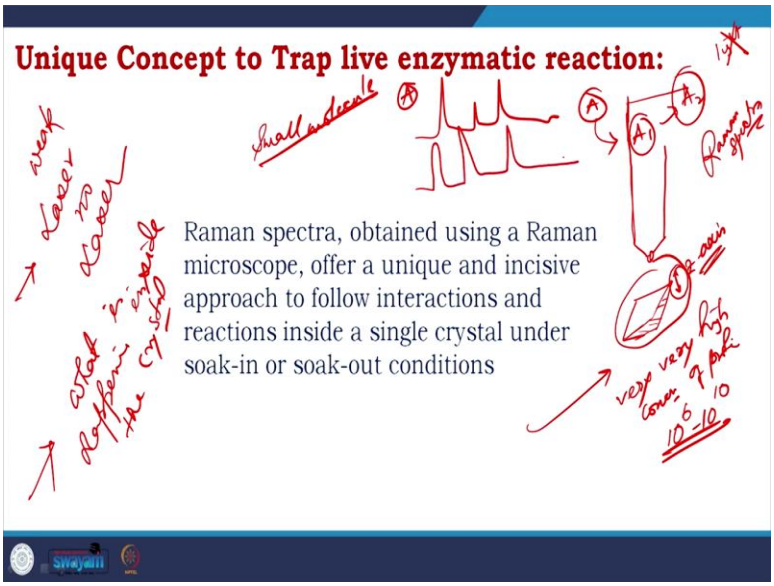

Coming to Raman crystallography, as I told it is a unique concept to trap live enzymatic reaction. The Raman spectra obtained using a Raman microscope offer a unique and intelligent approach to follow interactions and reactions inside a single crystal under soaking or soak out condition. First understand the concept, you have a crystal, the crystal offer a 3D environment. Now you have a setup of confocal microscope where you could scan the z axis.

Now imagine you are not taking the light you are taking the Raman spectrum by putting a Raman monochromator and receiving that. So, what is happening? Now you are replacing a liquid sample with a crystal. What is the advantage? First advantage is very high concentration of protein. If you compare between a solution of protein and the same concentration to get a crystal at least you get 10^6 to 10^{10} time enhancement, first advantage.

Second advantage is the use very weak laser or no laser. Third advantage, you are only scanning the z axis. So you only look at what is happening inside the crystal. Think about you have a small molecule (A). Now you have characteristic spectra of this (A) molecule. Now you set

everything, you set a protein crystal that affects (A) if (A) would be changing to A1, A2 there is a change of bond, there would be the change of spectra.

So, here you align and this is a different you understand. So, what you do, you have a high-resolution CCD camera. So, the ray is coming out, and you get the thing live. The data you are getting here, you have the baseline, baseline of the intact compound, and whatever is coming through, you are aligning it. So, whenever you get a change you know that this is the time when the bond is breaking or making.

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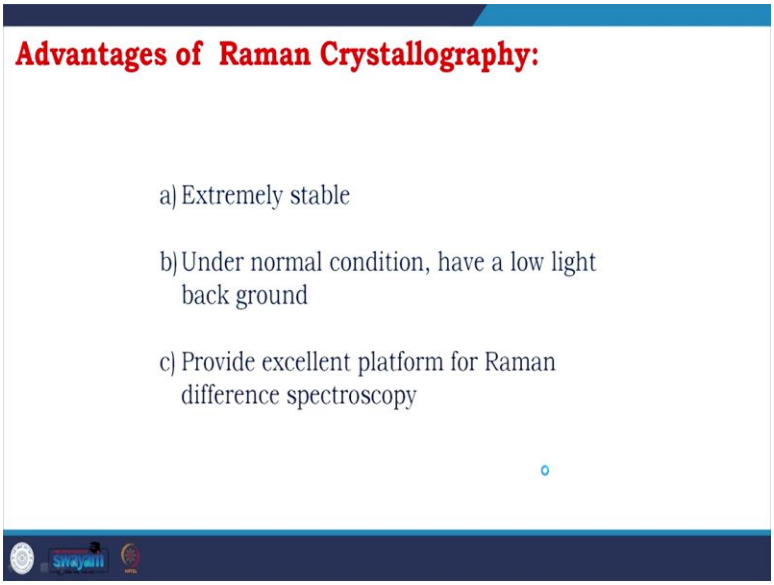

So, first advantages of Raman crystallography is extremely stable sample because you are using a weak laser or no laser. Under normal condition a low light background provides an excellent platform for Raman different spectroscopy. What is Raman difference as I was talking about the spectra of compound A. Now A is changing (suppose it is a amide let us say it is changing to amine) will change in the characteristic frequencies.

