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Scientific

JOBIN YVON Technology

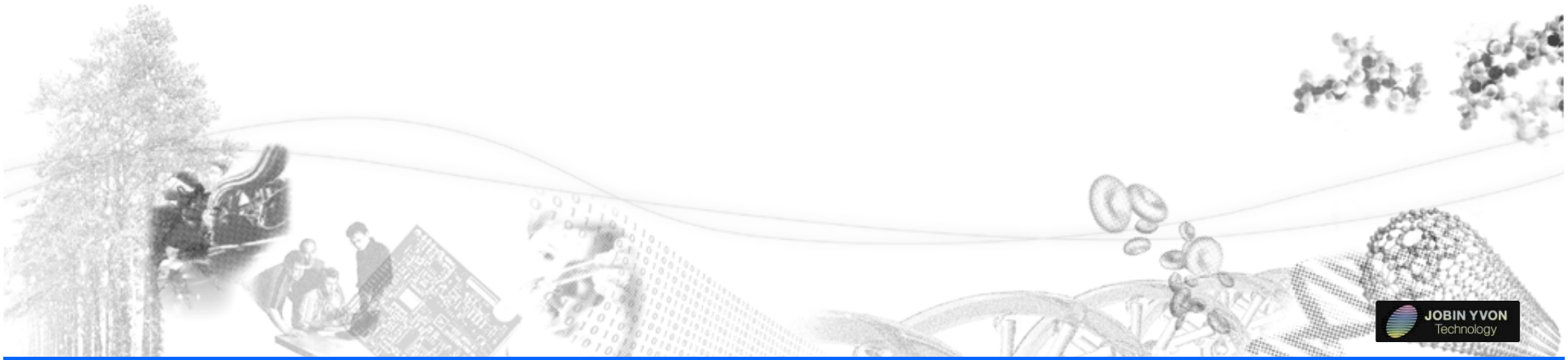
Raman Spectroscopy for proteins

Catalina DAVID Ph.D.– application scientist

Outline

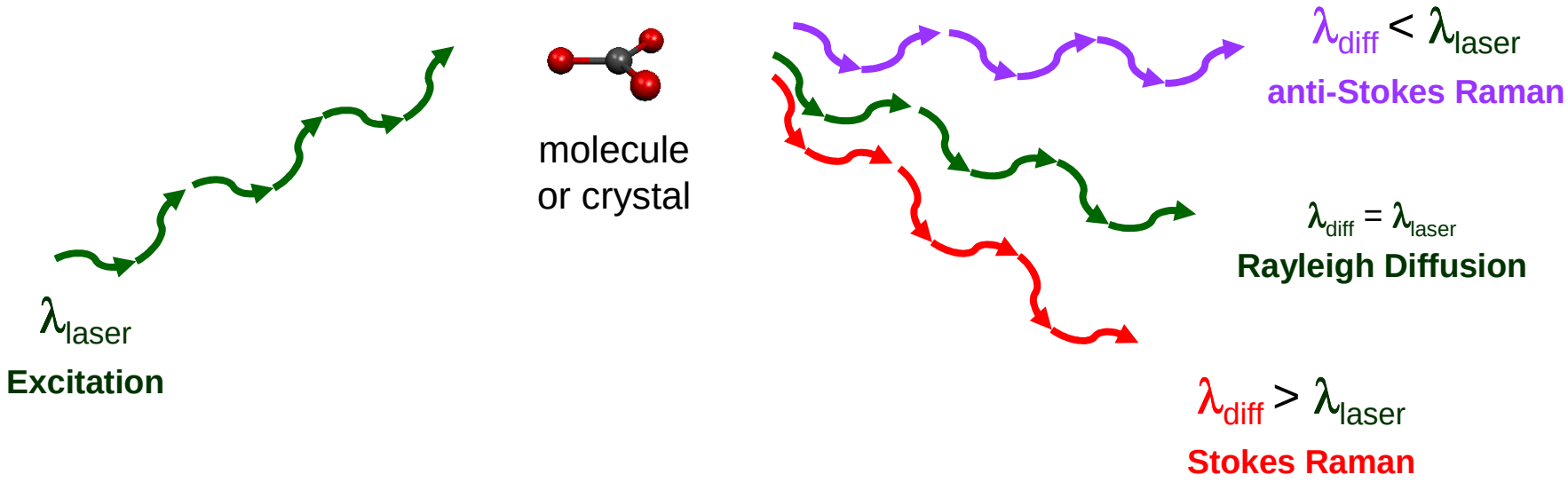
- Raman spectroscopy in few words
  - What is Raman spectroscopy ?
  - What is the information we can get?
- Basics of Raman analysis of proteins
  - Raman spectrum of proteins
  - Environmental effects on the protein Raman spectrum
  - Contributions to the protein Raman spectrum
  - UV Resonances Raman for proteins
  - Polarization measurements for proteins
  - Low-frequency measurements for proteins
- Conclusions

Raman Spectroscopy in few words



What is Raman Spectroscopy

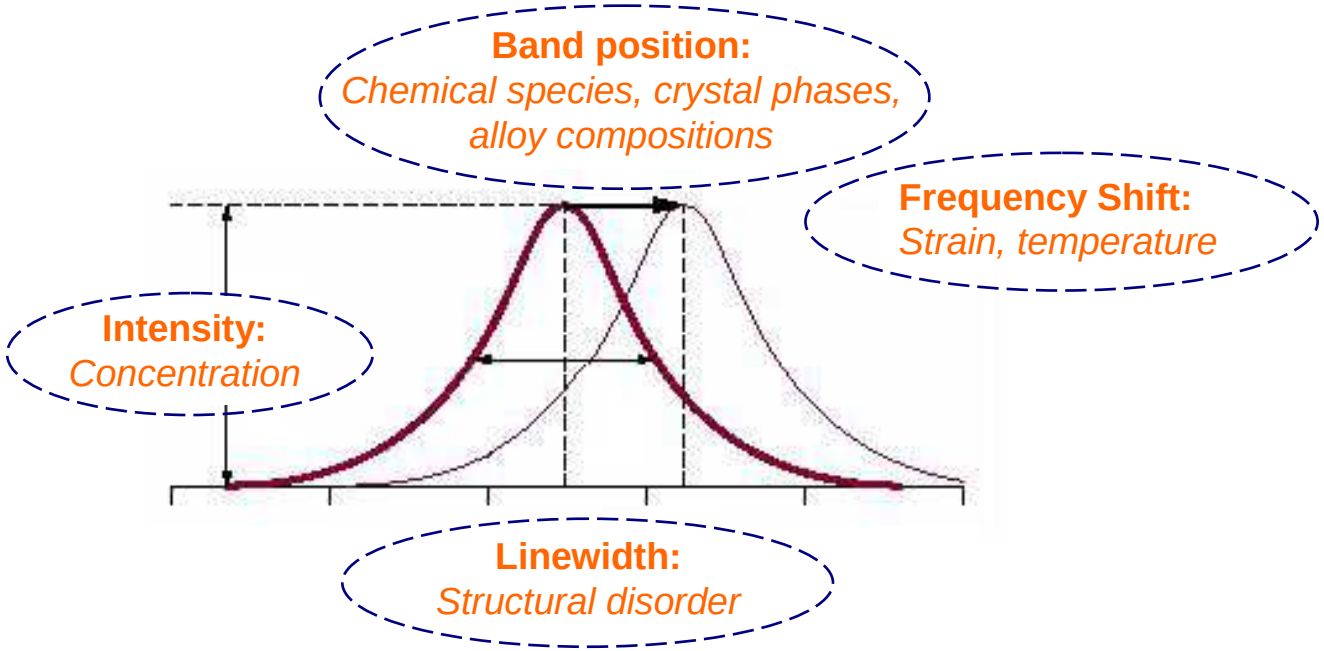
Raman effect = Inelastic Light Scattering



The frequency ($\nu = 1/\lambda$) difference between the incident and the scattered light characterises the molecule vibration.

