# VIBRATIONAL SPECTROSCOPY

*LITERATURE*


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## Videos

https://www.youtube.com/watch?v=46FMOc2msDM&list=PLm9edRZ1r8wVT2KTSwE87g3zmaYuDvhsQ

## How to read my handouts

Some of my handouts contain supplementary information. These sections are indicated by gray print and background. They represent additional information for those who are interested, but are not required for the examination.

## Essential knowledge

1. Answer the following questions regarding ***infrared*** spectroscopy:
   a) What component(s) of the electromagnetic radiation interact(s) with the molecule (electric field, magnetic field, or both)? (0.2 p)
   b) Are the incoming photons absorbed, elastically scattered, or inelastically scattered in the interaction? (0.2 p)
   c) Which of the following particles are moved by the electromagnetic radiation: electrons, nuclei, electrons and nuclei? (0.2 p)
   d) Which of the following particles have a different energy before and after the interaction with the electromagnetic radiation: electrons, nuclei, electrons and nuclei? (0.2 p)
   e) Describe the interaction according to the classical view. Your answer should relate to the mass on the spring model and state the moving mass. (2 p)
   f) Describe the interaction using the quantum mechanical view of the interaction. Draw a scheme that shows the potential energy as a function of the distance between two nuclei in the harmonic approximation, and the energy levels relevant for infrared spectroscopy. Don't forget to explain curves, lines, symbols, and other items that you draw. (1.6 p)
   g) Indicate transitions relevant for infrared spectroscopy in the plot from f). Which of these is/are most important? (1 p)
   h) What molecular property determines whether the transition probability is high or low? The property should be relevant only for infrared spectroscopy, not for spectroscopy in general. (0.6 p)
2. Answer the following questions regarding ***Raman*** spectroscopy:
   a) What component(s) of the electromagnetic radiation interact(s) with the molecule (electric field, magnetic field, or both)? (0.2 p)
   b) Are the incoming photons absorbed, elastically scattered, or inelastically scattered in the interaction? (0.2 p)
   c) Which of the following particles are moved by the electromagnetic radiation: electrons, nuclei,

electrons and nuclei? (0.2 p)
d) Which of the following particles have a different energy before and after the interaction with the electromagnetic radiation: electrons, nuclei, electrons and nuclei? (0.2 p)
e) Describe the interaction according to the classical view. (2 p)
f) Describe the interaction using the quantum mechanical view of the interaction: draw a scheme that shows the energy levels relevant for Raman spectroscopy and relevant transitions. Don't forget to explain curves, lines, symbols, and other items that you draw. (1 p)
g) Indicate transitions relevant for Raman spectroscopy in the plot from f). Which of these is/are most important? (0.6 p)
h) Plot a spectrum of the detected radiation for a molecule with 3 Raman active vibrations. Describe what you have plotted. Comment on the relative intensities of the signals that you plot. (1 p)
i) What molecular property determines whether the transition probability is high or low? The property should be relevant only for Raman spectroscopy, not for spectroscopy in general.(0.6 p)

## Examples for general knowledge

1. What transitions are observed in infrared and Raman spectroscopy?
2. Which of the following properties change(s) when infrared light is absorbed by a molecule: electronic state, equilibrium distance, vibrational amplitude, vibrational frequency and/or dipole moment at equilibrium distance?
3. What constituent of molecules oscillates in the vibrations relevant for infrared and Raman spectroscopy?
4. Define stretching and bending vibration.
5. Draw a harmonic potential with the energy levels of an harmonic oscillator with correct labeling of the axes. Indicate the transition that is observed in most cases.
6. Describe the absorption of infrared light according to the classical view.
7. Describe the Raman effect.
8. What is band assignment in infrared spectroscopy?
9. What vibration gives rise to an absorption band that is often used to detect conformational changes? Which atoms move most in this vibration?
10. Describe the effect of isotope labeling on an infrared spectrum.
11. Name some advantages and disadvantages of infrared spectroscopy
12. Discuss influence of mass and force constant on the vibrational frequency.
13. List information that can be obtained from vibrational spectra.
14. State the selection rules for the absorption of infrared light and for Raman scattering.
15. Describe a Fourier transform spectrometer.
16. Describe secondary structure analysis using the amide I band of proteins.
17. Describe Fourier self-deconvolution.
18. Discuss the principle of reaction-induced infrared difference spectroscopy.

19. Define wavenumber and state its unit.
20. What is the advantage of reaction-induced infrared difference spectroscopy?
21. What is a difference spectrum and what do positive and negative bands in a difference spectrum mean?
22. What kind of information can be deduced from time-resolved infrared difference spectra without any band assignment?
23. What kind of information can be deduced from time-resolved infrared difference spectra when a certain band has been assigned to a particular molecular group?
24. What information can be obtained from the spectral position of the C=O band of protonated carboxyl groups?
25. Name some strategies for band assignment in infrared spectroscopy and give some examples.

## Examples for functioning knowledge

1. Compare the energy of a typical vibrational transition with thermal energy and decide whether most of the oscillators are in the ground state or not.
2. Predict changes in frequency due to changes in structure or environment.
3. Predict the relative frequencies of different chemical groups (i.e. group A has higher or lower frequency than group B) from general knowledge on the influence of mass and force constant on frequency.
4. Predict whether a vibration is a strong infrared absorber or not from your knowledge on the selection rules for absorption of infrared light.
5. Predict whether a vibration is a strong Raman scatterer or not from your knowledge on the selection rules for Raman scattering.
6. Draw the spectrum of light that is scattered in a Raman experiment.
7. Discuss problematic aspects of secondary structure analysis using the amide I band.
8. Construct a difference spectrum of a reaction from the absorption spectra before and after the reaction.
9. Interpret certain aspects of an infrared difference spectrum.
10. Suggest an infrared experiment to study a certain aspect of a biomolecule or a biological process.
11. Construct a difference spectrum from given absorption spectra.
12. Attribute positive and negative bands to reactants and products of a reaction.
13. Suggest and describe an experiment that proves whether a certain amino acid gets protonated in the course of a protein reaction.
14. Draw conclusions from infrared difference spectra using the fingerprint approach.

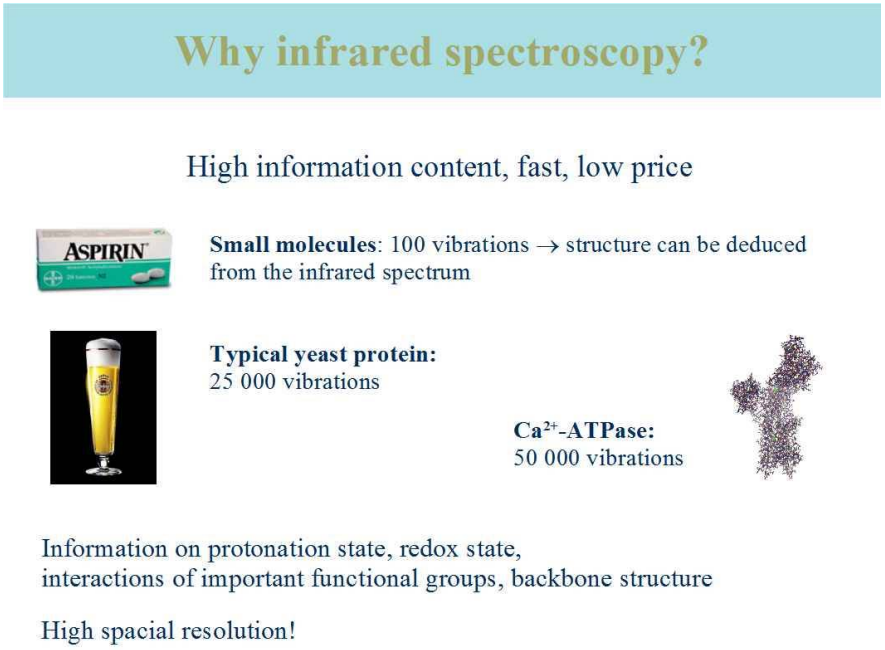
## Introduction

We will consider here two forms of vibrational spectroscopy: infrared spectroscopy and Raman spectroscopy. The physical process that gives rise to the spectroscopic signal is different for the two techniques but the infor-

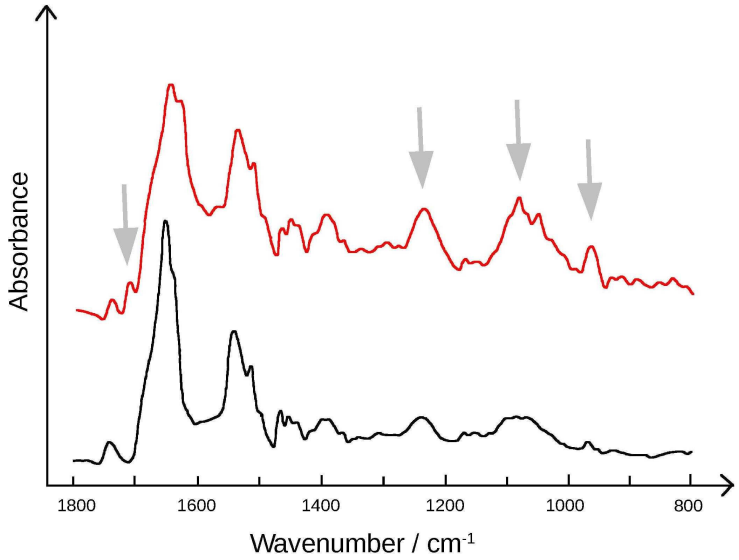
mation that can be obtained from the spectrum is the same. Therefore we will concentrate mainly on infrared spectroscopy but keep in mind that Raman spectroscopy provides the same kind of information.

## Why Infrared spectroscopy?

An infrared spectrum contains enough information to deduce the structure of small molecules from the spectrum. For biological systems this is no longer possible because they are too complicated. However, certain aspects of structure and interaction can be followed in a time-resolved way. It is possible to follow the fate of single amino acids in a large protein during a protein reaction, for example one can observe how the environment of this group changes, or how the protonation state changes.