You collect the video and keep the new or upcoming spectra aligning with the base spectra whenever there is a change you could detect when the reaction is happening, what is the span of the reaction and everything.

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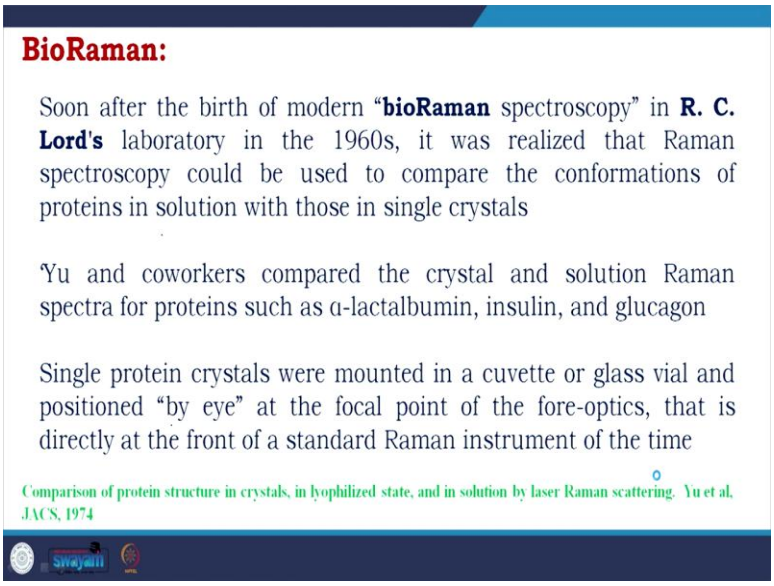

Soon after the birth of modern bioRaman spectroscopy in R. CL laboratory in 1960 it was realized that Raman spectroscopy could compare the conformations of proteins in solution with those in single crystals. Yu and coworker compared the crystalline solution Raman spectra for proteins such as alpha-lactalbumin, insulin and glucagon. So, they take it in the solution they take in the crystal and they compare.

Single protein crystals were mounted in a cubit or glass vial and positioned 'by eye' at the focal point of the fore optics directly at the front of a standard raman instrument.

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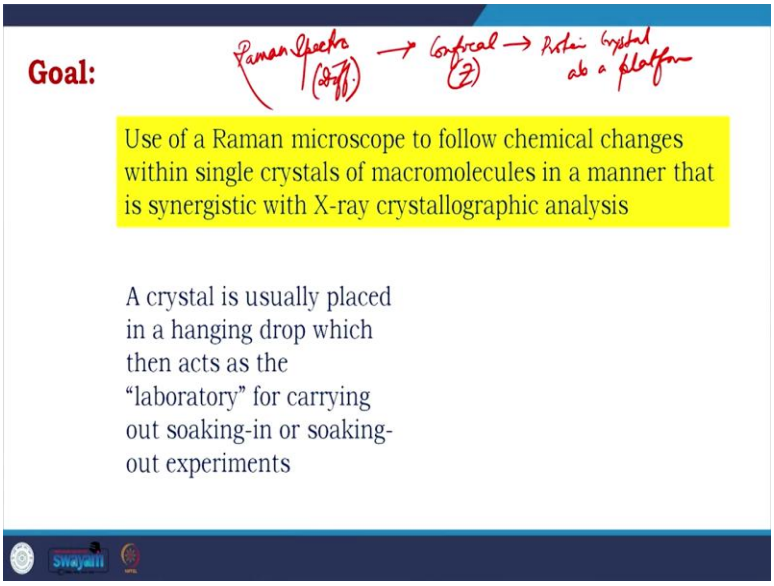

The goal of Raman microscope is to follow chemical changes within a single crystal of macromolecule in a manner that is synergistic with x-ray crystallographic analysis. So, we are using Raman spectra (especially the difference in Raman spectra). We are using a confocal microscope (we lock in z of the crystal), and we are using protein crystal as a platform for the experimental setup. So, Raman spectroscopy confocal and crystallography combined we name this unique technique as Raman crystallography. A crystal is usually placed in a hanging drop, which acts as the laboratory because this is the platform for soaking in or soaking out the experiment.