$$\nu_{scattered} = \nu_{laser} \pm \nu_{vibration}$$

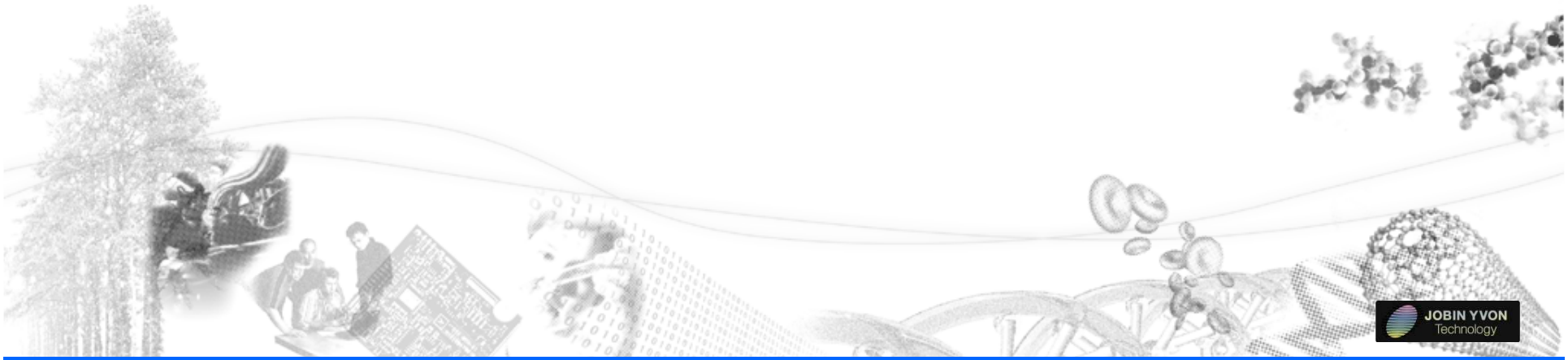
What is the information we can get



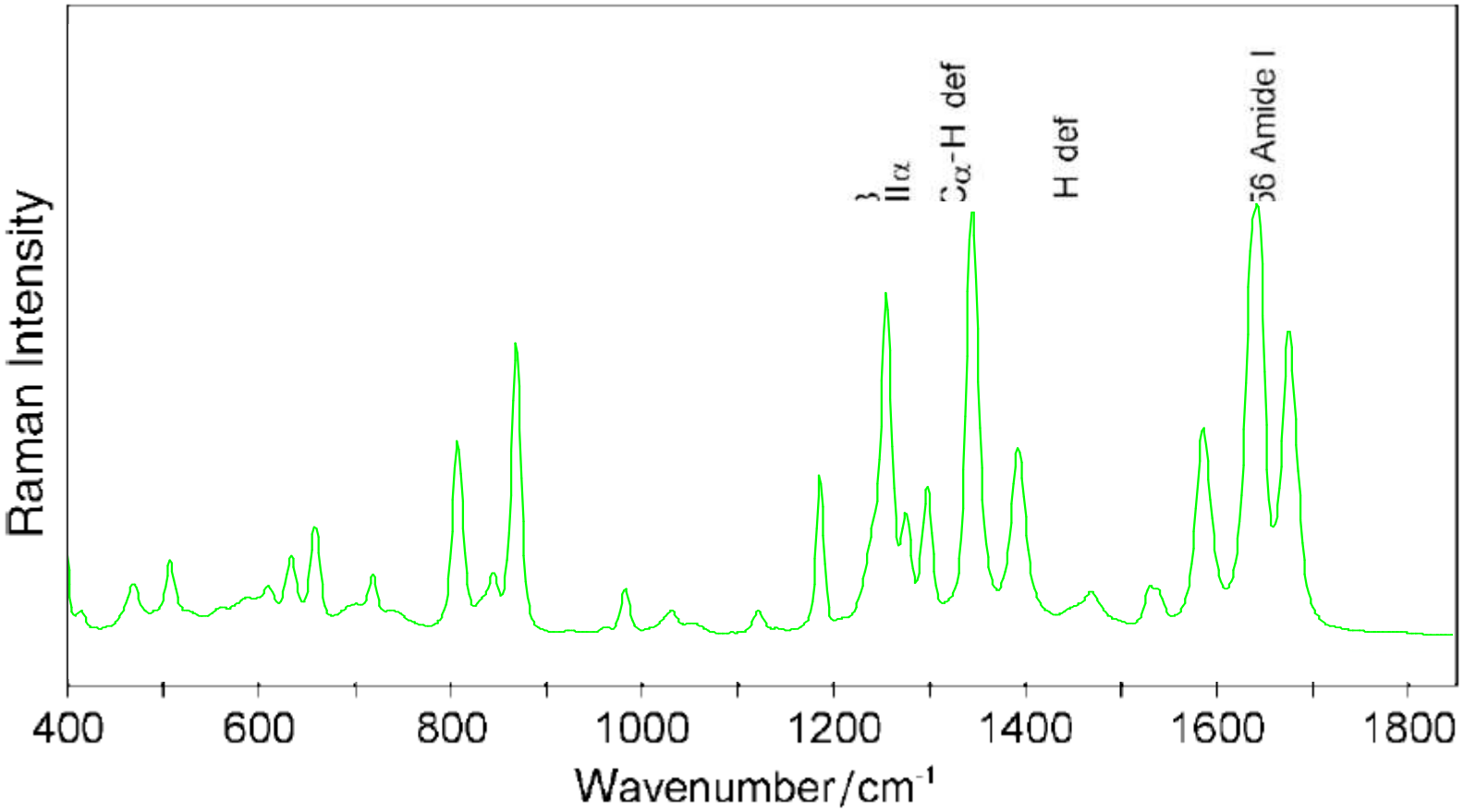
- A Raman spectrum provides a fingerprint which represents the set of bonds present in the material: vibrational frequencies are characteristic of chemical bonds or groups of bonds
- Vibrational frequencies are sensitive to details of the structure and local environment of a molecule, such as symmetry, crystal phase, polymer morphology, solvents, interactions, …
- Relative intensities corresponds principally to the species concentration but it can be related to the orientation of the material or molecule with respect to the incoming laser polarization.

Basics of Raman analysis of proteins

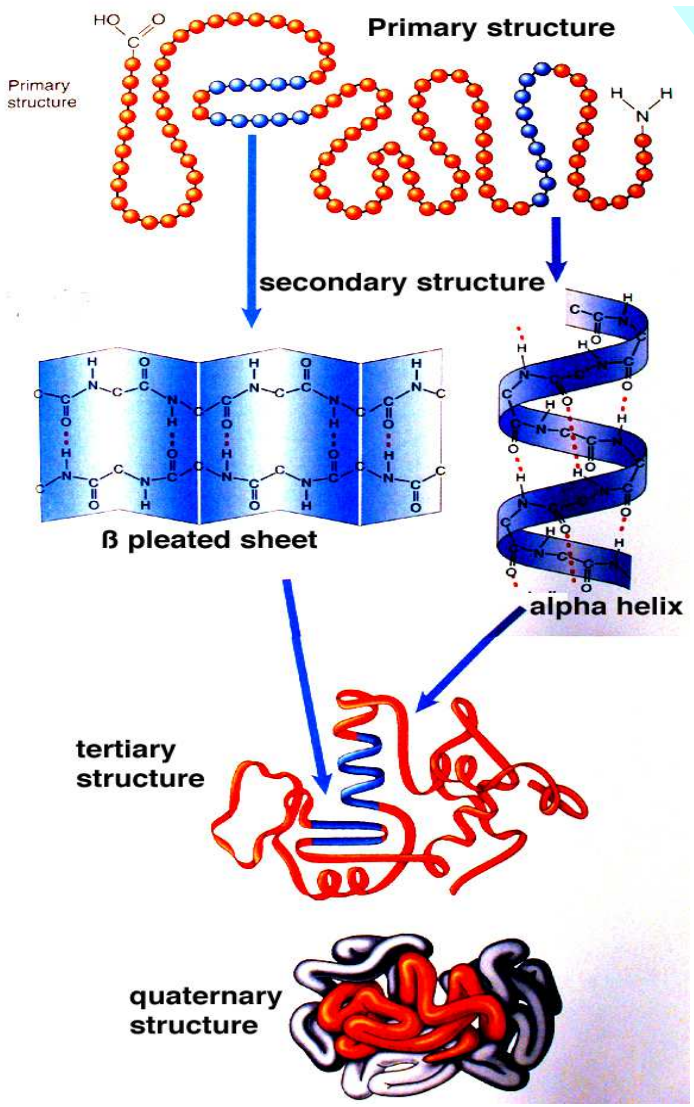
Raman spectrum of proteins



Raman spectrum of proteins



Raman spectrum of proteins

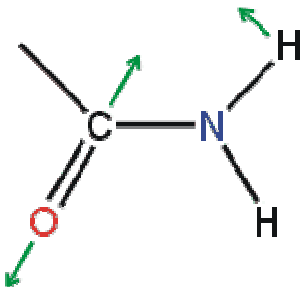


Secondary structure of the protein

Spatial arrangement of bonds (C=O)

Coupling between individual vibrations (C=O)

Amide I Raman band

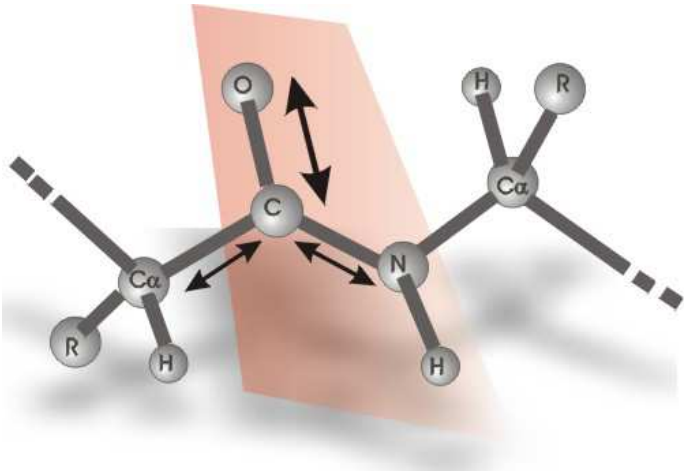


The precise positions of bands depend on inter and intra molecular effects, including peptide-bond angles and hydrogen-bonding patterns

Raman spectrum of proteins

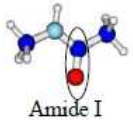
Secondary structure analysis

- Nine normal modes are allowed for the amide band of proteins.
- These are called A, B, and I-VII in order of decreasing frequency

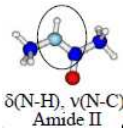


Amide Raman bands

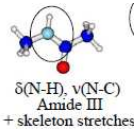
Amide I band 80% C=O stretch, near 1650cm^{-1}



Amide II band 60% N–H bend and 40% C–N stretch, near 1550 cm^{-1}

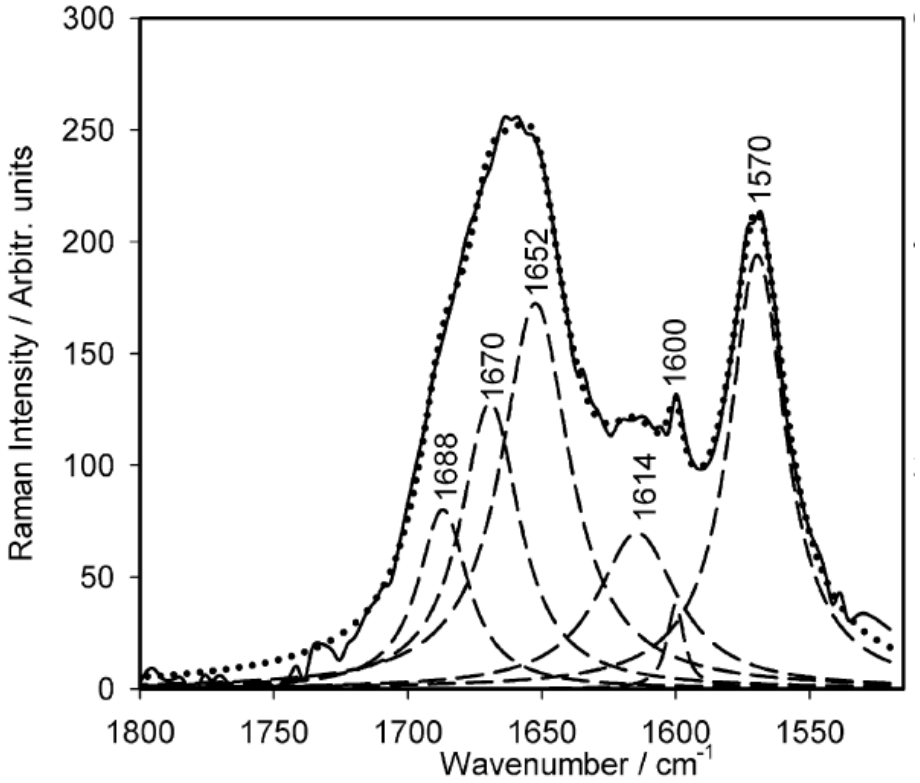


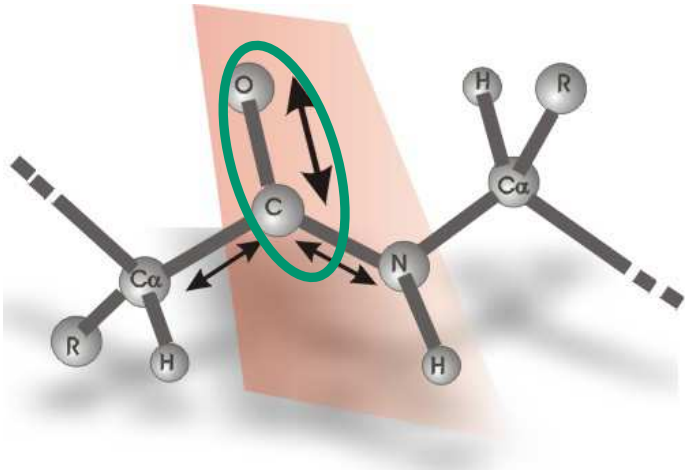
Amide III band 40% C–N stretch, 30% N–H bend, near 1300 cm^{-1}



Raman spectrum of proteins

Amide I bands





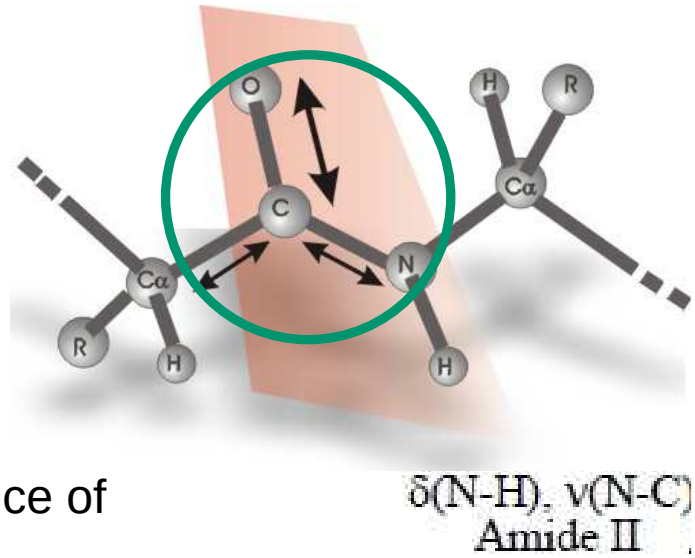
Principal Amide I Frequencies Characteristic of Protein Secondary Structures