Infrared spectroscopy is widely used in industry as an analytical method for example in quality control. It can also be used for more exotic purposes, for example to track down a driver who failed to stop after an accident from traces of paint left at the site of the accident. It is less used in industry for biological problems. However, there will be increasing application of IR spectroscopy due to the high information content. For example it is possible to identify bacterial strains from the infrared spectrum and to classify the relationship of bacteria. Or it is possible to diagnose diseased tissue. Here is an example: Shown are the spectra of healthy (black) and leukaemic lymphocytes (red). The spectra are clearly different indicating that diseases can be diagnosed using IR spectroscopy.




*Example for medical applications: Spectrum of healthy (black) and leukaemic (red) lymphozytes (redrawn from Jackson et al. 1997, Biophys. Chem. 68, 109-125, IR-Rev 6, Fig 5). The arrows point to spectral regions that indicate a higher nucleic acid content in the leukaemic cells. The protein absorption near 1650 $cm^{-1}$ indicates relative concentration changes and/or a different overall structure of the proteins.*

## Advantages - Disadvantages

+ high information content of the spectrum
+ applicable from small soluble to large membrane proteins
+ easy sample preparation for standard measurements
+ often short measuring time
+ high time resolution (µs with moderate effort, ns with pump-probe techniques)
+ not expensive (simple spectrometer for 25 000 Euros)
+ low amount of sample required (10 to 100 µg)
- absorption coefficients smaller than in the visible spectral region. Therefore high protein concentrations required for some applications
- high water absorbance requires a short optical pathlength and therefore high sample concentrations
- calculation of the absorption spectrum is difficult

## History

IR radiation was discovered in 1800 by the astronomer and musician F.W. Herschel (Sir William). In his experiment sunlight passed a prism and dispersed the spectrum into its spectral components. With a thermometer he measured the temperature in dependence of the wavelength. Interestingly the maximum of the temperature curve was outside the visible spectrum, beyond the red part of the spectrum. This was the first detection of infrared radiation and its name stems of course from the spectral position. The radiation appears warm and is also called heat radiation, since it is emitted from a body at a given temperature according to Planck´s radiation law. Herschel discovered already that water absorbs infrared radiation.

1835 the first spectrometer was built

1913 the first commercial spectrometer was built. Recording a spectrum was only interesting for some exotic scientists, since it required a lot of effort, took several hours and happened at night in dark and temperature-controlled cellar rooms.

The second world war saw an explosion of applications numbers for spectrometers in use were for example for the USA 1939 4 industrial spectrometers, 1945 400; for GB 1938 15 und 1947 500. From approx. 1950, infrared spectroscopy was used also for biological problems (sorry, this is just for me: IR-Allg/a1, IR-Rev 6). Since the introduction of Fourier transform infrared spectroscopy 30 years ago the numbers of application have increased dramatically.

## Energy of IR radiation

You will be familiar with spectroscopy in the visible and ultraviolet spectral range. In this range electronic transition are observed. The absorption of infrared radiation is the process that comes next at lower energies: the excitation of vibrational and rotational transitions. The energy required for a transition is approximately a factor of 10 smaller than for electronic transitions. The spectral region is adjacent to the visible spectral region and extends from 0.7µm to 1000µm, which is the infrared spectral region. In most cases the region from 2.5 to 25µm is used.

The process coming next at lower energies is the excitation of electronic angular momentum transitions in electron paramagnetic resonance (EPR or ESR) which needs 1000 to 10000 fold less energy. At even lower energies NMR transitions are observed. This is summarized below:

$$UV > Vis > IR > kT > EPR > NMR \quad \text{(with the thermal energy kT)}$$

IR spectroscopists do not use the wavelength in µm to plot their spectra but rather the inverse of the wavelength, the wavenumber $\tilde{\upsilon}$ in $cm^{-1}$). This quantity has the advantage of being proportional to the energy but at the same time the wavelength can easily be calculated. The region of 2.5 to 25 µm corresponds to 4000 to 400 $cm^{-1}$, which corresponds to $10^{13}$ to $10^{14}$ Hz. In the lecture we will only discuss vibrational transitions, since there are no rotational transitions observed in solution. However, in the infrared spectral region not only vibrational transitions can be excited but also low-energetic electronic transition. This is the case for some semicon-

ductors and this is exploited for infrared detectors. But also in biological systems there are low-energy electronic transitions. An example is the chlorophyll dimer of some photosynthetic reaction centers.

## Vibrations

*VIBRATIONAL FREQUENCY OF A 2-ATOMIC OSCILLATOR*

The vibrations that give rise to the absorption of infrared radiation are the vibrations of the atoms in a molecule. As these can be quite complicated, we will start with the most simple case: the vibration of a 2-atomic oscillator. We will first discuss the vibrational frequency and later the absorption of energy by the oscillator.

The two atoms have masses $m_1$ and $m_2$. The equilibrium distance between the two atoms is denoted by R and we can think of the molecule as two balls connected by a spring. We denote the force that holds the two atoms together by $F$ and the deviation of the actual distance from the equilibrium distance by $\Delta R$. In the harmonic approximation, Hooke's law is valid, which says that the force $F$ is proportional to $\Delta R$, the deviation from the equilibrium distance. The proportionality constant is the force constant and denoted by $k$. We obtain for the force $F$:

$$F = -k\,\Delta R$$

where $k$ is the force constant

Solving Newton's equation with this expression for force, we can calculate the the vibrational frequency $\nu$ of the two-atomic oscillator

$$\nu = (k/m_r)^{0.5}/2\pi$$

with the reduced mass $m_r$:

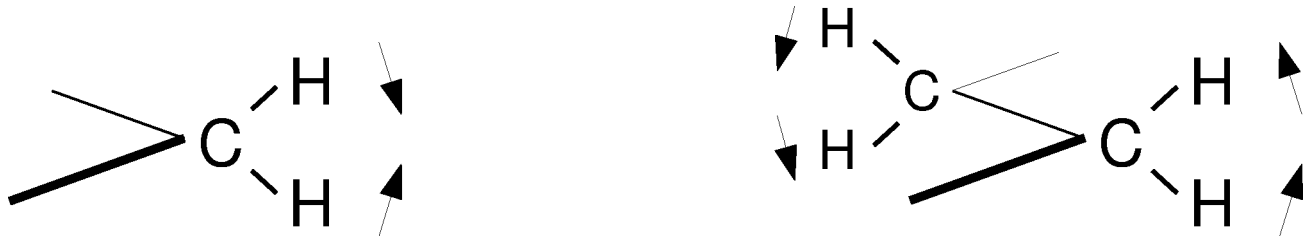
$$(1/m_r = 1/m_1 + 1/m_2)$$

The stronger the force constant, i.e. the stronger the bond between the atoms, the higher is the frequency. The smaller the masses, the higher is the frequency.

(The unit for the force constant is N/m with 100 N/m = 1 mdyn/Å)

*TYPES OF VIBRATIONS*

In a molecule with several atoms different types of vibrations are distinguished: stretching vibrations, bending vibrations and torsional vibrations. In stretching vibrations (abbreviated ν) the length of the bond changes, in bending vibrations (abbreviated δ) the bond angle of a 3-atomic fragment of the molecule (think of scissors) and in torsional vibrations (often abbreviated τ) the dihedral angle of a 4-atomic fragment of the molecule (think of twisting a rod or an eraser).




*Examples for bending vibrations (left hand side, $CH_2$ scissoring vibration) and a torsional vibration (right hand side, $CH_2$ rocking vibration)*

*SOME VIBRATIONS OF ACETYL PHOSPHATE*

The videos of this lecture illustrate some of the vibrations of a small molecule: acetyl phosphate.

*NORMAL MODES*

General description

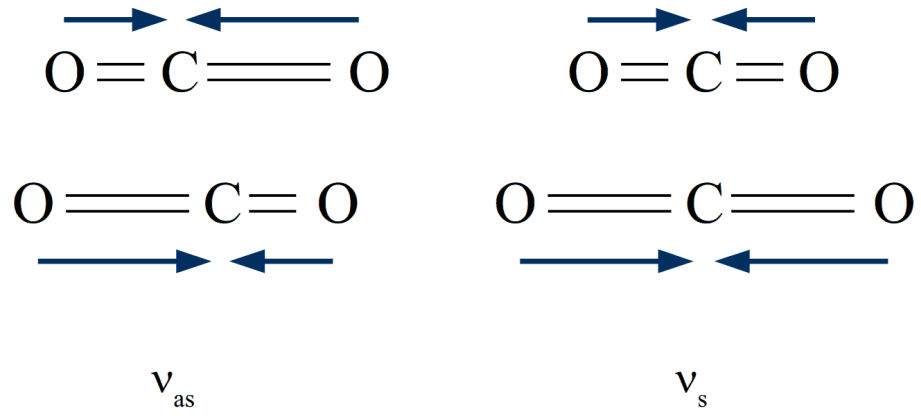
Bond lengths and angles are called ***internal coordinates*** of a molecule. Usually, several of them are coupled: they oscillate together with the same frequency and pass through their equilibrium position at the same time. A motion like this is called a ***normal mode of vibration***. Approximately, a normal mode vibrates independently from all other normal modes. Some normal modes are localized on a small part of the molecule, for example on a C=O bond. They are called ***group vibrations***. Others involve many atoms of a molecule and their frequencies characterize the chemical structure and conformation of the entire molecule like a fingerprint. A system with $N$ atoms has 3 $N$ degrees of freedom (3 for every atom). 3 degrees of freedom describe translation of the whole molecule, 3 rotations of the whole molecule and the remaining $3N$-6 degrees of freedom are vibrational degrees of freedom. Linear molecules have $3N$-5 vibrational degrees of freedom because the rotation around the symmetric axis does not count as a rotational degree of freedom because the nuclei do not change their position. Every vibrational degree of freedom can be described by a normal mode (of vibration). This gives 20 000 normal modes for an average *E. coli* protein and to nearly $10^9$ normal modes for *E. coli* DNA.