So, you have the crystal in the drop you add the small molecule it goes inside and it would be only counted (it would be only visible) if it comes inside because (in the instrumental setup we are only scanning the z axis). We are not accepting any other rays any other information which are coming from other sources. We are only locked at the z axis using the key concept of pinhole which is the key concept of confocal.

So, this is the crystal we put here; now, gradually, the small molecule would be going inside the protein crystal. Now the enzyme will react that would change the molecule as I was talking. Whenever there is a change there would be a change in the spectra, that change would be identified by the difference Raman which is continuously going through by the detection of the CCD camera. So, whenever you see a change you know that this is the moment there is going a change.

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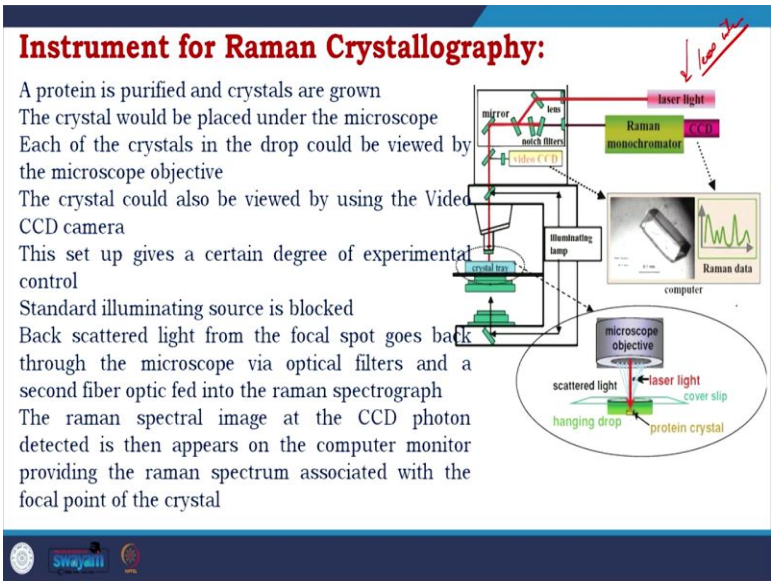

So, this is the crystal tray you put your crystal here, and you have the video CCD. You have the Raman Monochromator, you have laser light. You could use laser in less intensity or do not use it. You could see the position of the crystal, and you could control it. Just like you see the microscope objective, you have the crystal here and the scattered light is coming (the laser light)

A protein is purified and crystals are grown. So, you could do that only if you have the protein crystallography set up.

The crystal could also be viewed by using the video CCD camera as you see it is shown here. This setup gives a certain degree of experimental control. Standard illuminating source is blocked as I told earlier back scattered light from the focal spot goes back through the microscope via optical filters and a second fiber optic fade into the Raman spectrograph. So, the Raman spectrograph is continuously generated.

The Raman spectral image at the CCD photon detected then appears on the computer monitor providing the Raman spectrum associated with the focal point of the crystal.