Conformation	H_2O	D_2O
α-helix	1650–1657	1647–1654
Antiparallel β-sheet	1612–1640; 1670–1690 (weak)	1628–1635
Parallel β-sheet	1626–1640	
Turn	1655–1675	
	1680–1696	
Unordered	1640–1651	1643

The different types of secondary structures are characterized by amide I bands slightly different in position and shape

Raman spectrum of proteins

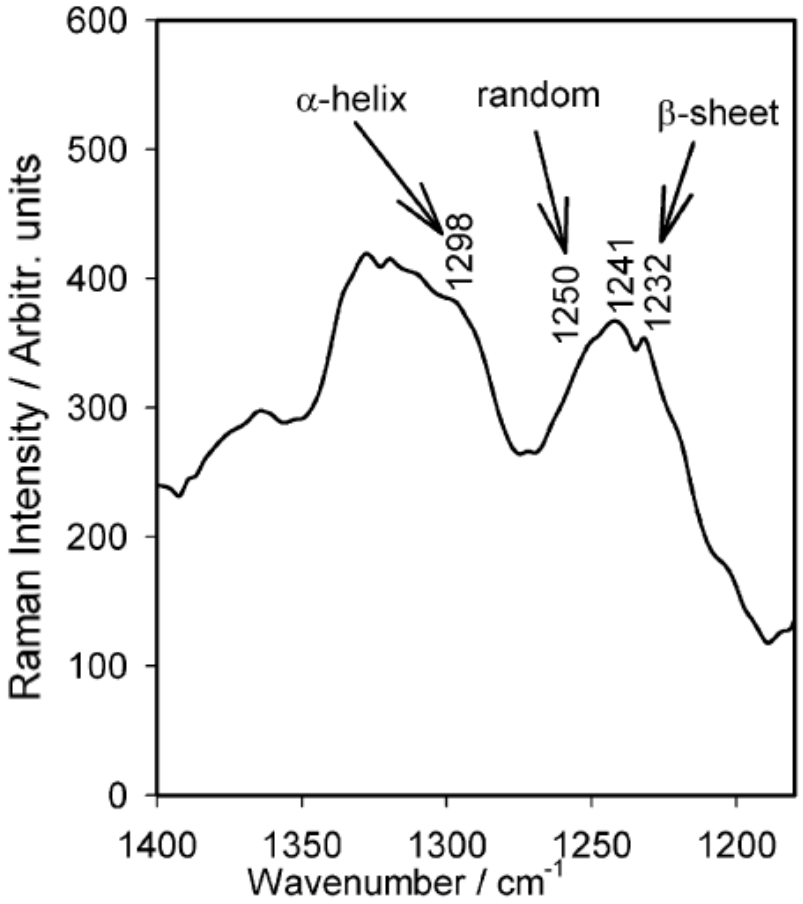
Amide II band

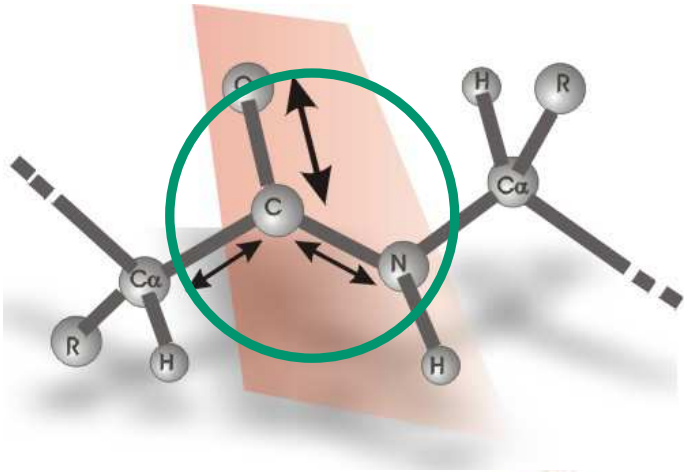


- Parallel / antiparallelβ- sheet structure ~ 1550 cm^{-1}
- It is a weak band It can not be observed in the absence of resonance excitation
- It is hardly affected by the side-chain vibrations but the correlation between secondary structure and frequency is less straightforward than for the amide I vibration.
- It can be sensitive to H/D exchange

Raman spectrum of proteins

Amide III bands





- Assignment

α-helix : 1270-1300 cm^{-1}
Random coil : 1243-1253 cm^{-1}
β-sheet : 1229-1235 cm^{-1}

- The structure of amide III band can be correlated to the amide I band = complementary structural information on the protein structure and in this way it is possible to get some additional details to the amide I

Raman spectrum of proteins

Other important spectral features in proteins spectra

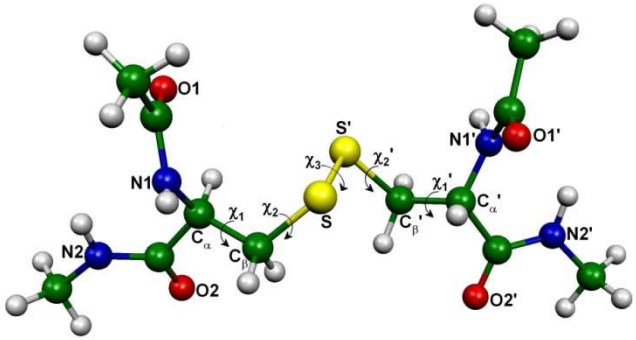
- Disulphide Bridges (S-S bonds)
- Aromatic aminoacids (Phenylalanine - Phe, tryptophan - Trp, tyrosine - Tyr, hystidin - His)

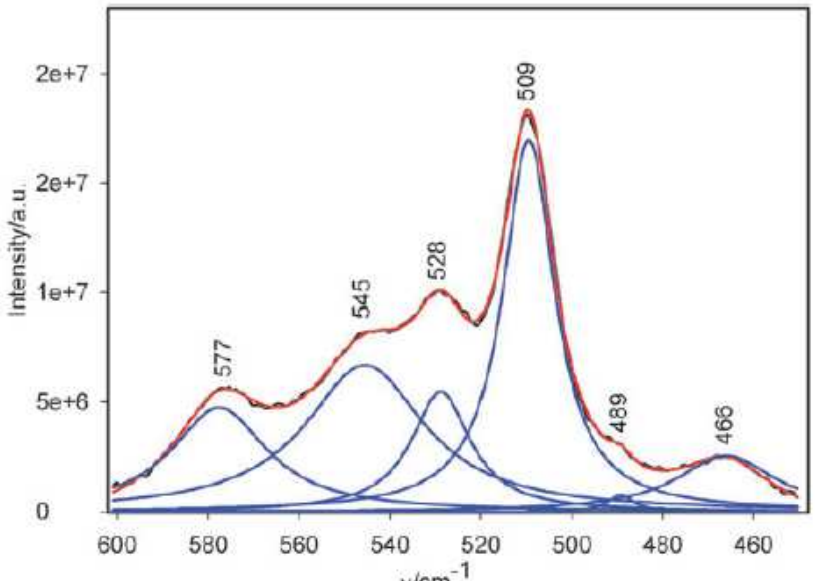
Raman spectrum of proteins

S-S bond stretching

- Experimental studies, shows that for the proteins whose structure contains S-S bridges, the S-S Raman bands are located in the spectral range 500-550 cm^{-1}.

- The factors affecting the frequency of vibration are: the relative conformation of atoms C_α-C_βS-S'-$C'_\beta C'_\alpha$ around C_β-S and C_β-S' bonds, the mode coupling and the hydrogen bonds





The analysis of the lysozyme Raman spectrum in the 450–600 cm^{-1} spectral range using Lorentzian functions. Experimental spectrum in black and simulated spectrum in red (band decomposition in blue)

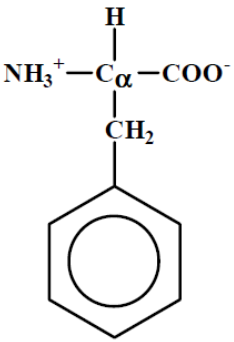
David et al, PCCP, 2009

Raman spectrum of proteins

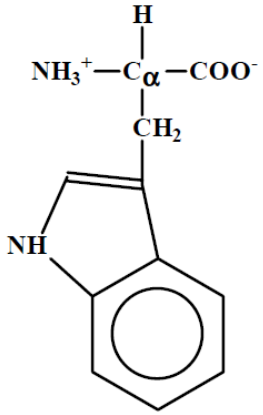
Aromatic aminoacids

- Some of the vibrational bands of tyrosine (Tyr), tryptophan (Trp) or phenylalanine (Phe) are sensitive to the microenvironment

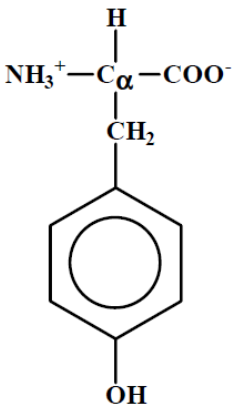
- Theirs band positions may vary up to 5 cm^{-1} in the Raman spectra of proteins.



Phenylalanine



Tryptophan



Tyrosine

Aromatic residues	*Mean frequency (cm^{-1})*
Phe	620
Tyr	640
Trp	750
Tyr	830, 850
Phe	1000, 1030
Trp	1011
Tyr, Phe	1170-1200
Trp	1340-1360
Trp	1582
Phe, Trp	1584
Tyr	1590
Phe	1605
Trp, Phe, Tyr	1610-1616
Trp	1618-1621
His	3110-3160

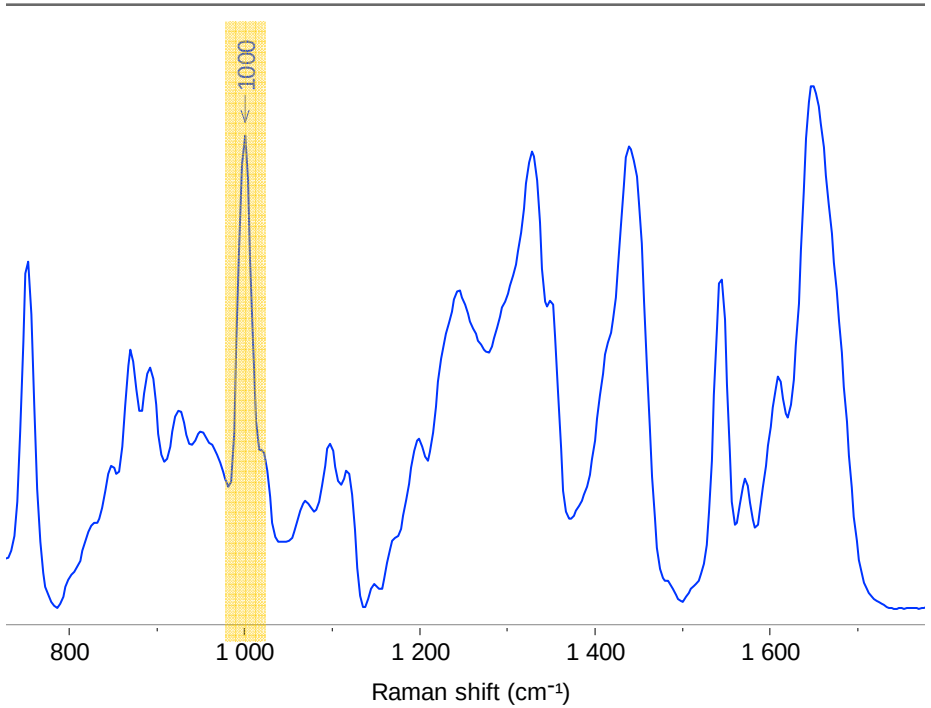
Important Raman modes of aromatic aminoacids within the protein structure

Raman spectrum of proteins

Aromatic aminoacids - Phenylalanine

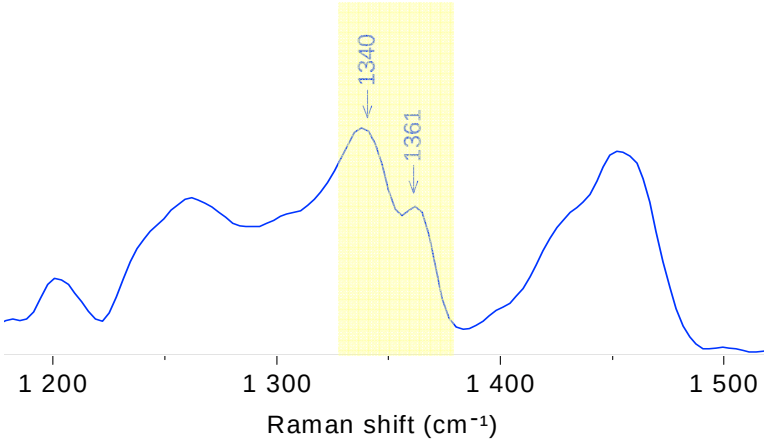
- Phe shows very intense band around 1000 cm^{-1}
- This band is not sensitive to conformational changes of protein and therefore can be used for normalization of the Raman spectra of protein

Breathing mode



Raman spectrum of proteins

Aromatic aminoacids - Tryptophan



■ The components of the Fermi doublet of Trp : 1340 and 1360 cm^{-1}.