Normal modes of $CO_2$

I would like to illustrate now some of the above with an examples. We first examine $CO_2$. How many vibrations do we expect? $CO_2$ is linear, so we expect $3N$-5 = 4 vibrational degrees of freedom or normal modes: 2 stretching vibrations and 2 degenerated bending vibrations.

The two stretching vibrations are illustrated below. Both the symmetric and the antisymmetric stretching vibration are normal modes. Each normal mode consist of two coupled stretching vibrations. In other words, two internal coordinates, that is the bond lengths of the two C=O bonds, are coupled in each normal mode. In the symmetric vibration, the two stretching vibrations are in phase. In the antisymmetric vibration, the two stretching vibrations are 180 degrees out of phase. The two normal modes have different frequencies, that of the anti-

symmetric vibration is higher than that of the symmetric vibration. The frequency of a single C=O bond would be in between these two frequencies.



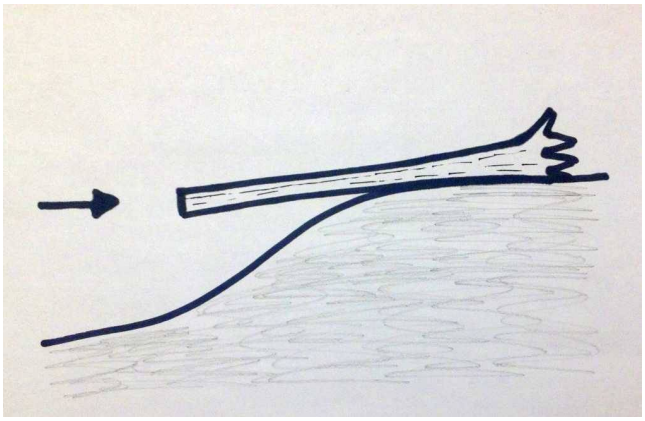

*Stretching vibrations of $CO_2$. Shown are the two extreme positions of the vibrations for the two stretching vibrations (antisymmetric stretching vibration $\nu_{as}$ and symmetric stretching vibration $\nu_s$) as well as the equilibrium positions of the atoms. When the equilibrium positions are shown, the arrows indicate the movement of the oxygen atoms.*

Why are the antisymmetric and symmetric stretching vibrations normal modes, but the stretching vibrations of the individual C=O bonds not? The answer is that normal modes vibrate independently from each other. When one vibrates, the other is not affected. This is not true for the individual C=O bonds. The are coupled by the movement of the central carbon atom. When one C=O bond starts to vibrate, the movement of the central carbon atom will also make the other C=O bond vibrate. Therefore, these vibrations are not independent and are not normal modes.

Tree trunk

The concept of normal modes is not limited to molecules. Sometime one can observe them in unexpected locations, for example in a forest. In the videos of this lecture I show some vibrations of a tree trunk that was disrooted by a storm. The stem was lying on a slope and the end was free to oscillate as indicated in the image.

First I bent the stem downwards and let it oscillate. So the initial vibration was vertically, it became then circularly, then horizontally, later again circularly and so on. Obviously, inducing the vertical vibration generates other movements as well, in particular the horizontal vibration. This shows that there is an interaction between vertical vibration and horizontal vibration, in other words both are coupled and they are not independent from each other. Therefore, the vertical vibration is not a normal mode of vibration for this stem.



When I bent the stem horizontally a similar sequence of vibrations occurs. When the horizontal vibration is initiated, other movements are generated with time, in particular the vertical vibration. Therefore, also the horizontal vibration is not a normal mode of vibration. So what are the normal modes of this stem?

Next I bent the stem upwards and to the side at the same time to induce a diagonal vibration. This vibration does not change with time and it is therefore not coupled to any other vibration. Therefore the diagonal vibration is a normal mode of the system. It can be thought to be a superposition of the horizontal vibration and the vertical vibration. Similarly, the normal modes of molecules are composed of vibrations of several internal coordinates.

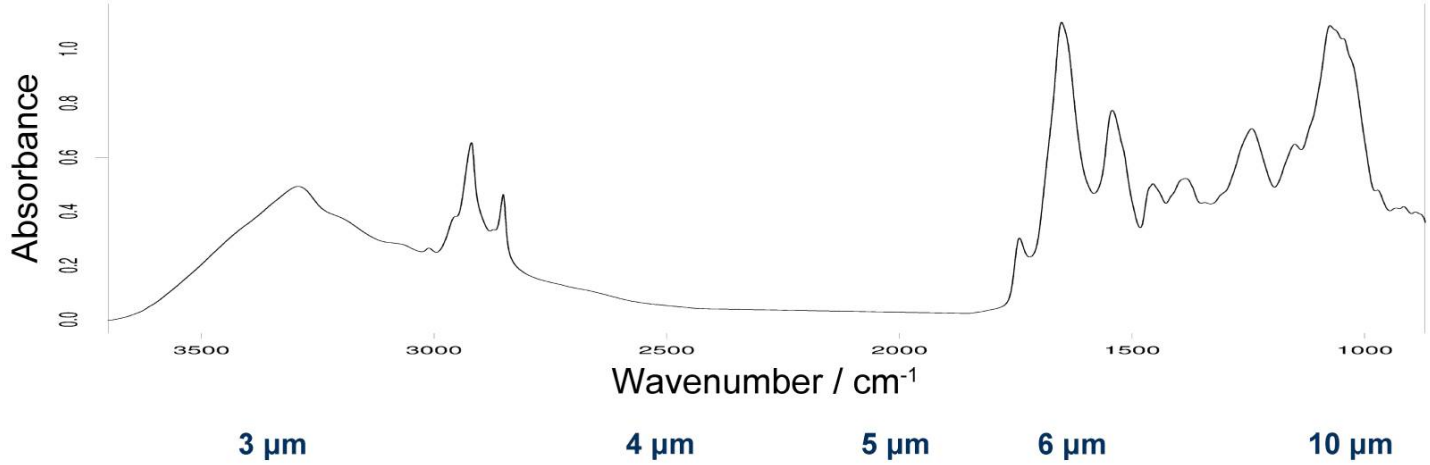
The general motions of such a system of coupled oscillators can be described by a superposition of the normal modes, even the complicated motions of the tree trunk that were described above.

## *INFLUENCES ON THE VIBRATIONAL FREQUENCY*

### Absorption regions at the example of a microalga

We will now look into the different factors that influence the vibrational frequency of a normal mode. We will see later that this frequency corresponds to the frequency of the absorbed infrared light. Therefore, the vibrational frequency determines where in the spectrum the absorption band of that vibration will be found. That is the reason why infrared spectroscopy is one of the methods of vibrational spectroscopy. A further method is Raman spectroscopy. Because Raman spectroscopy detects also vibrations, generally the same information can be obtained from a Raman spectrum. We will discuss Raman spectroscopy a bit later. But for now we will continue with the discussion of what influences the vibrational frequency and we use an infrared spectrum of a dried microalga to illustrate the general principles. The infrared spectrum is plotted against the quantity wavenumber in units of reciprocal centimeters. I have also indicated the corresponding wavelengths below the wavenumber scale. The wavelength range of the plotted spectrum spans from just above 2 μm to 10 μm. Note again, that plotting from high to low wavenumbers is equivalent to plotting the spectrum from short to long wavelength.

The spectral range plotted here belongs to the mid-infrared range which is used in most bioanalytical studies. In the vertical direction, the quantity absorbance is plotted. This quantity does not have a unit.




*Infrared spectrum of a micro-alga. Spectrum recorded by J. Andersson.*

Influence of the masses

As already mentioned, there are two main influences on the vibrational frequency: the force constant and the masses of the vibrating atoms. Both factors lead to the effect that vibrations of certain molecular groups appear in defined spectral regions.

We discuss first the effect of the mass. Hydrogen is the atom with the lowest mass and therefore stretching vibrations involving hydrogen have the highest frequencies and the highest wavenumbers (3700-2800 $cm^{-1}$). Stretching vibrations involving two heavier atoms, for example CO, CN, or CC stretching vibrations are found at lower wavenumbers (for single and double bonds below 1800 $cm^{-1}$). They are also found at lower wavenumbers outside the spectral range shown here.

The mass effect does not only influence the positions of an absorption band in the spectrum. It is also the basis for an important interpretation tool, as it is used to assign absorption bands to specific vibrations. First the infrared spectrum of the sample is recorded. Then, the experiment is repeated with a sample where one has introduced an isotopic substitution at a specific position. This does not change the force constant but only the masses. The vibrational frequency shifts and this identifies the absorption band or bands to which the substituted atoms contribute.

A simple isotopic exchange experiment is the use of deuterium oxide, $D_2O$ ($^2H_2O$), instead of ordinary water as a solvent. This makes acidic groups like NH, OH, and SH groups exchange their proton for a deuteron. As a consequence, the absorption bands in the infrared spectrum shift and reveal the participation of these groups in the corresponding vibrations.

A second benefit of using $D_2O$ is to shift the strong absorption band of ordinary water at 1640 $cm^{-1}$ to ~1200 $cm^{-1}$, since the region around 1640 $cm^{-1}$ is of special interest for secondary structure analysis of proteins.

Influence of electron density

For stretching vibrations, the force constant depends on the electron density in the vibrating bond. The higher the electron density, the higher the force constant and the higher the vibrational frequency. For double and triple bonds the force constant is approximately twice or three times that of a single bond. Therefore double and triple bonds absorb at higher wavenumbers than single bonds. Single bonds absorb at the lower end of the spectrum shown above, double bonds near 1600 $cm^{-1}$ and triple bonds near 2200 $cm^{-1}$. You do not need to remember these numbers, but you should remember the general trend. Note also that the vibrational frequency, and thus the wavenumber, both depend on the square root of the force constant. Therefore the wavenumber of double bonds is approximately 1.4 times higher than that of single bonds.

The electron density may change due to environmental effects for example when an enzyme "prepares" the substrate for the catalytic reaction. These changes are detectable in the infrared spectrum and are important clues for the understanding of the catalytic mechanism.