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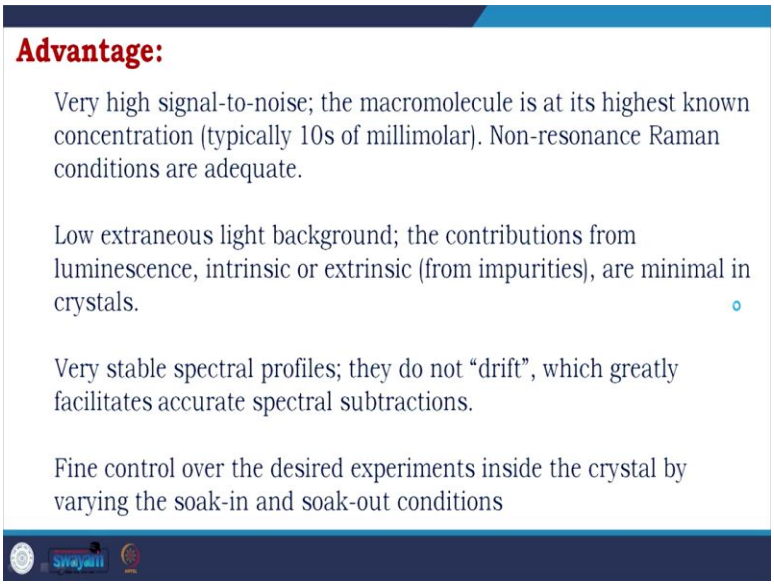

So, advantage very high signal to noise, the macromolecule is at its highest known concentration typically tens of millimolar. Non resonance Raman conditions are adequate. Low extraneous light backgrounds, the contributions from luminescence intrinsic or extrinsic (from impurities) are minimal in the crystal. Very stable spectral profile, they do not drift which greatly facilitate accurate spectral subtractions in the different Raman spectra.

Find control over the desired experiment inside the crystal by varying the soak in and soak out condition. So, you could change the concentration of the small molecule, and you have another platform to do a beautiful range of experiments.

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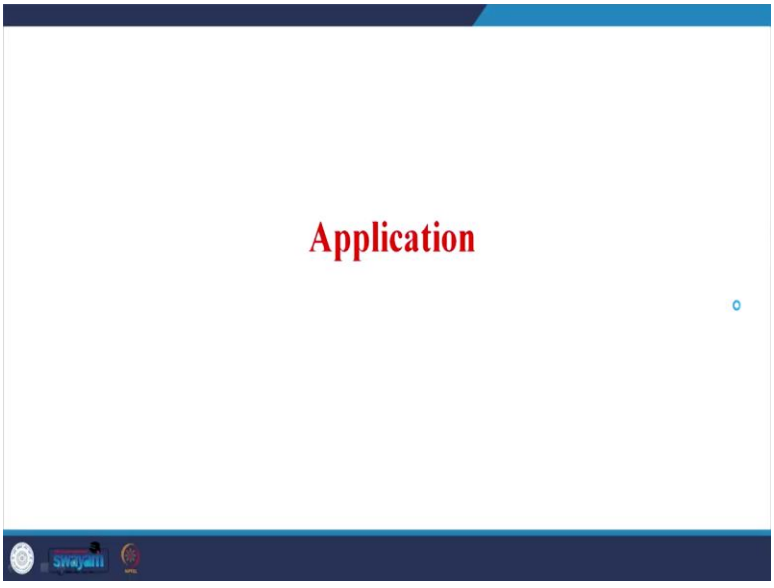

Application, you could see the live reaction. But going into more detail, I have taken an example of enzyme beta lactamase, because bata lactamase is one of the rare enzyme which works when I say work, I mean reacts with the substrate covalently.

If you have gone through my protein studies and started understanding enzymology, you know that enzymes just take part in the reaction make the reaction faster and make the reaction possible but do not take part directly in the reaction. Exception is this enzyme beta lactamase or penicillin binding proteins, they directly take part in the reaction (reacting with the bata lactam). So, that is why this technique for tracking a live reaction is the best possible vehicle.

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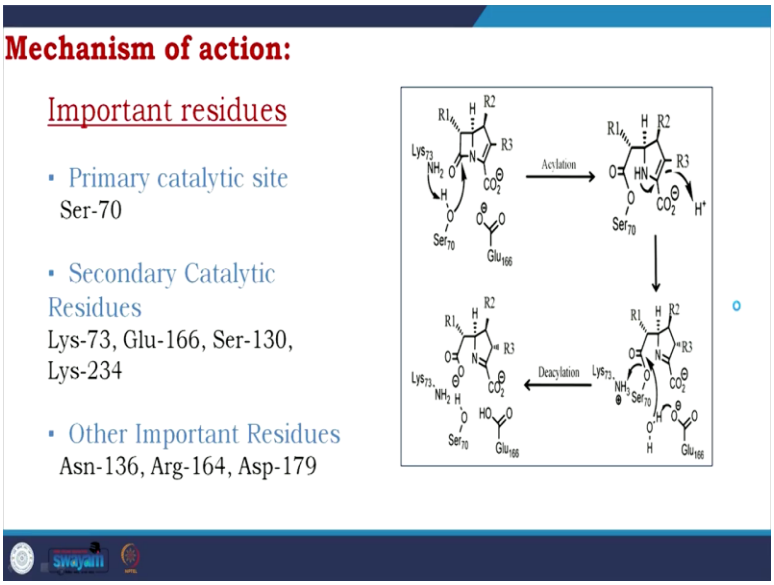