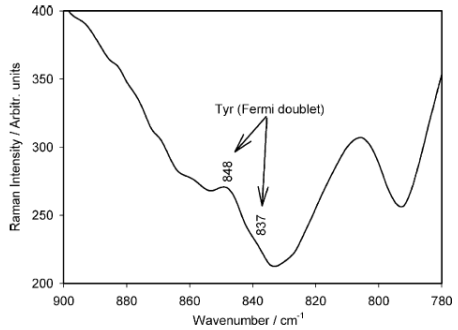
- I_{1360}/I_{1340} serves as a hydrophobicity marker.
- The 1360 cm^{-1} band is strong in hydrophobic solvents ($I_{1360}/I_{1340} > 1.1$)
- The 1340 cm^{-1} band is stronger in hydrophilic environment ($I_{1360}/I_{1340} < 0.9$)

■ The 1010 cm^{-1} band is sensitive to the strength of van der Waals interactions of the Trp ring with surrounding residues

- ν near or below 1010 cm^{-1} indicates weak or no van der Waals interactions
- ν near 1012 cm^{-1} or higher reflect stronger van der Waals interactions

Raman spectrum of proteins

Aromatic aminoacids - Tyrosine



- Tyrosine doublet Raman bands near 830 and 850 cm^{-1}.

- They are caused by Fermi resonance between the in-plane breathing mode of the phenol ring and an overtone of out-of-plane deformation mode

- The intensities of these two bands depend on the hydrogen bonding condition of the phenol side chain.

Relative intensity of sensitive Raman bands in rabbit lenses with induced cataract

		Rabbit lenses	
		Normal	Cataractous
H_2O	I_{3390}/I_{2935}	0.40	0.50
S–H	I_{2580}/I_{2730}	1.53	1.38
TYR	I_{832}/I_{855}	0.92	0.96
TRP	I_{880}/I_{760}	0.78	0.66
Amide	I and III		No change
TYR/PHE	I_{644}/I_{624}	1.77	1.44

Journal of Molecular Structure, 214 (1989) 111–117

- The ratio I850/I830 is often analyzed

  - 6.7 corresponds to non-hydrogen bonde
  - 2.5 the OH group of tyrosine is a strong
  - 0.3 corresponds to tyrosine as a donor of a
  - 1.25 shows that the OH group serves both as an acceptor and a donor of a hydrogen bond.

Raman analysis of proteins

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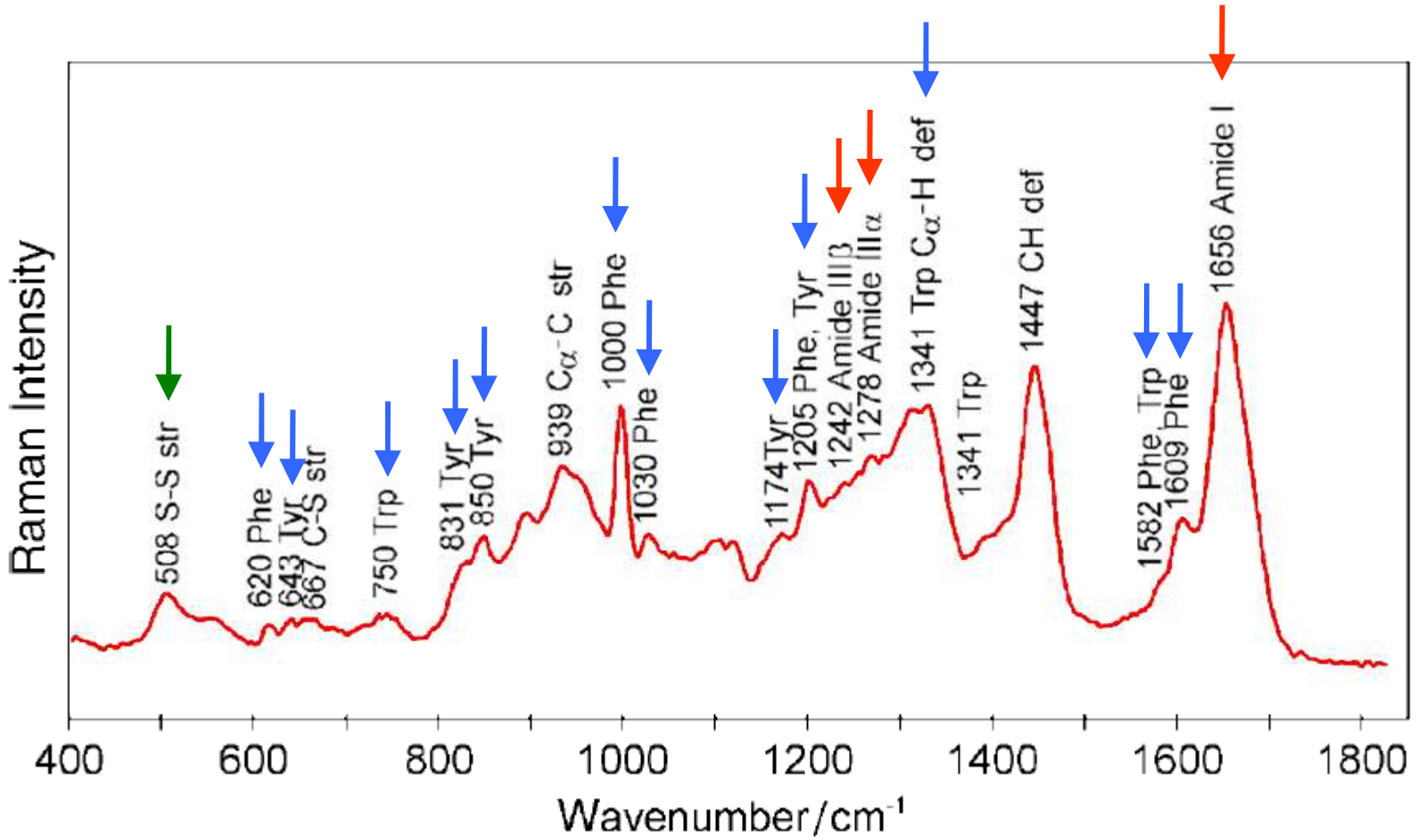
- Size of proteins makes spectrum complex

  - Polypeptide backbone
    - Secondary structure – amides bands
    - Tertiary structure – background, aromatic aminoacids

  - Aminoacids in side chains
    - H-bonding
    - Environment
    - Intermolecular interactions
    - Aromatic aminoacids are sensitive to micro-environment

Raman analysis of proteins

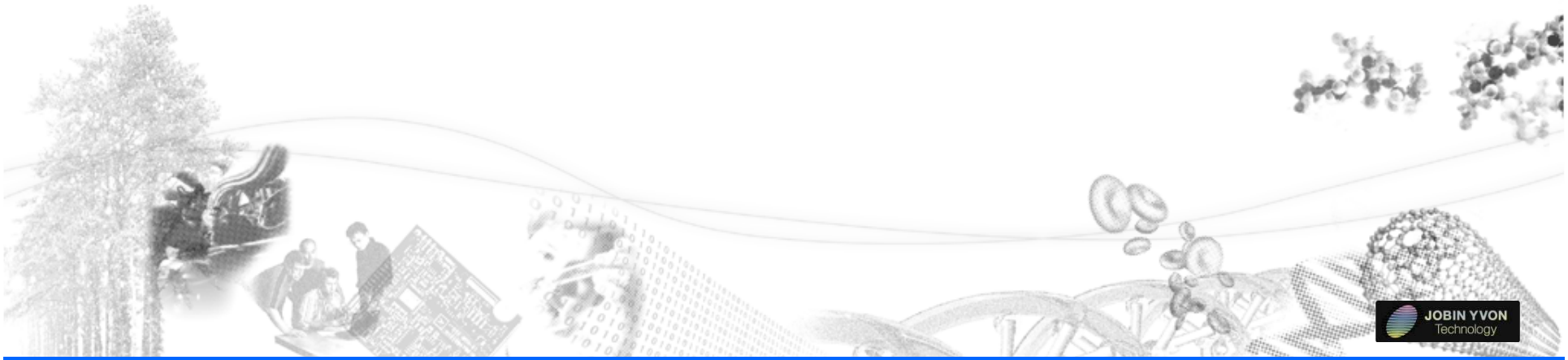


backbone **amino acids** **S-S**

Basics of Raman analysis of proteins

Environmental effects on the protein

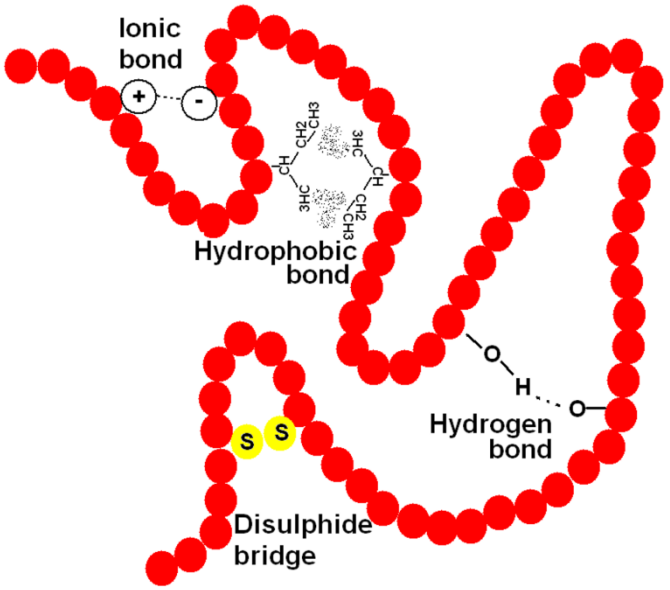
Raman spectrum



Environmental effects on the proteins Raman spectrum

■ Four major types of interactions stabilize the native structure of proteins :

- The hydrogen bonds
- The covalent disulphide bonds
- The ionic bonds
- The hydrophobic effect



■ These interactions can be disturbed by reactions with external agents, solvents, pH, temperature, salt concentration…thus the protein can suffer some conformational changes