Influence of the type of vibration

It is easily imagined that shortening or elongation of a bond meets stronger resistance than a movement perpendicular to the bond. Therefore the force constant of stretching vibrations is typically a factor of 10 larger than that of bending vibrations and stretching vibrations have the higher frequency. For example, stretching vibrations involving hydrogen are found in the 3000 $cm^{-1}$ range, whereas the corresponding bending vibrations absorb below 1700 $cm^{-1}$.

Group vibrations

Normal modes are in general composed of the vibrations of several internal coordinates, like bond stretching or band angle vibrations. However, in many cases, a normal mode involves mainly the vibration of one or of a few internal coordinates. These vibrations are relatively independent from the rest of the molecule and are called ***group vibrations***. A good example are C=O double bonds which absorb between 1800 and 1600 $cm^{-1}$. In general, the region of group vibrations is found above ~ 1500 $cm^{-1}$ and involves stretching vibrations of double bonds and of groups involving hydrogens.

In contrast, below ~1300 $cm^{-1}$ many vibrations are strongly coupled to other vibrations and the position of an absorption band strongly depends on a large part of the structure of a molecule. Therefore this region is called ***fingerprint*** region. It is characteristic like a fingerprint for the molecular structure. The fingerprint region is therefore very important for the determination of structures of small molecules by infrared spectroscopy.

Summary

In summary, the approximate position of an infrared absorption band is determined by the vibrating masses, the bond strength (single, double, triple), and the type of vibration. For the biological sciences, the effect of the environment is often the most interesting since it gives clues on the catalytic mechanism of enzymes.

Assignment

After explaining these general properties of vibrations, we will now discuss the main bands in the shown infrared spectrum of a microalga and reveal the vibrations that cause these absorption bands. In other words, we will assign the observed absorption bands to the vibrations that cause them. Accordingly, this analysis of the spectrum is called band assignment.

As already mentioned, we find the absorption of XH stretching vibrations at the high wavenumber end of the mid-infrared spectrum. OH and NH stretching vibrations absorb above 3000 $cm^{-1}$. They provide information on the water content of the sample and on the hydrogen bonding strength to these groups. As this spectrum is from a dried sample, the water content is very low. For a sample in aqueous solution, this water band would be the strongest absorption band of the sample.

At lower wavenumbers - around 2900 $cm^{-1}$ - a complicated band profile can be seen. This is still in the region of XH stretching vibrations and can be assigned to CH stretching vibrations. These vibrations are abundant in lipids and therefore the CH stretching bands provide information about the lipid content, the conformational disorder of the lipid chains and they can be used to study lipid phase transitions.

Below the CH stretching vibrations, there is a large region in the spectrum with very weak absorption from biological samples. The next band (~1740 $cm^{-1}$) is found in the region of CO double bond stretching vibrations and the first band here stems again from lipids. As for the CH stretching band, this band can be used to study lipid content, lipid phase transitions, but also the hydrogen bonding to the lipid carbonyl group.

The next band in the CO double bond region is very prominent and also very important one for protein analysis. It is the amide I band of proteins (1700 - 1600 $cm^{-1}$) which is caused by the so called amide I vibrations of the polypeptide backbone. Several internal coordinates contribute to this normal mode, but the main contribution is the CO double bond stretching vibration of each peptide group. This band provides information about protein content, but also on the secondary structure of proteins and in consequence also on protein aggregation. In the same region, also the HOH bending vibration of water is absorbing. In the shown spectrum, there is very little contribution from water absorption because the sample was dried. However, in spectra of proteins in aqueous solution, the water absorption is usually by far the dominating contribution in this spectral region. This is unfortunate and restricts the experimental conditions as we will discuss later.

The next band at lower wavenumbers is again a protein band (~1550 $cm^{-1}$). This band is called amide II band and stems from the so called amide II vibrations of the protein backbone. This normal mode consists mainly of the NH bending vibration and the CN stretching vibration of each peptide group. Also this band provides information about protein content and protein secondary structure. It can also be used to study hydrogen bond stability.

The next band (~1455 $cm^{-1}$) is also assigned to bending vibrations, in this case to the bending vibrations of methyl and methylene groups. This band is little used for analysis. This is also true for the following band (~1385 $cm^{-1}$) which can be assigned to another bending vibration of methyl groups.

At lower wavenumbers (~1240 $cm^{-1}$), the antisymmetric stretching vibration of $PO_2^-$ groups gives rise to a prominent band. $PO_2^-$ groups are found in polynucleotides and in lipids. It is sensitive to the interaction of the phosphate groups with the environment and depends also on the conformation of DNA.

Finally, the intense and broad band (1200-1000 $cm^{-1}$) at the lower end of the spectral range shown in this spectrum stems mainly from CO and CC single bond vibrations found in carbohydrates. These vibrations couple well because the frequencies of the isolated bonds are similar. Thus they give rise to delocalized normal modes that extend over a larger part of the molecule. These normal modes depend on the structure of the molecule and are thus different for different carbohydrates. Therefore the absorption in this spectral range can be used to study the carbohydrate composition of a sample. Not surprisingly, the carbohydrate band is found in the fingerprint region of the infrared spectrum where the absorption is characteristic of the molecular structure as a fingerprint is characteristic of a person.

There is also a contribution to this band from the symmetric stretching vibration of $PO_2^-$ groups but in the case shown here, this contribution is minor. Nevertheless this contribution illustrates an important point. In most regions of the infrared spectrum of complex biological samples, several groups from different molecules contribute to a particular absorption band. Thus the assignments I have just discussed, are assignments to the groups and molecules that dominate the absorption in a particular spectral region. This does not exclude that other groups and other molecules also absorb in that region.

In this section I have given you an overview about the main features in an infrared spectrum of biological samples. It is not necessary that you learn all these features by heart, but I recommend you to recapitulate how the assignments in this section fit with the general principles that we discussed in the section before.

## Information that can be derived from the infrared spectrum

*IN GENERAL*

Structure and geometry of the vibrating group and the electron density distribution determine the vibrational frequency. Both are influenced by the environment. Therefore, the following information can be derived from the infrared spectrum.

*CHEMICAL STRUCTURE*

The chemical structure of a molecule is the dominating effect that determines the vibrational frequencies via the strengths of the vibrating bonds and the masses of the vibrating atoms. This effect may seem to be of minor relevance to biophysicists since the chemical structure of a large biomolecule cannot be deduced from the vibrational spectrum and will be often inert in biophysical investigations. However this is not always the case and I will name a few examples for structural changes that occur in protein studies.

Changes to the protonation state of side chains is an important example. Protonation and deprotonation reactions are often essential steps in a catalytic mechanisms. Here, vibrational spectroscopy seems to be the method

of choice since the protonation state of most side chains is reflected in the spectrum, whereas X-ray crystallography usually can not detect the protonation state of side chains.

Some examples for protonation and deprotonation reactions are given:

- protonation of Asp and Glu residues accompanies proton pumping by bacteriorhodopsin,

- proton transfer reactions are often coupled to electron transfer reactions,

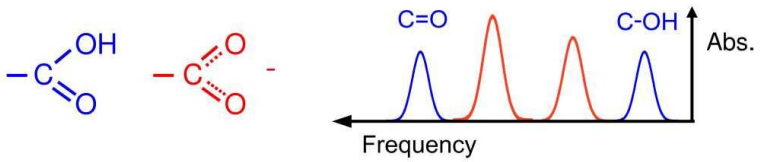
- protonation is a mechanism for charge compensation when a positive ion is released from negatively charged protein residues.

The following  illustrate how protonation reactions can be detected in the infrared spectrum.

**Information from the spectrum:**

**Chemical structure**

Example 1: Protonation / deprotonation of carboxyl group




A protonated carboxylic acid has a C=O double bond and a C-OH single bond, which oscillate with high and low frequency, respectively. The deprotonated form has two bonds with intermediate electron density (between single bond and double bond); the density in both bonds is the same. This makes the force constants in the two bonds equal and because of this the two vibrations couple as in $CO_2$. Accordingly, there are two bands for the deprotonated form, one for the antisymmetric stretching vibration $\nu_{as}$ and one for the symmetric stretching vibration $\nu_s$.

Because the electron density in the CO bonds of the deprotonated carboxyl group is intermediate between those of a single and a double bond, the average frequency of its two vibrations is between that of the C=O vibration and that of the C-OH vibration of the protonated carboxyl group.

Other examples for an alteration of chemical structure are protein modifications like phosphorylation and the monitoring of the chemical reactions that are catalyzed by enzymes.

*REDOX STATE*

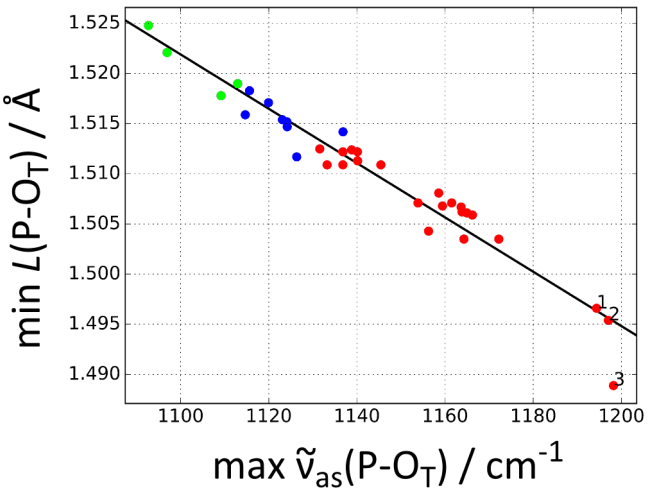
Redox reactions are the basis of the energy delivering processes photosynthesis and respiration in living organisms. They affect the electron density distribution of a given molecule. This will modify the force constants between the atoms and thus will alter its vibrational spectrum. Because of this sensitivity, redox-active cofactors involved in photosynthesis could be investigated. These studies could assign signals in the protein spectra to specific functional groups of the cofactors and in consequence statements about their protein environment.