So looking at important residues the primary catalytic residue is Ser-70, the secondary catalytic residues are lysine 73, glu 166, Ser 130, lysine 234, other important residues are Asparagine 136, Arginine 164 and Aspartate 179. If you are new to the field, you might think that what I am talking about is a number because if you get the same family of enzyme, you find that they have different numbers of the residues.

In the field of antimicrobial resistance, beta lactamase or PVP binding proteins are the major target and people have developed a language to talk about this which is called Amblers classification.

What is Amblers classification? When you talk about class A beta lactamase, you take the enzyme TEM from E. coli record its residue numbers and talk about others you will refer to this number.

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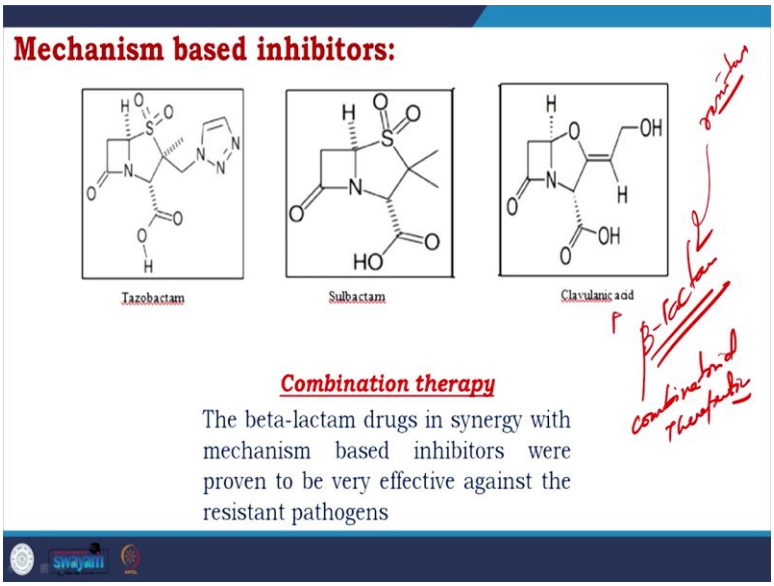

And more interestingly a group of drug called mechanism based inhibitors or MBIs. They are tazobactam, sulbactam and clavulanic acid. If you look at them, they have mostly similar structure. So, these MBIs take very important role in beta lactam based therapeutics because we all know that classic beta lactams are ineffective if there is resistance against them. But when you use them with this (MBIs) it is called combinatorial therapeutics.

Combinatorial therapeutics are still working for them and specially for resistant pathogens we use them.

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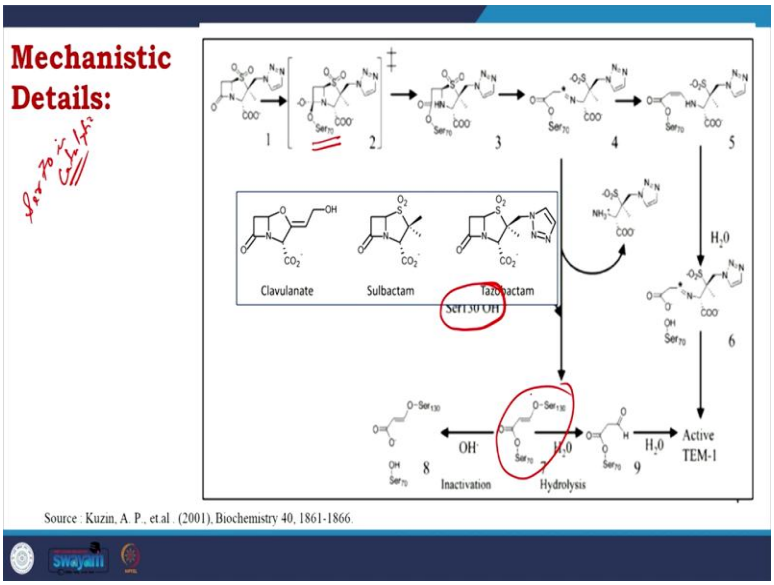