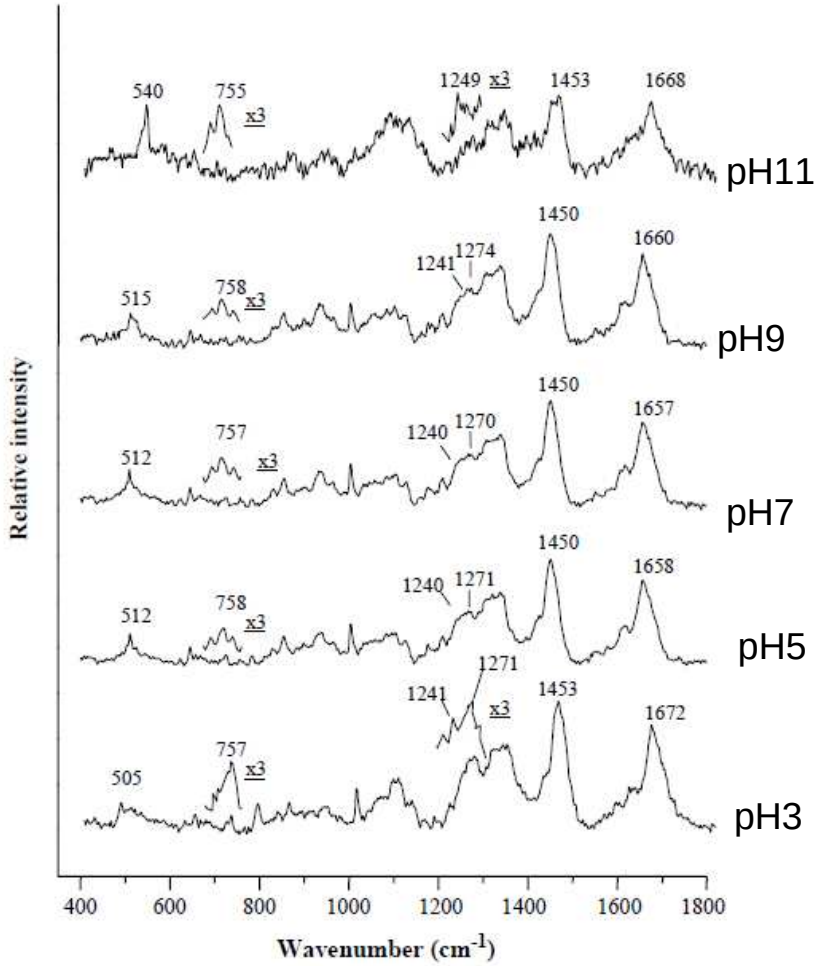
■ The conformational changes can be often observed and followed on the Raman spectrum

■ Raman spectroscopy allows the study of folding / unfolding processes in proteins

Environmental effects on the protein Raman spectrum

pH effect

- changes in the ionization of proteins side chains
- hydrogen bonds can be strongly disturbed
- The native structure of the protein can be affected
- Representative example is the study done by SW Ellepola et al. on rice globulin protein, for different pH values
- They found a slightly shift of amide I and amide III bands indicating a transition from α-helical structure to β-sheets and disordered structures under different pH conditions



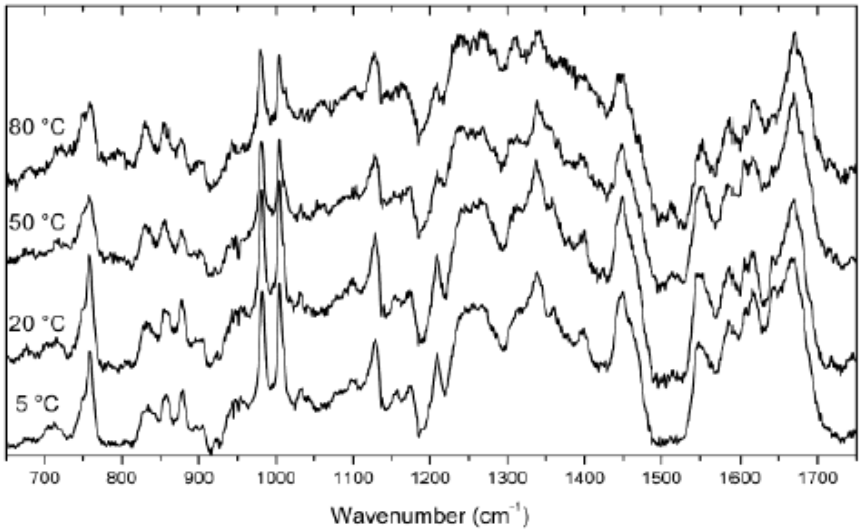
Raman spectra of rice globulin under different pH conditions.

S.W. Ellepola et al. / Journal of Cereal Science 43 (2006) 85–93

Environmental effects on the protein Raman spectrum

Temperature effects

- Very high or very low temperatures can lead to the weakness or even broking of bonds inside the protein structure – Secondary structure is destabilized - Tertiary structure will be affected
- Example below : Study of the thermal denaturation of human haptoglobin (a glycoprotein from a blood serum which plays an important role in immune system and in response to shock conditions)



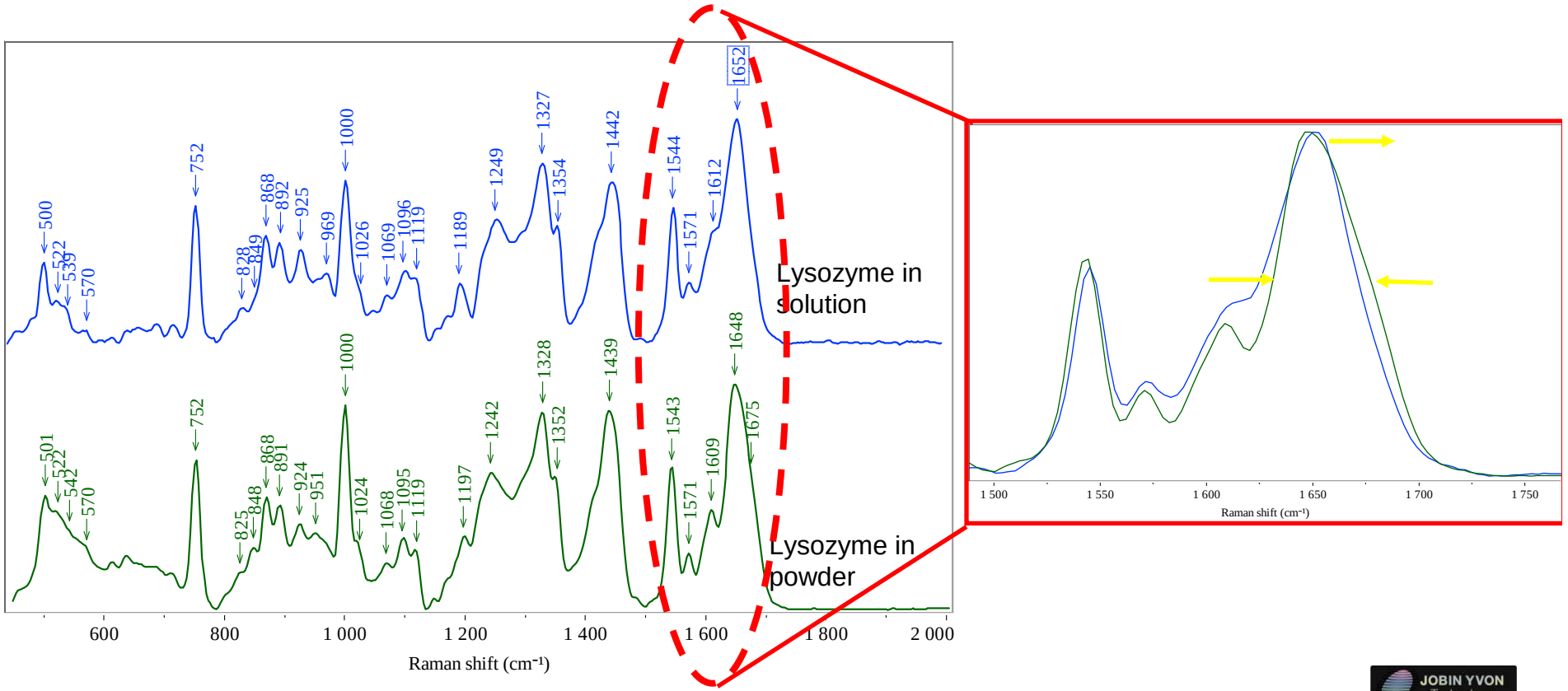
T. Pazderka et all., Proceeding

Raman spectra of an irreversible thermal denaturation of Hp 1-1

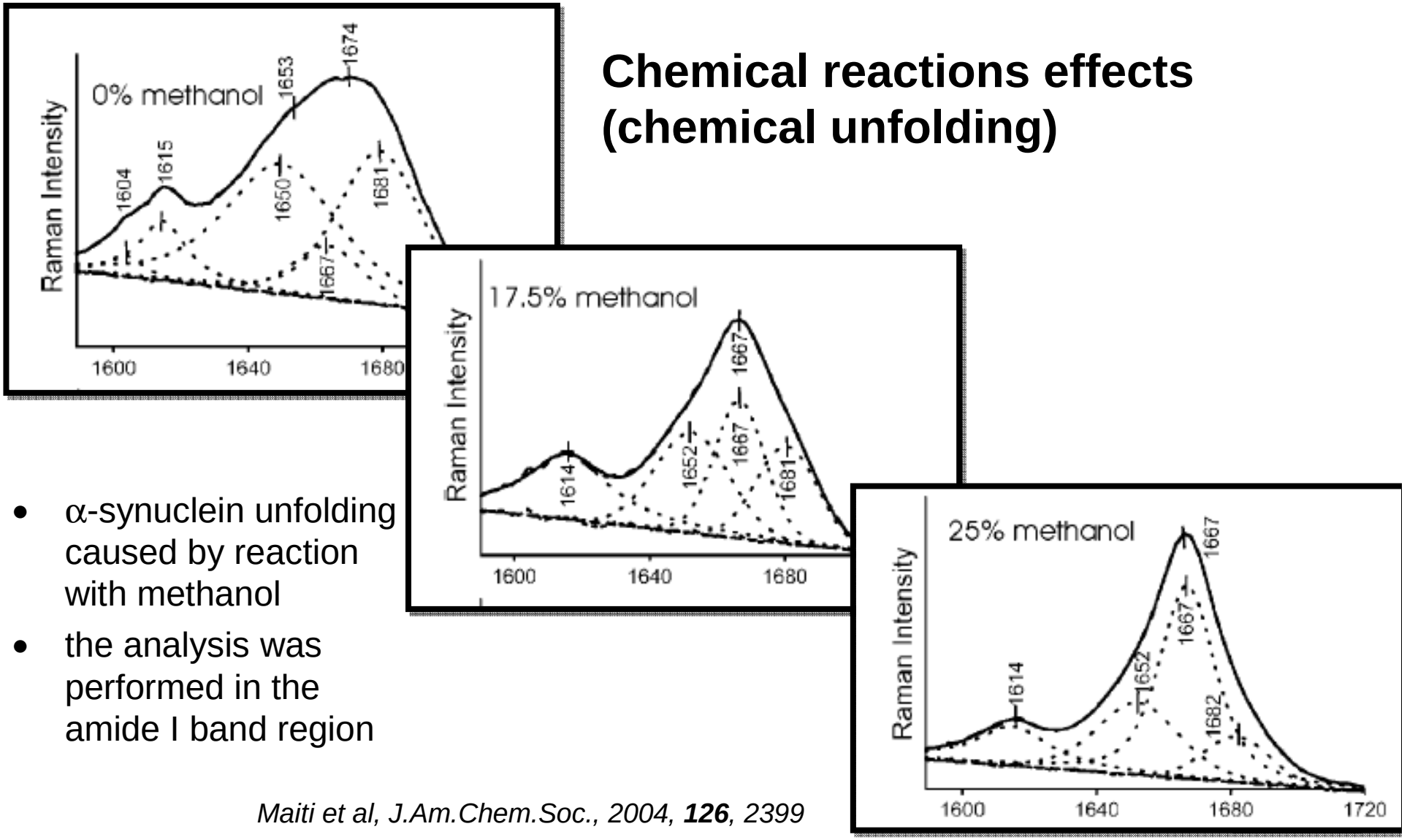
Environmental effects on the protein Raman spectrum