*BOND LENGTHS AND BOND STRENGTH*

Vibrational frequencies are correlated with bond length and bond order of the vibrating bonds. These correlations are valuable for the understanding of the catalytic mechanism of enzymes since they reveal how an enzyme perturbs the bonds of the catalytically active groups.

A very good correlation is shown in the figure. It correlates a particular phosphate bond length and one particular phosphate vibration. This particular PO bond length can be determined with an amazing accuracy of 0.2 pm from the vibrational spectrum.

*Example for a correlation between structure and vibrational spectrum. It is based on density functional theory calculations (done by M. Rudbeck) on models of phosphorylated amino acids. The correlation is between the shortest PO bond of phosphate groups and the wavenumber of the asymmetric $-PO_3^{2-}$ stretching vibration (P. Pettersson, A. Barth, RCS Advances 2020).*




An example where such a correlation was applied to protein studies is pyruvate binding to lactate dehydrogenase which leads to a downshift of the pyruvate C=O band of 35 $cm^{-1}$. This large shift corresponds to a change in bond length of only 0.02 Å or 2 pm (Callender & Deng Annu. Rev. Biophys. Biomol. Struct. 1994)!

Note what small differences in bond length can be measured by vibrational spectroscopy. This "spatial resolution" is higher than that of other methods and provides insight into the molecular details of the catalytic mechanism. On the other hand, not all bonds can be predicted with the same accuracy as in the example shown.

*BOND ANGLES AND CONFORMATION*

Vibrations are often coupled and this coupling depends on details of the molecular geometry. Therefore, coupling often provides insight into the three-dimensional structure of molecules. A simple example are the two coupled CO vibrations in the $COO^-$ group. Their coupling and thus the frequency of the two stretching modes (normally observed near 1400 and 1570 $cm^{-1}$) depends upon the electron density in and the angle between the two CO bonds. In the hypothetical extreme cases of the angles of 90° and 180°, coupling is zero for 90° but is strongest for 180°. In addition, coupling is strongest when the two bonds oscillate with the same frequency and therefore depends on the electron density distribution in the carboxylate group. As a consequence, the frequencies of the two modes may shift considerably upon cation chelation (Deacon & Phillips 1980; Tackett 1989;

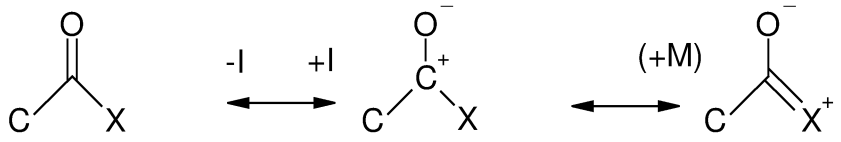
Nara et al. 1994) which can be explained by changes of bond lengths and angles (Nara et al. 1996). The effects depend upon the mode of chelation and have been valuable in studies of several $Ca^{2+}$ binding proteins (Nara et al. 1994; Fabian et al. 1996; Mizuguchi et al. 1997a).

A second example are the amide groups of the protein backbone. The Coulomb interactions between them couple the vibrations of one amide group to the same vibrations of other amide groups. This coupling depends on the three-dimensional structure of the protein backbone. As discussed in more detail later, this coupling makes the absorption of the amide groups sensitive to the secondary structure.

*INFORMATION ON NEIGHBORING GROUPS WITHIN THE MOLECULE VIA MESOMERIC AND INDUCTIVE EFFECTS*

When we study the vibration of a given bond in a molecule, its electron density will be influenced by the neighboring groups in the molecule and this will have an effect on the vibrational frequency. An example is a keto group (C=O) with different neighbors.

The C=O bond is polar which can be described by two mesomeric structures (left and middle structure in the Fig. below). These mesomeric structures are used when a molecule cannot be represented by a valance bond structure. The mesomeric structures have no physical meaning as such, they are not two structures in equilibrium, but they represent limiting cases. The real structure is a weighted average of the mesomeric structures. How much each structure contributes depends upon the substituents. They exert two types of effects: mesomeric and inductive effects.



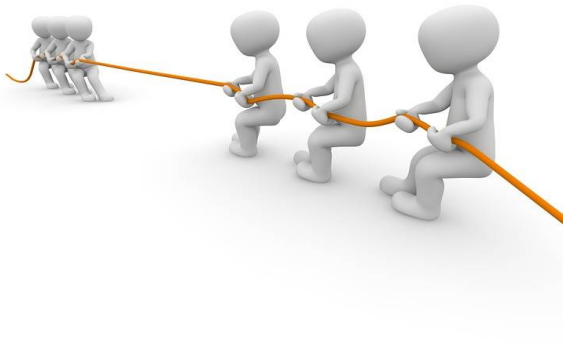

The *mesomeric effect* is due to the delocalization of π electrons. According to IUPAC it is "The effect (on reaction rates, ionization equilibria, etc.) attributed to a substituent due to overlap of its p- or π-orbitals with the p- or π-orbitals of the rest of the molecular entity. Delocalization is thereby introduced or extended, and electronic charge may flow to or from the substituent." A group that attracts electrons out of the bond has a -M effect and is an electron acceptor and a group that can donate electrons into a neighboring bond has a +M effect and is called electron donor.

The *inductive effect* is an electrostatic effect caused by differences in electronegativity of the atoms. In IUPAC's golden book, the inductive effect is defined as "an experimentally observable effect (on rates of reaction, etc.) of the transmission of charge through a chain of atoms by electrostatic induction." The inductive effect makes bonds polar (positive and negative partial charges on the atoms) which reduces the electron density in these

bonds. Positive and negative inductive effect are defined with respect to an aliphatic C-H bond. Electronegative atoms have a -I effect and pull electrons towards them.

Inductive and mesomeric effects make that the C=O bond of keto, ester and amide groups absorbs at different wavenumbers (see Problems and study questions).

*[Pixabay]*



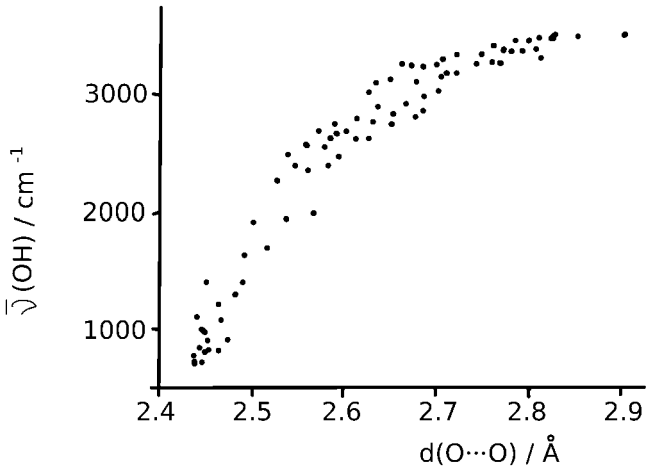
Electron withdrawing (-I) substituents stabilize the mesomeric structure with the C=O double bond (left structure in the above figure), because they compete with oxygen for the electrons. This is like a rope contest where the middle of the rope indicates the center of the π electrons and the two teams on both sides of the rope are the carbonyl oxygen and atom X. If only the carbonyl oxygen is pulling and X does not put up resistance, the electrons will end up at the carbonyl oxygen. But if both atoms pull, then the electrons will not be moved to the carbonyl oxygen and stay in the middle of the C=O bond. The effect is that the C=O bond becomes stronger.

Electron donating (+I) substituents stabilize the polar structure $C^+$-$O^-$ because they don´t put up resistance against the electron pull by oxygen. The effect is that the C=O bond becomes weaker.

Substituents with a +M effect also stabilize the polar structure because they donate an electron pair into the C-X bond which restores the normal number of four bonds around the carbon atom.

*HYDROGEN BONDING*

The next influence on the vibrational spectrum is hydrogen bonding. Hydrogen bonds stabilize the structures of proteins and DNA and are essential for catalysis. Vibrational spectroscopy is one of the few methods that directly report on the strength of hydrogen bonds. As a general rule, hydrogen bonding lowers the frequency of stretching vibrations, since it decreases the electron density in the covalent bonds which lowers the restoring force. But hydrogen bonding increases the frequency of bending vibrations since it produces an additional restoring force. Typically, formation of a single hydrogen bond leads to a downshift of the C=O stretching




*Correlation between frequency and O-O distance in OH···O hydrogen bonds (redrawn by C. Baronio from*

band by 20 $cm^{-1}$ and the enthalpy of hydrogen bonding and the distance of hydrogen bond acceptor and donor can be quantified using experimental correlations.

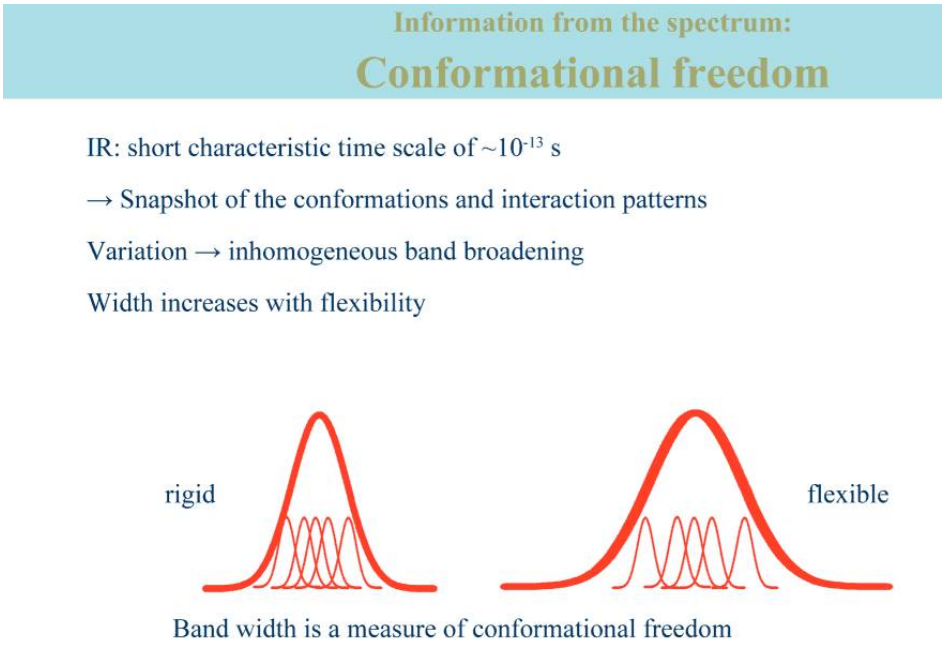
*T Steiner: Angew. Chem. Int. Ed. 2002, 41, 48-76)*

*ELECTRIC FIELDS*

Similar to hydrogen bonding, the electric field produced by the environment modifies the electron density distribution of a given molecule. A strong electric field has been detected for example in the active site of dehalogenase where it strongly polarizes the product of the catalytic reaction (Carey 1998). For carboxyl groups in the absence of hydrogen bonding (bands above 1740 $cm^{-1}$), there is an inverse correlation of the C=O stretching frequency with the dielectric constant $\varepsilon$ (Dioumaev & Braiman 1995).