So, here is the mechanism and looking at the mechanism, serine 70 is involved, but we know that serine 70 is catalytic residue. So, involvement of serine 70 is understandable but more interestingly this MBIs involve a second serine which is serine 130 that is the beauty of how these mechanism-based inhibitors work. They involve the catalytic serine 70 and a secondary catalytic residue serine 130, making a bridge between them. So, it is very difficult to develop resistance.

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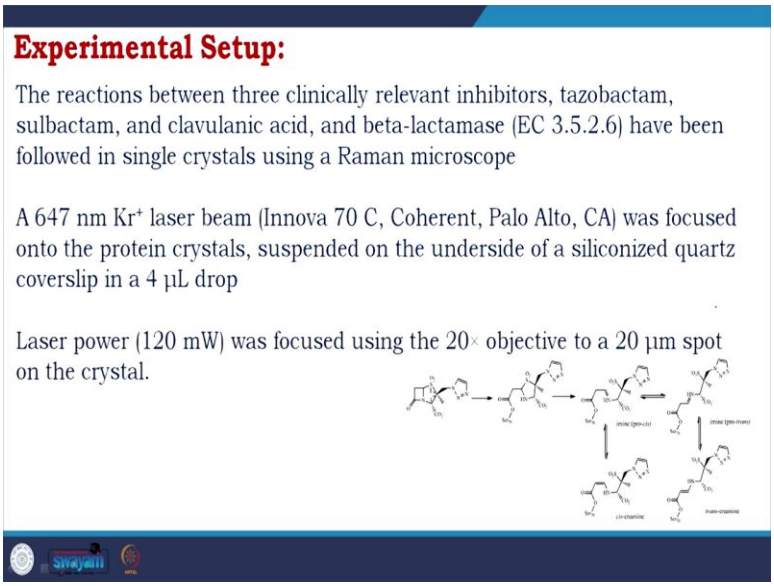

So, what experimental setup is developed between 3 clinically relevant inhibitors and batalactamase has been followed in single crystal using Raman microscope or Raman

crystallography apparatus. A 647-nanometer krypton laser beam was focused onto the protein crystal suspended on the underside of a siliconized quartz coverslip.

So, the presence of siliconized quartz coverslip reduces the intensity. Laser power 120 megawatts was focused using a 20x objective to a 20-micrometer spot on the crystal.

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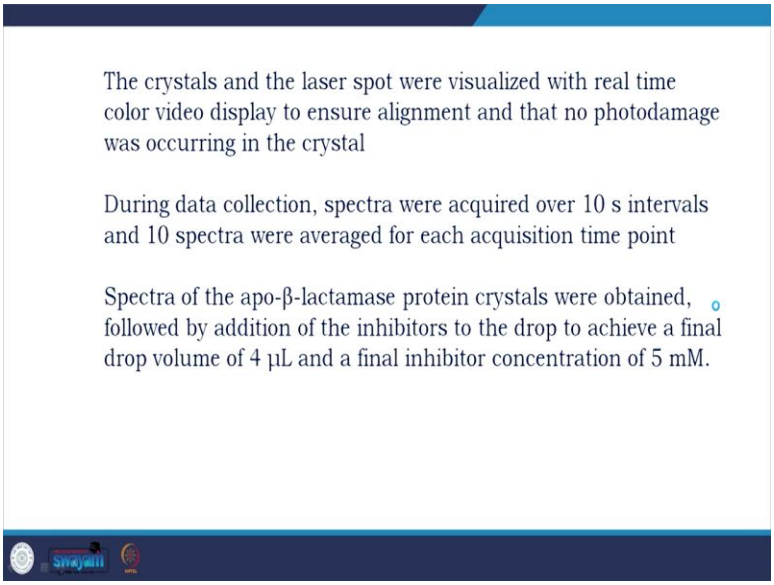

The crystals and the laser spots were visualized with real-time color video display to ensure alignment. No photodamage was occurring in the crystal during data collection. Spectra were acquired over 10-second intervals and ten spectral averages for each acquisition time point. So, it is giving accuracy. Spectra of the apo beta-lactamase protein crystals were obtained where there was no small molecule.