Solvent effects

- The interaction of the protein with the solvent often induces broadening of bands and shifts of their maxima



Environmental effects on the protein Raman spectrum



Chemical reactions effects (chemical unfolding)

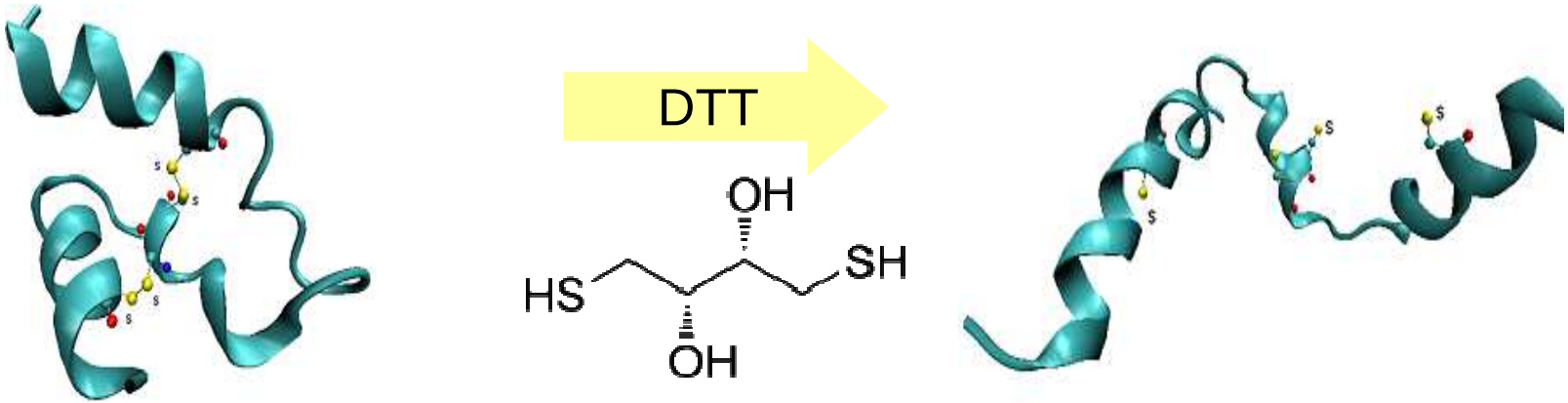
- α-synuclein unfolding caused by reaction with methanol
- the analysis was performed in the amide I band region

*Maiti et al, J.Am.Chem.Soc., 2004, **126**, 2399*

Environmental effects on the protein Raman spectrum

Chemical reactions effects (reductive unfolding)

- The tertiary structure of the BSA is very well stabilized by 17 disulphide bridges (S-S).
- When the protein will react with a reducing agent, as DTT for example, the S-S will be cleaved. Thus the structure will be perturbed thus the protein unfold occurs

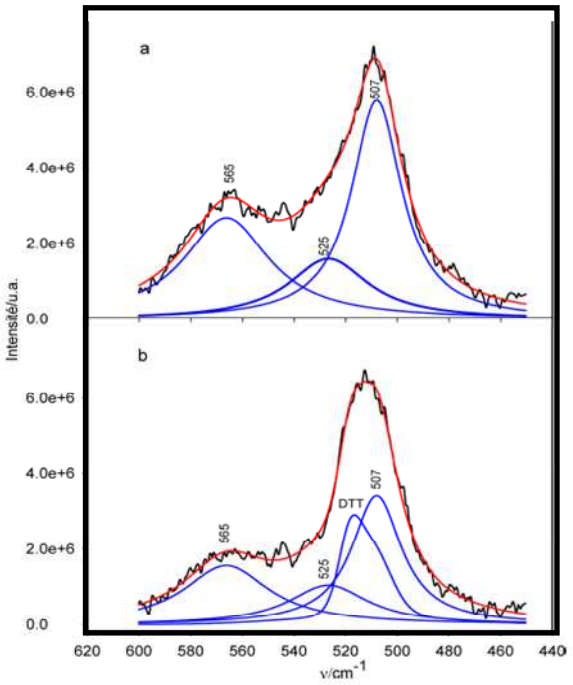


- The kinetic of the reaction is monitored by measuring Raman spectra over time

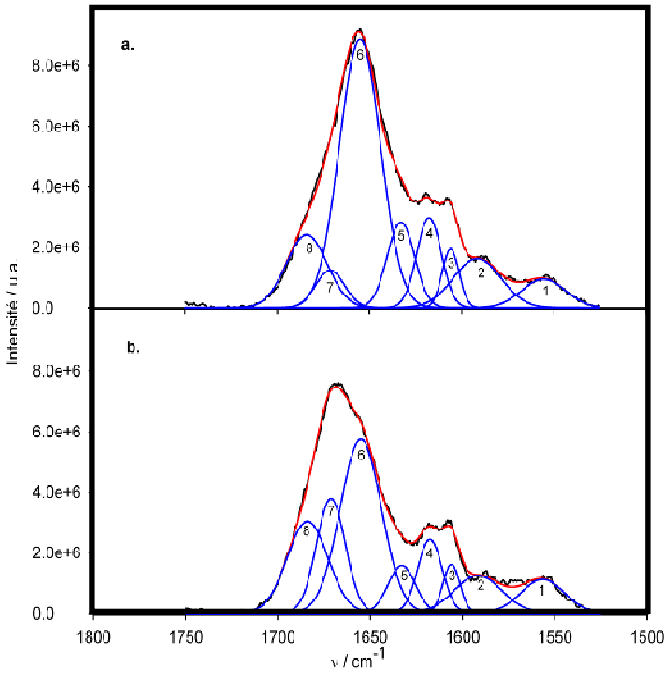
- Two spectral regions can be analyzed
  - the S-S Raman band – cleavage of the bond
  - the amide I Raman band

Environmental effects on the protein Raman spectrum

Chemical reactions effects (reductive unfolding)



S-S band before/after reaction



Amide I band before/after reaction

- Contributions of the different peaks are calculated after deconvolution
- Physical parameters, such as reaction rate, free enthalpy, activation energy can be calculated – quantitative results

C. David et al. 2008, Biopolymers

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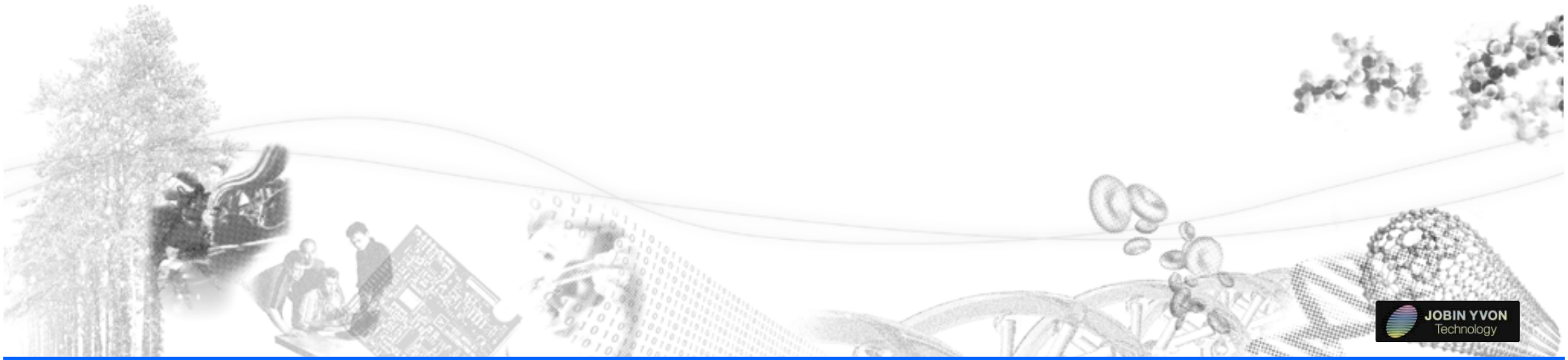
Basics of Raman analysis of proteins

Contributions to the protein Raman spectrum



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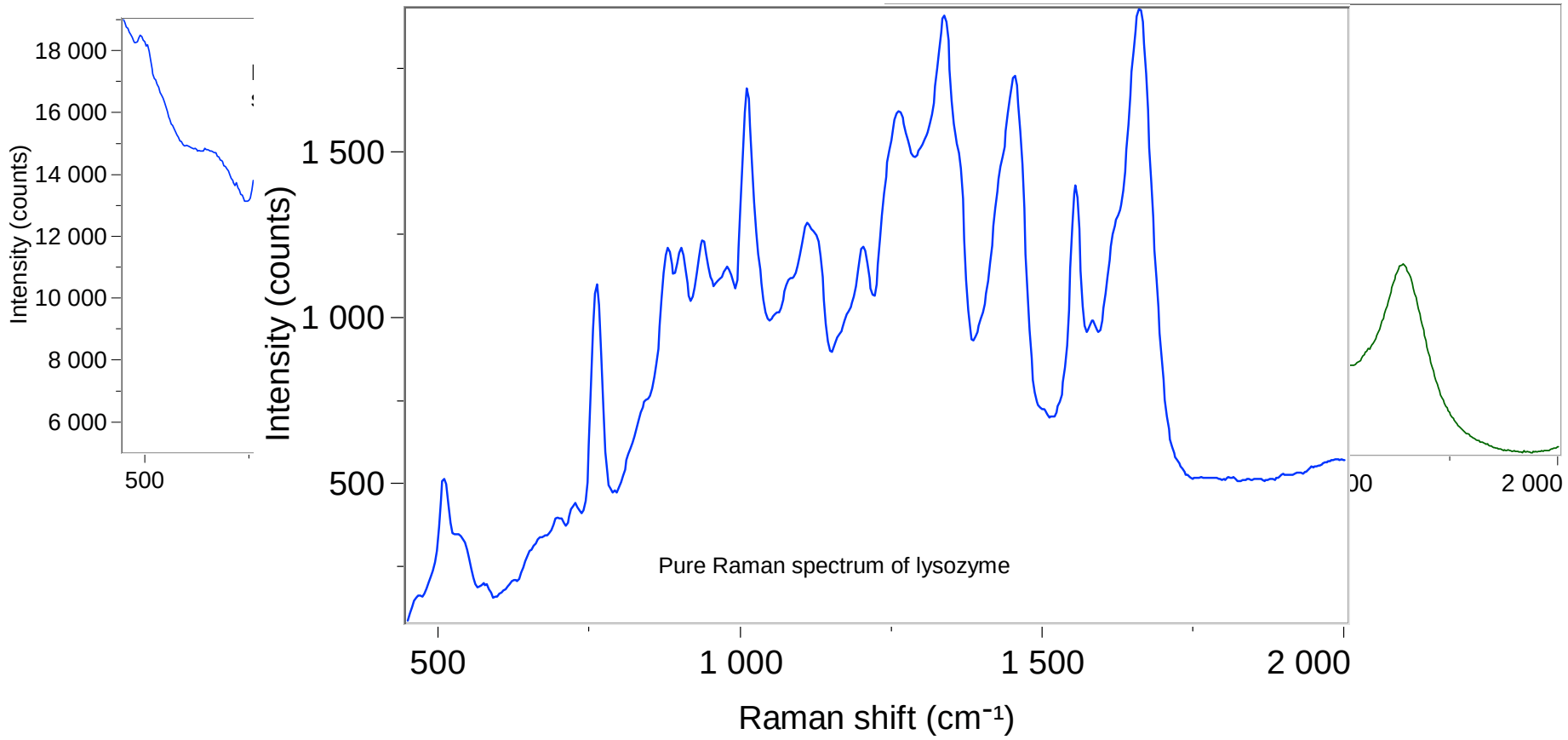
Contributions to the protein Raman spectrum

The solvent

- The reactions in which are involved the proteins occur mostly in solution : water or buffers
- The buffers as well as the water have their own Raman signature composed of one or more bands.
- Theirs bands may overlap to the protein ones – the Raman quantitative analysis become difficult
- Raman spectra of buffers should be measured separately and used as references
- The most convenient solvent in terms of spectral contribution is water. Water it is a weak Raman scatterer, leading to little or no interference from water in Raman spectra.