*CONFORMATIONAL FREEDOM*

Besides band position and band intensity, the third spectral parameter, the band width, is also useful for a molecular interpretation. Due to its short characteristic time scale on the order of $10^{-13}$s, vibrational spectroscopy provides a snapshot of the sample conformer population. As the band position for a given vibration usually is slightly different for every conformer, Inhomogeneous band broadening is the consequence. Flexible structures will thus give broader bands than rigid structures and the band width is a measure of conformational freedom. It is possible to relate band width with entropy and thus to quantify entropic effects in catalysis.



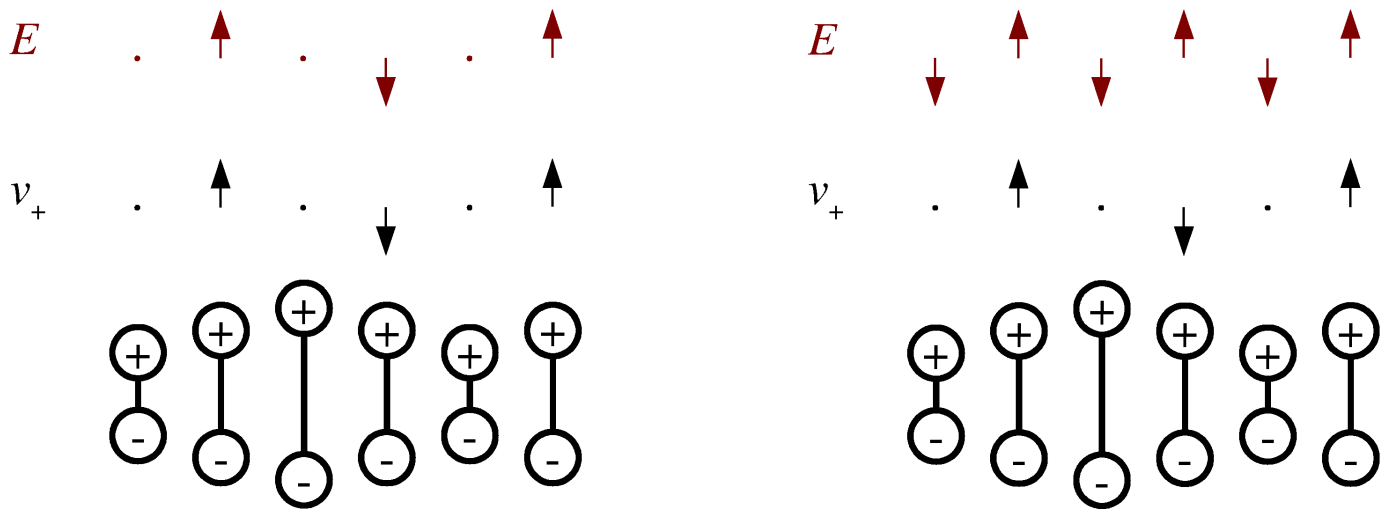

*Infrared absorption of (for example) a protein vibration. The small bands are the absorptions of indivudual protein molecules. The large bands are the sum of all individual absorptions. The individual band positions differ slightly because each vibrating bond interacts with the protein environment slightly differently than the others. If the protein is rather stiff (left hand side), then the variation in interaction strengths will be less than for a flexible protein (right hand side). This will lead to a narrower band for the stiff protein.*

For molecules that bind to proteins, the restriction of conformational freedom is a natural consequence of binding. This often reduces the band width by a factor of two. For example, phosphate bands of GTP become sharper when the nucleotide binds to Ras and ubiquinone is in a more rigid environment when bound to cytochrome $bo_3$.

## Vibrational transitions

*CLASSICAL VIEW OF THE INTERACTION*

The interaction between a vibrating bond and infrared radiation is mediated by the electric field of the electromagnetic wave. More specific: the interaction is between oscillating partial charges of the vibrating bond and the electric field. When the vibration and the electric field of the radiation oscillate with the same frequency and when the electric field is in phase with the velocity of the moving charges, then this velocity will increase and the vibration absorbs energy from the radiation. A velocity increase means also that the oscillation amplitude increases. Note however, that the frequency of the oscillation does not change. The effect is illustrated in the following figure.




*The interaction between the oscillating electric field E of an electromagnetic wave and a vibrating bond. The bond is assumed to be polar and the partial charges of the two atoms are indicated. The electric field vector indicates the direction of the force exerted on positive charges. $v_+$ is the velocity of the positive partial charge.*
*Left: Electric field E and the vibration oscillate both with the same frequency. As discussed in the mass on a spring chapter in Introduction to Spectroscopy, the driving force (= electric field E in this case) is is phase with the velocity of the oscillating mass (= atom with partial charge), when absorption occurs. Then the driving force increases the maximum velocity of the mass, which increases the oscillation amplitude. When the frequency of the driving force is the same as that of the mass, the electric field "supports" the vibration at all times and increases the amplitude of the vibration.*
*Right: the electric field oscillates faster than the vibrating bond. Now the electric field "supports" the vibration at some times, but impedes it at other times. Thus there is no net effect over a longer time period.*

As we have seen, the interaction between infrared radiation and molecular vibrations depend on the existence of oscillating partial charges. When there is no partial charge, the electric field has no "handle" to grip the molecule and there is no interaction. When the partial charges are large, then the interaction is strong and the absorption is strong.

Two partial charges of opposite sign form a dipole, which can be described by a dipole moment. The dipole moment is just the product of the positive partial charge $q$ and the distance between the partial charges, which is the bond length $L$.

$$\mu = qL$$

Thus, a prerequisite for the absorption of infrared radiation is an oscillating dipole moment. The absorption probability (calculated in a quantum mechanical calculation) is proportional to the square of the change of dipole moment when the oscillator passes through its equilibrium position. This is one of the ***selection rules*** for the absorption of infrared radiation. The larger the partial charges $+q$ and $-q$, the larger the dipole moment and the larger the change in dipole moment.

The change in dipole moment with respect to the bond length is independent from the distance and is equal to q:

$$\partial\mu/\partial L = \partial\mu/\partial L(L_0) = q$$

That is: the larger the oscillating partial charges the stronger the absorption. According to a thumb rule polar bonds are strong infrared absorbers, apolar bonds weak absorbers or infrared inactive (no absorption). For example C=O is a strong absorber, C=C absorbs weekly in $HFC{=}CH_2$, or not at all in $H_2C{=}CH_2$.

We have seen that the interaction between light and oscillation leads to an increase in oscillator amplitude which means that the maximal potential energy increases and therefore also the total energy of the oscillator. In the classical world this increase can occur continuously. However, this is not what happens in the real quantum mechanical world as described below.

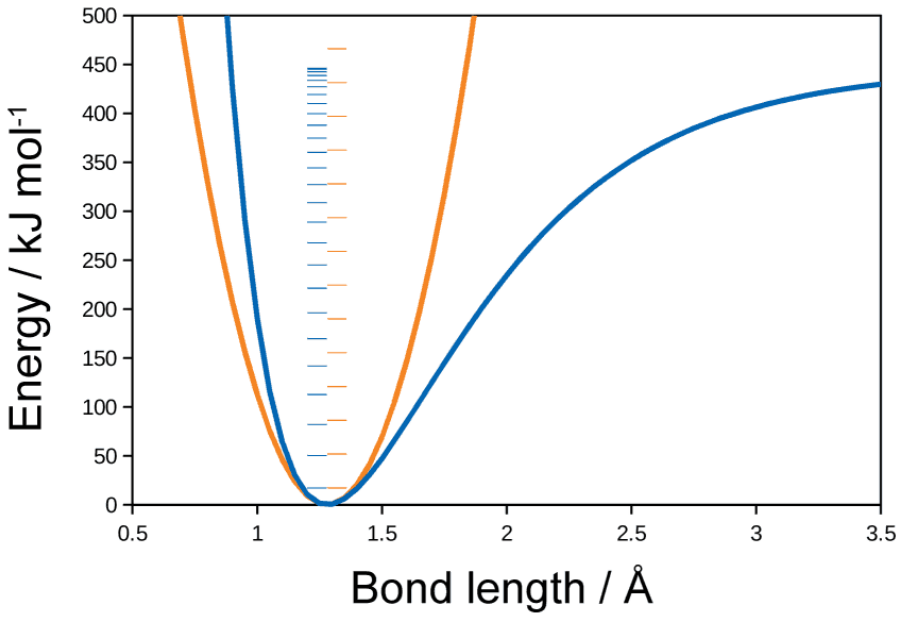
*ENERGY LEVELS OF THE HARMONIC OSCILLATOR*

*Comparison of a Morse potential and the potential energy of a harmonic oscillator. The potential and the vibrational energy levels were calculated for the HCl molecule with parameters given in Physical Chemistry by Engel & Reid and in Introduction to infrared and Raman spectroscopy by Colthup, Daly, & Wiberley.*

*Equilibrium bond length: 1.28 Å*

*Bond energy from the bottom of the potential: 446 kJ/mol.*

*Frequency of vibration: $8.65\times10^{13}$ $s^{-1}$.*



The potential energy of a harmonic oscillator is described by a harmonic or parabolic potential where the potential energy of the oscillator is equal to half of the force constant multiplied with the squared deviation of the bond length from the equilibrium bond length.

$$E = ½\ k\ \Delta L^2.$$

The harmonic potential is shown as orange line in the figure. The vertical axis is the potential energy, the horizontal axis the distance between the nuclei, for example for a two-atomic molecule. The plot is based on the parameters for the HCl molecule.