Followed by the addition of the inhibitors to the drop to achieve a final drop volume of four microliter and a final inhibitor concentration of five millimolar.

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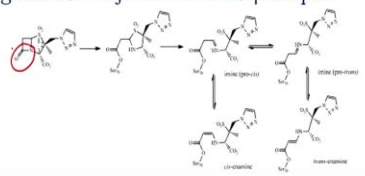
Experimental Setup:

The reactions between three clinically relevant inhibitors, tazobactam, sulbactam, and clavulanic acid, and beta-lactamase (EC 3.5.2.6) have been followed in single crystals using a Raman microscope

A 647 nm Kr^+ laser beam (Innova 70 C, Coherent, Palo Alto, CA) was focused onto the protein crystals, suspended on the underside of a siliconized quartz coverslip in a 4 µL drop

Laser power (120 mW) was focused using the 20× objective to a 20 µm spot on the crystal.



Spectra were then acquired serially every two to 3 minutes following addition of the inhibitor for experiment in D2O hepes buffer. An apo beta lactamase spectrum was subtracted from the inhibited protein spectra varying time interval following addition of inhibitor according to this equation.

The difference spectrum = [protein] + [inhibitor] – f[protein]

where f is a subtraction factor selected to minimize the protein amide one band from the apoprotein in the different spectra.

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assignment	tazobactam frequency ($cm^{-1} \pm 5$ cm^{-1})	sulbactam frequency ($cm^{-1} \pm 5$ cm^{-1})	clavulanic acid frequency ($cm^{-1} \pm 5$ cm^{-1})
enamine O–C=C–N symmetric stretch	1592 in H_2O (1582 in D_2O)	1599 in H_2O (1581 in D_2O)	1605 in H_2O (1587 in D_2O)
other enamine band	969	972	
	787	727	799
			521 in H_2O (514 in D_2O)

Raman difference spectra reveals an intermediate enamine band at 1605 cm^{-1} in water, which shifts to 1587 cm^{-1} in D_2O due to NH exchange with N ↔ D

The corresponding features for sulbactam and tazobactam are seen at 1599 cm^{-1} (1581 cm^{-1} in D_2O) and 1592 cm^{-1} (1582 cm^{-1} in D_2O), respectively

So, the assignments Raman difference spectra reveal an intermediate enamine band at 1605 cm^{-1} in water which shifts to 1587 cm^{-1}in D2O due to NH exchange with this buffer, the corresponding features of sulbactam and tazobactam are seen at 1599 cm^{-1}.

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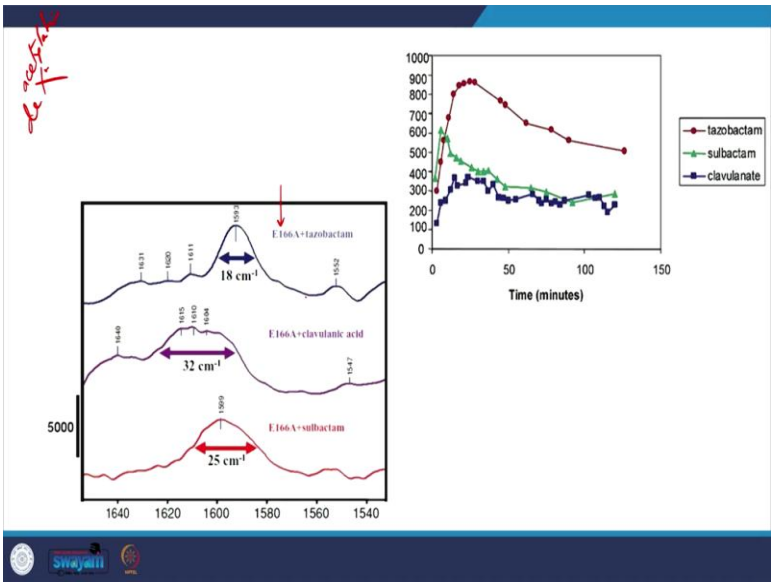