Contributions to the protein Raman spectrum

The solvent - example



Contributions to the protein Raman spectrum

The fluorescence

■ The fluorescence can strongly affect the Raman spectrum - it can cause significant background noise

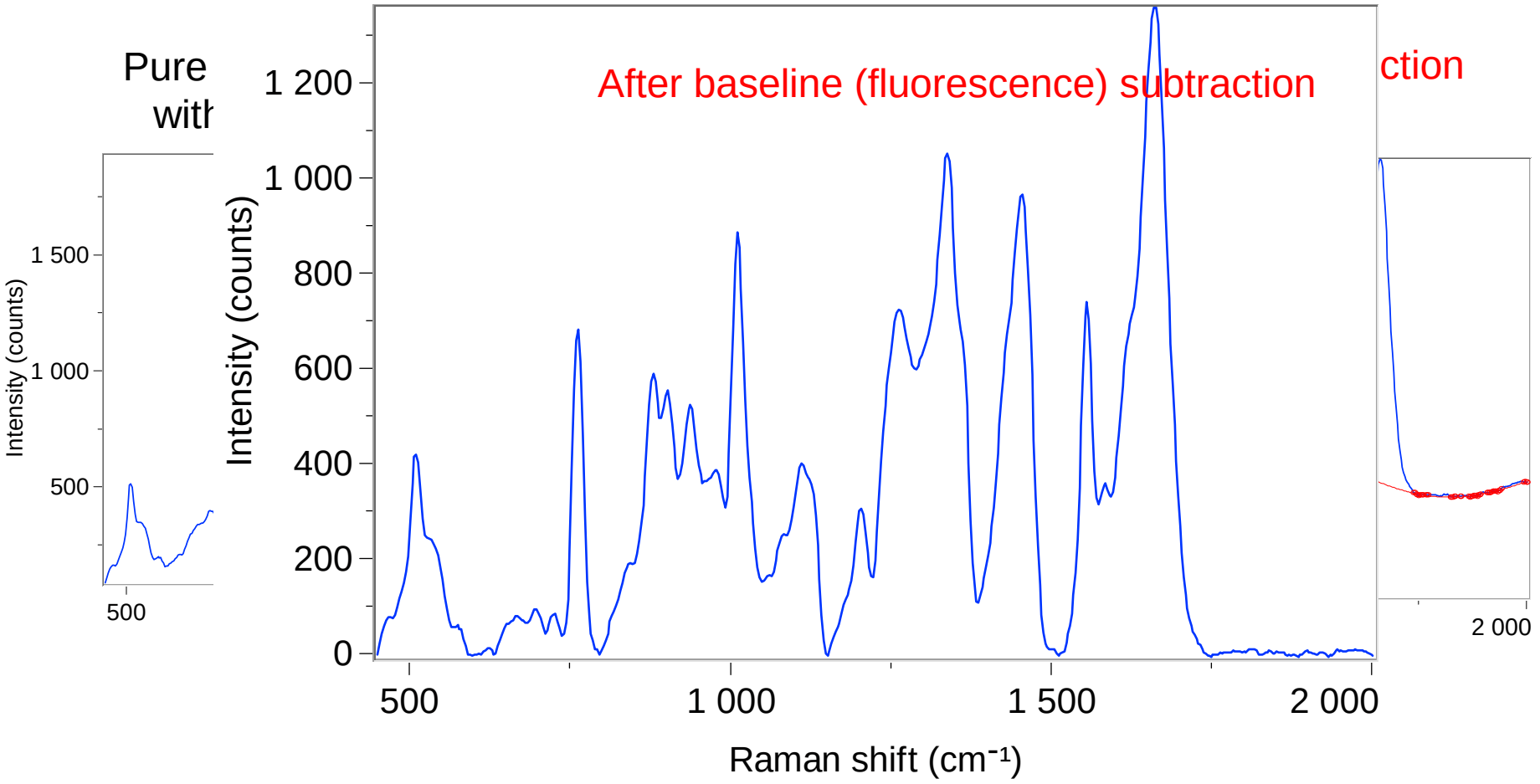
- **Intrinsic fluorescence** (caused by the presence of some aromatics aminoacids)
  - it is possible to remove the fluorescence by selecting a suitable wavelength

- **Extrinsic fluorescence** (caused by impurities, solvents, buffers…)

  - biological solutions should be pure as much as possible
  - photobleaching can be used to decrease the fluorescence background (pay attention to the exposure time and the subsequently the thermal degradation of molecules within the sample solution)

Spectra analysis and the contributions to the baseline

The fluorescence - example



Contributions to the protein Raman spectrum

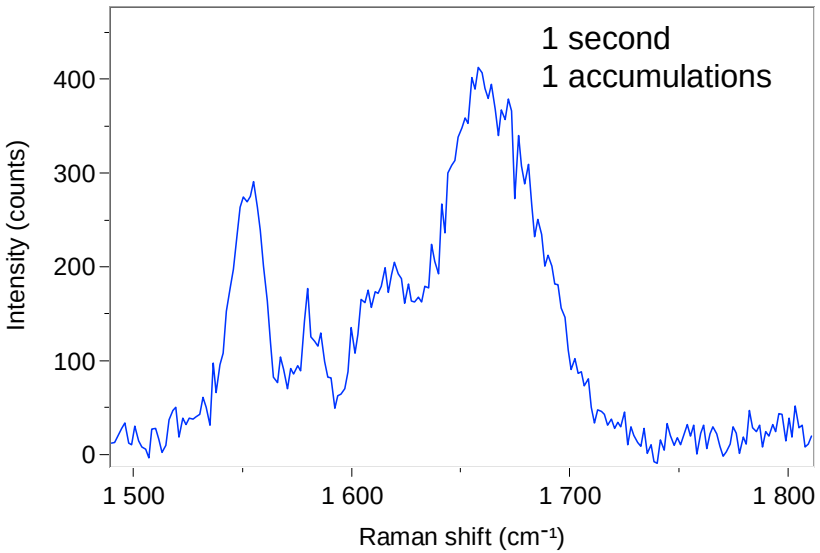
The signal-to-noise ratio

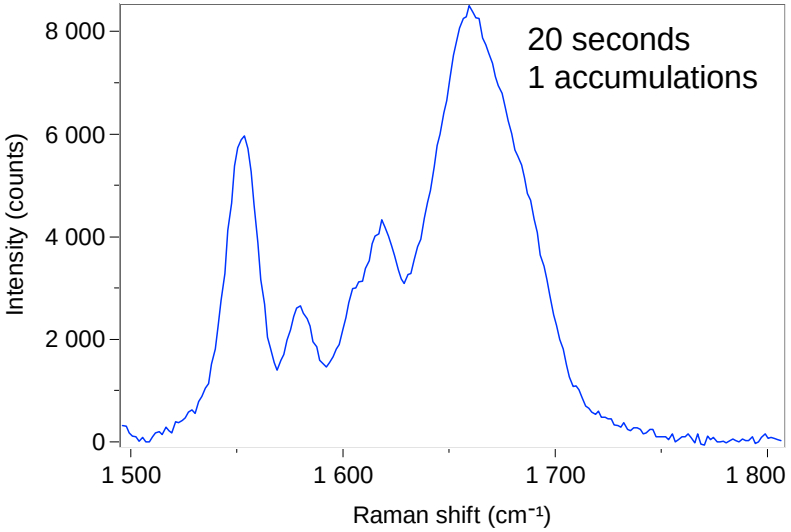
- A quantitative spectral analysis based on Raman spectroscopy requires a Raman spectrum with minimal signal-to-noise ratio.
- Longer acquisitions in optimal conditions could be requested
- The signal averaging is also a way to improve signal-to-noise ratio.
- For the reproducible measurements, several spectra accumulations can be performed, thus improving the signal-to-noise ratio.

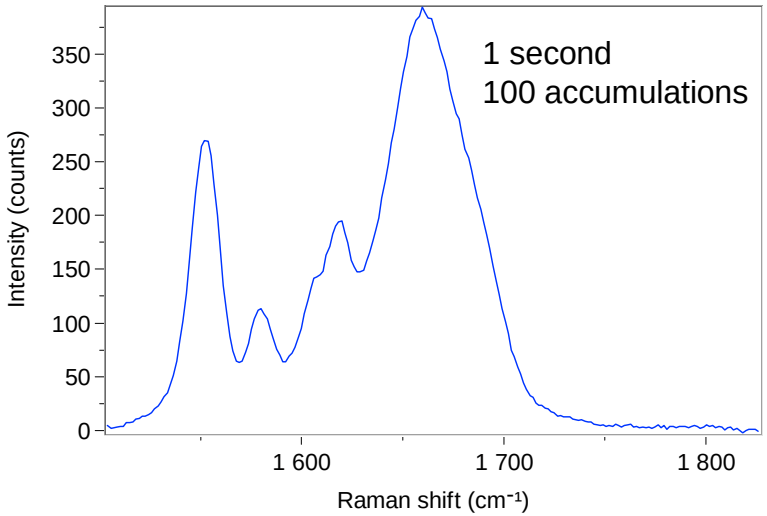
for *n* accumulations, the signal-to-noise ratio will be improved by a factor $\sqrt{n}$

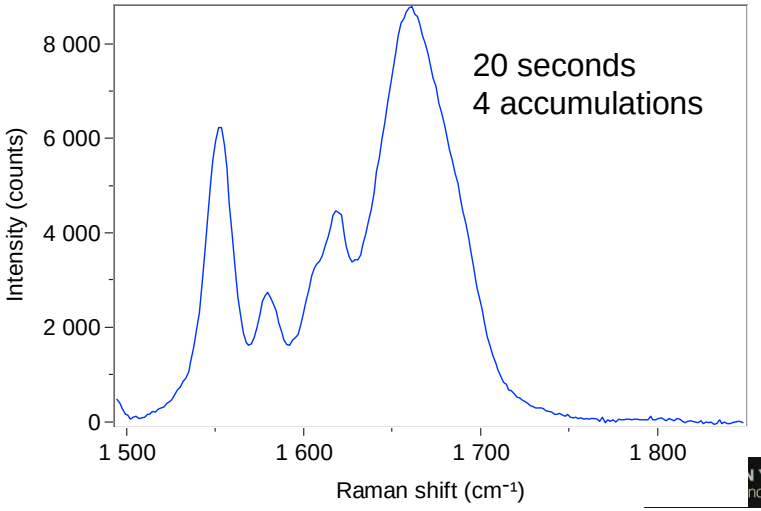
Contributions to the protein Raman spectrum

The signal-to-noise ratio - Example









Basics of Raman analysis of proteins

UV Resonances Raman for proteins

UV Resonances Raman for proteins

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■ Deep UV resonance Raman spectroscopy (DUVRRS) usually employs excitation wavelengths in the 190nm to 300nm range

■ The peptide bond strongly absorb at 190 nm – strong resonances are created and Raman signal coming from the peptide chains (proteins) can be efficiently and selectively enhanced

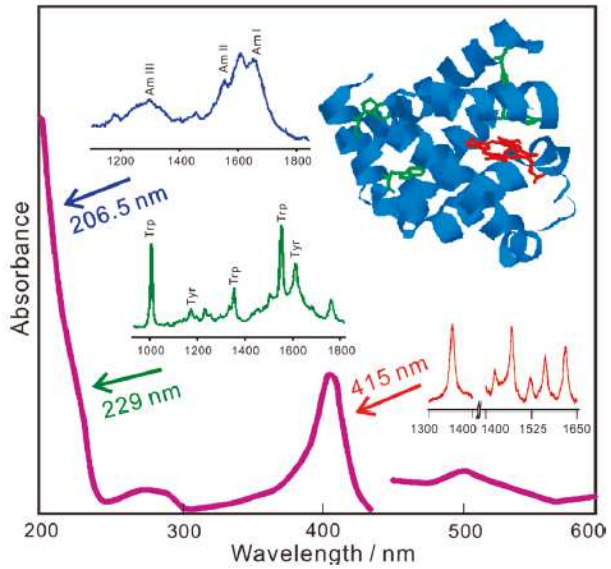

Figure 1. Selectivity of resonance Raman spectral measurements of myoglobin showing the protein absorption spectrum and the different resonance Raman spectra obtained with different excitation wavelengths.