With this potential, the movement of the oscillator is harmonic, meaning that it can be described by a single sinus function with a frequency that depends on the width of the potential.

During the vibration, the oscillator moves up and down the parabolic curve and exchanges potential for kinetic energy and vice versa. In the minimum, the oscillator has no potential energy but maximum kinetic energy. The total energy remains constant and is equal to the maximum potential energy.

In the classical world the total energy can assume any value, however this is not the case in quantum mechanics where the energy levels are discrete. The energy levels are shown as orange lines in the figure. The spacing between the levels is Planck's constant times the vibrational frequency. The spacing to the next levels is the same no matter which level we are considering.

Another difference from the classical world is the existence of a ground state energy. In classical mechanics, the oscillator can be right in the minimum of the potential energy curve. It has then no kinetic energy, does not move, and the distance is the equilibrium distance. In quantum mechanics the energy of the oscillator can never be lower than half of the energy spacing between the energy levels.

Energy spacing and ground state energy together result in the equation for the energy levels of the harmonic oscillator shown here, where the counting index n runs from zero over all natural numbers.

$$E = (n + ½)\ h\nu$$

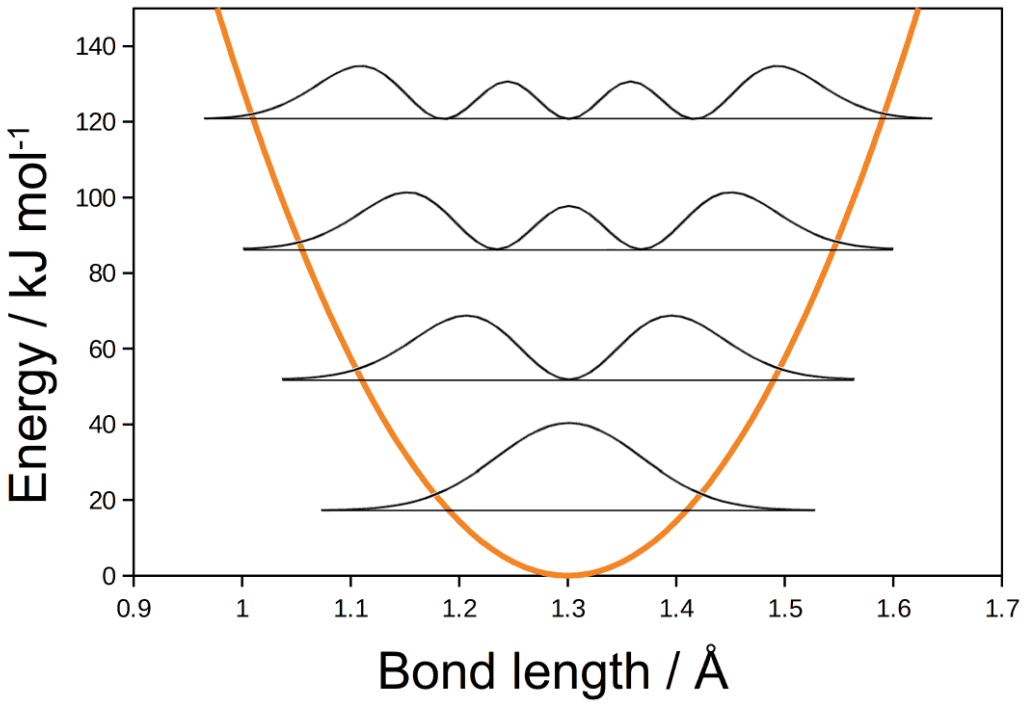
The harmonic potential is an approximation of the Morse potential which describes the potential energy curve of a covalent bond much better than the harmonic potential. For short bond distances, the Morse potential is steeper, meaning that the repulsion between the atoms is stronger, whereas it is shallower at longer bond length. Importantly it levels off and becomes constant for large distances between the atoms because there is no interaction between the atoms when they are far away.

The Morse potential is shown in blue. It is an example for an anharmonic potential. This means that the oscillator movement can no longer be described by a simple harmonic movement. Instead it has to be described by several sinus functions with different frequencies.

The energy levels of the anharmonic oscillator are also shown in the figure. As you can see, these energy levels are no longer equidistantly spaced. The higher the level, the smaller is the energy difference to the next level. When compared to the energy levels of the harmonic oscillator, those of the anharmonic oscillator are lower.

We return now to the harmonic oscillator because it provides a satisfactory explanation for most features in an infrared spectrum.

The figure below shows again the potential curve of an harmonic oscillator. The total energy is represented by the horizontal lines in the figure for several vibrational states, starting from the vibrational ground state $n = 0$ up to $n = 3$.




*Energy levels and probability functions for the harmonic oscillator. The equilibrium bond length was 1.3 Å*

A classical oscillator moves between the two intersections of the horizontal line for the total energy with the potential curve. For example, a classical oscillator with a total energy that corresponds to the second energy level would oscillate between a bond length of a bit more than 1.1 Å to a bit less than 1.5 Å. It cannot move beyond these limits because then it would move up the potential curve and would need an energy that is larger than its total energy.

This is different in the quantum world. For the quantum mechanical oscillator the probability of finding the oscillating bond at a given bond length is shown by the curves on top of the horizontal lines. It can be seen that the quantum mechanical oscillator has a larger freedom to move than the classical oscillator as the bond length can be found beyond the limits given by classical mechanics.

In the ground state the probability of the quantum mechanical oscillator is highest around the equilibrium position. This corresponds to a classical oscillator which is at rest and which therefore can be found only at the equilibrium position. But the quantum mechanical oscillator is never at rest, i wobbles around the equilibrium position. This is a consequence of the uncertainty principle which says that one can never exactly determine mo-

mentum and position at the same time. Because the quantum mechanical oscillator is never entirely at rest, the energy of the ground state is higher than the minimum of the potential curve.

For the excited states of the harmonic oscillator, the most probable bond lengths are those close to the turning points of the vibration. This is true also for the classical oscillator because the movement of the atoms is slowest around the turning points and thus they spend most time there. In the quantum mechanical description this property is the more pronounced the higher the quantum number $n$ is.

When the oscillator gets higher energy, the oscillation amplitude gets larger. For example with a total energy that corresponds to the highest level shown, the minimum bond length is about 1 Å and the maximum bond length about 1.6 Å. It is important to note that the vibrational frequency is the same for all energy levels. What changes is the maximum amplitude.

*QUANTUM MECHANICAL VIEW OF THE INTERACTION*

Fermi's golden rule

The next step is to calculate the probability for a transition between the ground state and the first excited state. This will give us both selection rules that apply to the absorption of infrared light. To calculate the transition probability, we will use ***Fermi´s golden rule***, which was already mentioned in the lecture Introduction to Spectroscopy. According to this rule, the probability for the transition from state $|\Psi_0\rangle$ to state $|\Psi_1\rangle$ is proportional to $|\langle\Psi_1|\boldsymbol{V}|\Psi_0\rangle|^2$. $\boldsymbol{V}$ is the operator that describes the perturbation energy. In order to proceed we have to find expressions for the perturbation operator and to think about $|\Psi_0\rangle$ and $|\Psi_1\rangle$.

The absorption of photons is mediated by the interaction between the electric field of the electromagnetic wave and the charge distribution of the molecule. As in the lecture Introduction to Spectroscopy, we can therefore describe the perturbation $\boldsymbol{V}$ by

$$\boldsymbol{V}(t) = -\overline{\boldsymbol{\mu}}\,\overline{E}(t)$$

where $\overline{\boldsymbol{\mu}}$ is the dipole moment operator. $\boldsymbol{V}$ and $\overline{\boldsymbol{\mu}}$ are operators that describe the molecule that we are interested in and $\overline{E}$ is the oscillating electric field vector of the electromagnetic wave. (I found this interaction operator with and without the minus sign. The minus sign should be correct according to the classical interaction energy. But the sign does not matter for the further calculation because the electric field is oscillating between negative and positive values.)

Using Fermi's golden rule, we find then that the probability for $|\Psi_0\rangle \rightarrow |\Psi_1\rangle$ is proportional to

$$|\langle\Psi_1|\overline{\boldsymbol{\mu}}|\Psi_0\rangle|^2\, E^2 \cos^2\alpha$$

where $\langle\Psi_1|\overline{\boldsymbol{\mu}}|\Psi_0\rangle$ is the transition dipole moment and $\alpha$ is the angle between electric field and transition dipole moment. This is identical to what we found already in the lecture Introduction to Spectroscopy.

The transition dipole moment in infrared spectroscopy

We proceed with analyzing the transition dipole moment and the next step is to think about the state vectors $|\Psi_0\rangle$ and $|\Psi_1\rangle$. When infrared light is absorbed, only the nuclei change their vibrational state from state $n$ to state $m$, the electrons remain in their ground state. When we now use the Born-Oppenheimer approximation we can write the state vectors $|\Psi_0\rangle$ and $|\Psi_1\rangle$ as products of the state vectors of the electrons and of the nuclei. $|\Psi_0\rangle$ is the product of the electronic ground state vector $|\psi_0\rangle$ with the nuclear state vector for the vibrational state $n$ $|\phi_n\rangle$. $|\Psi_1\rangle$ is the product of — again — the electronic ground state vector $|\psi_0\rangle$ with an excited nuclear state vector for vibrational state $m$ $|\phi_m\rangle$.