And if you see, now we have used a mutant. why we use mutant? Because we want to stop the reaction. So, if you see the mechanism of beta-lactamase, there is an acylation step and de-acylation step. We stop the de-acylation by making this glutamate to alanine mutation. And we see that when we have recorded there is change in the span and change in the peak.

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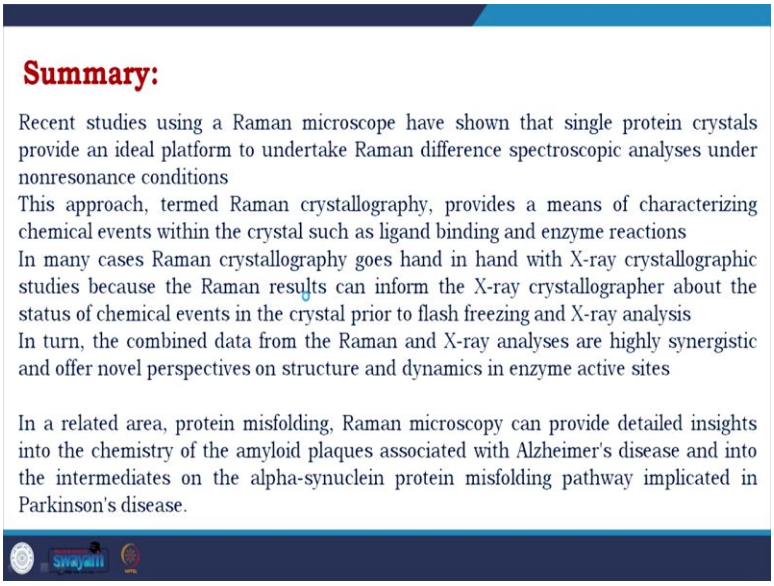

So, in summary recent studies using a Raman microscope have shown that single protein crystals provide an ideal platform to undertake Raman different spectroscopic analysis under non resonance conditions. This approach, in terms of Raman's crystallography, provides a means of characterizing chemical events within the crystal, such as ligand binding and enzymatic reaction. In many cases Raman crystallography goes hand in hand with x-ray crystallographic studies because the Raman results can inform the x ray crystallographer about the status of chemical events in the crystal prior to flush freezing and x-ray analysis.

So, what it is talking about sometime we do soaking and all and we have no idea what actually had gone inside. So, we do the entire procedure and then we find that the substrate did not go inside or the substrate did not get it properly. But now if you combine them, if you do a Raman crystallography analysis before going for the final data collection, you could know that your drug is there or not, because you see the characteristic spectra of your small molecule in the Raman monochromator.

In turn, the combined data from the Raman x-ray analysis are highly synergistic and offer novel perspective on structure and dynamics in enzyme active sites.
So, you see the normal amide bands, and then you see the alteration. Looking at the alteration you could comment. Very interestingly, this could have allowed you to develop live experiments. So, we are at the end of this module we have discussed the general principle of spectroscopy. We have talked about circular dichroism we have talked about UV visible spectroscopy.

We talked about fluorescence spectroscopy we talked about green fluorescence protein which amazing tool in modern age to trap live experiments to do many you know tagging experiments GFP is really a gift to human civilization and our ah advancement in identifying a lot of biological phenomena. Then we talk about infrared spectroscopy I agree that infrared is not much to do with protein.

But infrared and Raman as we have talked ah they are getting fingerprints they are getting significant interesting data which in future with modern setup moderated setup where you could study proteins would give us more information and we finish up with Raman. We talk about

Raman's effect on its characteristics spectra of protein but more importantly Raman microscopy and Raman spectroscopy the Raman crystallography.

I would finish this module the two techniques that people are looking for in coming days with that I would finish this module thank you very much for listening.