Asher et al., J. Phys. Chem. Lett., 2011

- Excitation at 415 nm results in an intense RR spectra which contains heme ring vibrations
- Excitation at 229 nm shows RR spectra dominated by Tyr and Trp aromatic ring side chain vibrations
- Excitation at 206.5 nm, shows RR spectra dominated by the peptide bond amide vibrations

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UV Resonances Raman for proteins

Advantages of deep UV Raman Resonance

- ☺ Raman signal enhancement : Rayleigh law enhancement ($1/\lambda^4$) and resonance enhancement ($\sim\sigma_s^2$ – Raman cross section)
- ☺ Raman Spectra Simplification : resonance enhancement occurs only within the Raman bands associated with the electronic transition – few bands
- ☺ Ambient Background Elimination : measurements in outdoor environments during full sunlight

UV Resonances Raman for proteins

Disadvantages of deep UV Raman Resonance

☹ Thermal damage of the sample : usually pulsed lasers are used

☹ Photochemical damage : depends on total photon dose and wavelength (photon/illuminated spot)

☹ DUVRRS systems require a high level of operator skill, require regular alignment and servicing of the systems and the laser - the cost can be high

Basics of Raman analysis of proteins

Polarization measurements for proteins

Polarization measurements for proteins

- In addition to the “normal” Raman, polarised Raman provides information about molecular orientation and symmetry of the bond vibrations

- All molecules can be more or less symmetric. Thus, the positioning of these molecules (or of the sample) with respect to the laser beam, can greatly influence the intensity of Raman bands

- The excitation of the molecule on one or another symmetry axes can lead to a different Raman scattering

Polarization measurements for proteins

- Proteins have no only one symmetry but several (cyclic, dihedral, cubic, helical..) and these symmetries are local and not global (as for smaller molecules)

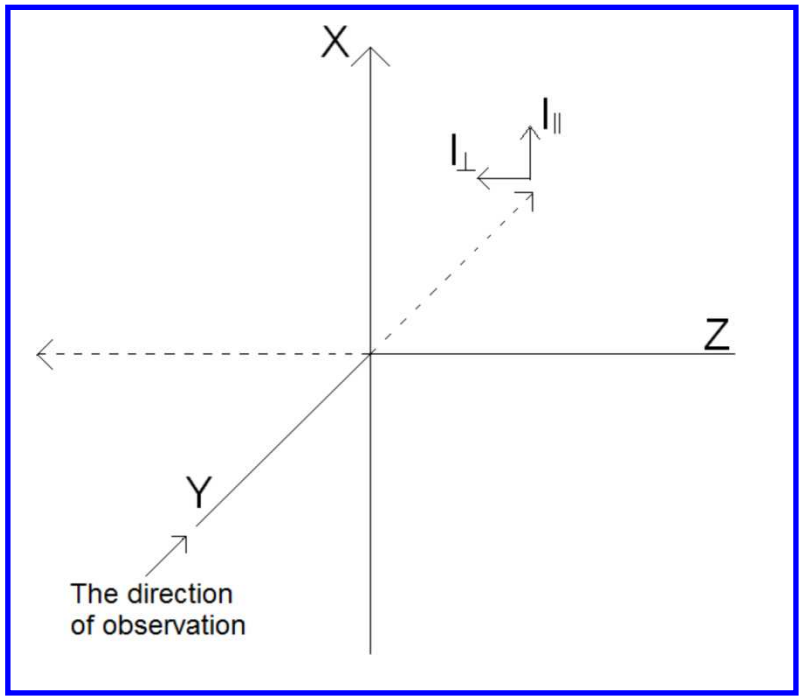
- Polarization measurements are possible – it is a practical way to identify the totally symmetric modes in the Raman spectra of molecules possessing a certain degree of symmetry

- Therefore, the interpretation of spectra is not an easy task to do due to the strong vibration modes coupling

Polarization measurements for proteins

Example : Polarization effect on the Raman spectrum of Lysozyme

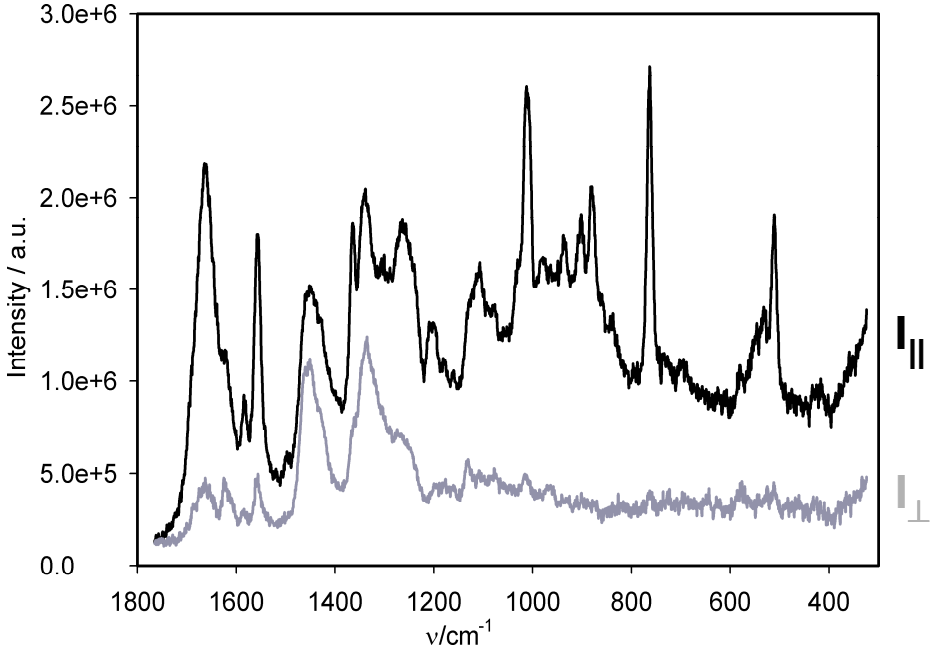
Experimental configuration



- Z is the direction of the incident radiation propagation
- Y is the direction of observation of the scattered light
- X is the direction of polarization of the electric vector of the incident beam

Polarization measurements for proteins

Example : Polarization effect on the Raman spectrum of Lysozyme



- $I_{||}$ is the intensity of scattered radiation with its electric vector polarized along the X axis

- $I_{\perp}$ is the radiation whose polarization is directed along the Z axis

- Depolarization ratio (ρ) can facilitate the assignment of unknown bands to molecular vibrations

$$\rho = \frac{I_{\perp}}{I_{II}}$$

Basics of Raman analysis of proteins

Low-frequency measurements for proteins

Low-frequency measurements for proteins

- The bio-macromolecules exhibit low-frequency motions
- These motions are collective motions of all atoms in a protein
- These vibrations are very sensitive to the microenvironment variations due to the : temperature, pressure, solvation, pH or ionic strength
- The Raman bands associated to these vibrations are very broad and difficult to assign. Thus, the experimental results are very often compared with the theoretical analysis (Normal Mode Analysis and Calculations)

Low-frequency measurements for proteins

Example : Low-frequencies modes of chymotrypsin

Proc. Nat. Acad. Sci. USA
Vol. 69, No. 6, pp. 1467–1469, June 1972

Conformationally Dependent Low-Frequency Motions of Proteins by Laser Raman Spectroscopy

(α-chymotrypsin/protein conformation)

K. G. BROWN, S. C. ERFURTH, E. W. SMALL, AND W. L. PETICOLAS

Department of Chemistry, University of Oregon, Eugene, Oreg. 97403

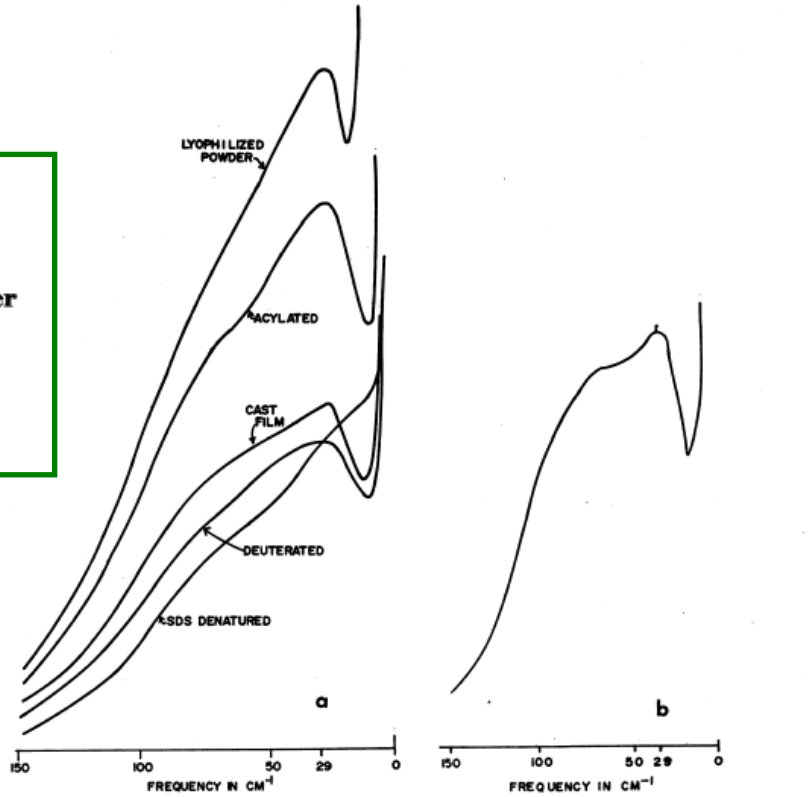


FIG. 1. (a) Low-frequency Raman spectra of α-chymotrypsin. The relative intensity of the Raman bands is of no significance as the spectra of protein samples have been separated for clarity. A band maximum at about 29 cm^{-1} occurs in every sample except that which was denatured with SDS. (b) Low-frequency Raman spectra of a single crystal of α-chymotrypsin.

Conclusions

Conclusions

- Raman spectroscopy can be successfully used as a method for probing the structure and conformation of native proteins

- Important structural information can be deduced from specific Raman vibrational bands as: amide I, amide II and amide III bands.

- The influence of chemical reactions mechanism involving proteins (folding/unfolding, oxidation, reduction, phosphorylation, and polymerization) can be monitored by following the evolution over the time of Raman bands: the disulphide bridges stretching, aromatics ring vibrations, protein side chain deformation etc

Conclusions

- The experimental results are usually submitted to complex processing which may offer the access to important physical and chemical parameters for the understanding of studied mechanisms

- Thus, it is possible to determine the reaction free enthalpy and free enthalpies for the unfolding of the protein structure.

- The study of the reaction kinetics in biological environment has allowed to accurately determining the rate constants of reaction.

Conclusions

- Widely used in Raman spectroscopy method, the polarization measurements and more precisely the depolarization ratio can facilitate the assignment of unknown bands to molecular vibrations

- Deep ultraviolet resonance Raman spectroscopy as well as the low-frequencies measurements are powerful tools for structural characterization of proteins. They can be applied for studying peptide and protein secondary structure and the dynamics of protein and peptide folding

Thank you

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