The Born-Oppenheimer approximation is possible because the nuclei are much heavier than the electrons and therefore move much slower. It assumes that the movement of the nuclei does not depend on the movement of the electrons, instead the electrons adapt instantaneously to the position of the nuclei. One consequence of the Born-Oppenheimer approximation is that the vibrational energy levels are calculated without considering the vibrational kinetic energy of the electrons (i.e. the kinetic energy of the electrons due to the vibrational motion together with the nuclei). The vibrational energy levels correspond therefore to the movement of the nuclei only. This is different from the classical view, where we have said that the moving masses are the atoms, meaning the nuclei plus those electrons that faithfully follow the movement of the nuclei. However, the contribution of the electrons to the vibrational energy will be very small because of their small mass, so when it comes to the question what are the moving masses in vibrational spectroscopy, you can answer either "the nuclei" or "the atoms" and I will consider both answers as correct: that the vibrational levels correspond to the movement of he nuclei or that they correspond to the movements of the atoms.

The transition dipole moment (TDM) for a transition from the vibrational level $n$ to level $m$ within the electronic ground state $\psi_0$ can be written as given below using the Born-Oppenheimer approximation that separates the nuclear wavefunctions $\phi_n$ and $\phi_m$ from the electronic wavefunction $\psi_0$ and $V = \overline{\boldsymbol{\mu}}(t)\overline{E}(\mathrm{t})$ for the interaction potential $V$, where $\overline{E}(t)$ is the electric field of the electromagnetic wave and $\overline{\boldsymbol{\mu}}(t)$ the *operator* of the dipole moment.

$$\overline{\mathrm{TDM}} = \langle \psi_0\phi_m \,|\, \overline{\boldsymbol{\mu}} \,|\, \psi_0\phi_n\rangle.$$

This is the same transition dipole moment that is relevant for the absorption of UV/vis light. The only difference is that the electrons are in their ground state also after the transition. Further calculation shows that this transition dipole moment is zero when it is evaluated at fixed positions of the nuclei. But when we consider that the dipole moment operator changes when the nuclear positions change, the result is different from zero. The transition dipole moment can then be factored into two terms that each gives rise to one selection rule: The right hand term in the expression below represents the selection rule that vibrational transitions only occur to the next vibrational level $\Delta n = \pm 1$; which is strictly valid only for the harmonic oscillator.

This selection rule limits the number of transitions that are relevant for infrared spectroscopy considerably. In the mid-infrared spectral range and at room temperature, we have to consider only the transition from the vibra-

tional ground state to the first excited state because the large majority of oscillators are in the vibrational ground state before absorption. The reason for this is that the thermal energy is smaller than the energy gap to the first excited state.

For this transition from $n = 0$ to $n = 1$ of a diatomic oscillator from the vibrational ground state to the first excited state the transition dipole moment is finally

$$\overline{\mathrm{TDM}} = \overline{\langle \partial \boldsymbol{\mu} / \partial R(R_0) \rangle} \, (h/8\pi^2 m_r \nu)^{0.5},$$

where $\overline{\langle \partial \boldsymbol{\mu} / \partial R(R_0) \rangle}$ is the (expectation value of the) change of dipole moment when the oscillator passes through the equilibrium positions $R_0$ of the nuclei (for a simple stretching vibration: when the oscillator passes through the equilibrium bond length $L_0$), $h$ is the Planck´s constant, $m_r$ the reduced mass of the diatomic oscillator ($1/m_r = 1/m_1 + 1/m_2$) and $\nu$ the frequency of oscillation.

The term on the right hand side is calculated only from the nuclear state vectors or wave functions. It is a factor that depends on the reduced mass of the oscillator and its frequency. It has different values for different vibrational transitions. In particular, it is zero for $\Delta n \neq \pm 1$. Therefore it is responsible for the selection rule $\Delta n = \pm 1$, as mentioned above.

The left term has contributions from only the electrons [CS]. It describes the change of dipole moment when the nuclei move through the equilibrium position $R_0$.

Striktly speaking, this term should be written as $\overline{\langle \partial \boldsymbol{\mu}_E / \partial R(R_0) \rangle}$ because the scalar product is calculated with the state vectors (wave functions) of the electrons only and because only the dipole moment operator of the electrons $\overline{\boldsymbol{\mu}_E}$ acts on the electronic state vectors. The dipole moment operator of the nuclei $\overline{\boldsymbol{\mu}_N}$ has no effect on the state vectors of the electrons. $\overline{\langle \partial \boldsymbol{\mu}_E / \partial R(R_0) \rangle}$ corresponds therefore to the movement of the center of the electron charge distribution, which accompanies the vibration.

The electronic term $\overline{\langle \partial \boldsymbol{\mu} / \partial R(R_0) \rangle}$ determines the direction of the transition dipole moment. It gives rise to the selection rule that infrared absorption only takes place when the dipole moment of the molecule changes with the vibration. The larger the change, the stronger the absorption. Often a large change is correlated with a large bond polarity, i.e. a large difference in the electronegativities of the bonded atoms. This is the same conclusion that we obtained with the classical view.

We return for a moment to the selection rule

$$\Delta n = \pm 1$$

which says that the quantum number $n$ changes only by plus minus 1. These are the so called ***fundamental transitions***. This selection rule is strictly valid only for the harmonic oscillator. For the anharmonic oscillator, more transitions are allowed, for example those where $n$ changes by ±2. These are called ***overtones*** and produce usually only weak bands in an infrared spectrum.

The vibrational frequency is different for different vibrations. When the vibrational frequency is different, then also the energy spacing between the levels is different, as this is proportional to the vibrational frequency. A

transition can now be induced by infrared radiation when the energy of the photon matches the energy gap between the vibrational levels (Bohr's frequency rule), in other words when

$$h\nu_{\text{photon}} = h\nu_{\text{vibration}}.$$

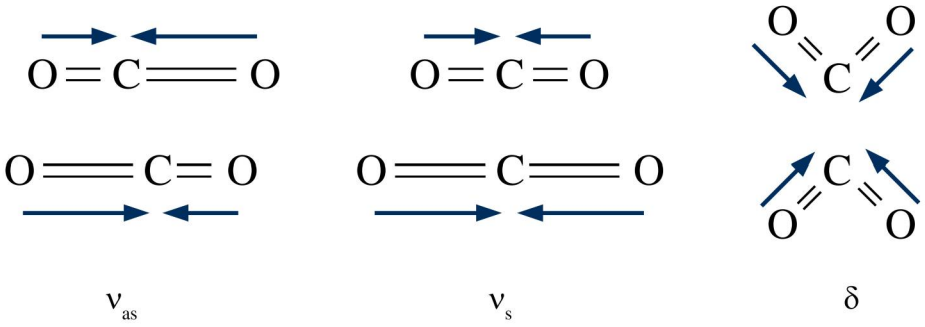
This is very similar to the classical description where the electric field needed to have the same frequency as the vibration in order to increase the amplitude of the vibration. Under this condition we can induce a transition from one level to the next level. Other transitions are not allowed for an harmonic oscillator.

Most of the transitions relevant for infrared spectroscopy are between the vibrational ground state and the first excited state. Why? The reason is that the distance between the energy levels is larger than the thermal energy, therefore 99% of all oscillators are in the ground state at room temperature, only 1 % in the first excited state.

*SELECTION RULE "CHANGE OF DIPOLE MOMENT REQUIRED" AT THE EXAMPLE OF $CO_2$*

Vibrations of $CO_2$

One of the selection rules states that the dipole moment has to change during the vibration for absorption to occur. I would like to illustrate this selection rule at the example of $CO_2$. The figure shows again the normal modes of $CO_2$ now with arrows representing the dipole moments of the two bonds.




*Vibrations of $CO_2$. Shown are the two extreme positions of the vibrations for the two stretching vibrations $\nu_{as}$ and $\nu_s$, and for one of the bending vibrations. The second bending vibration is the same movement but rotated by 90° around a horizontal axis in the paper plane. The arrows are the vectors of the dipole moments of individual bonds (not the movements of the atoms!). They add to the total dipole moment.*

Oxygen in $CO_2$ has a negative partial charge, C a positive. One can dissect the partial charge on the C atom into two parts and construct vectors of the dipole moment for the individual bonds (direction from – to +, from O to C). We assume that the partial charges do not change during the vibration. Then the dipole moment depends only on the separation between positive and negative partial charge, in other words on the bond length. It is large, when the bond is elongated and small when the bond is contracted.

The dipole moments of both bonds add up to the total dipole moment. For the equilibrium structure, the total dipole moment is zero because both bonds have the same length and their dipole moments have the same magnitude but point in opposite directions.

- In the antisymmetric stretching vibration the dipole moments of the two C=O bonds show in different directions. When one of them is small, then the other is large and vice versa. Therefore there is a resulting total dipole moment and the direction of it is different at the two extreme positions. It points to the left in the top structure and to the right in the bottom structure. When going from the top structure through the equilibrium structure to the bottom structure, the direction of the dipole moment changes from pointing to the left to pointing to the right. Therefore there is a change in the total dipole moment with the vibration and this vibration is infrared active and absorbs at 2349 $cm^{-1}$. Note that this change of dipole moment occurs although the molecule has no permanent dipole moment.
- In the symmetric stretching vibration the dipole moments of the individual C=O bonds have the same magnitudes at all times. As they have opposite directions, the resulting total dipole moment is zero at all times. This vibration is infrared inactive, which means that it does not absorb infrared light.
- In the bending vibration, the magnitude of the dipole moments does not change, but their directions. This gives a total dipole moment that points downwards in the top structure and one that points upwards in the bottom structure. Therefore the dipole moment changes with the vibration and the vibration is infrared-active. It absorbs at 667 cm-1.

## Spectrum recording

*CLASSICAL DISPERSIVE IR-SPECTROMETER*

A dispersive IR spectrometer is similar to a vis spectrometer (vis = visible, for the visible spectral region) with one important difference: the monochromator is placed between sample and detector to minimize the detection of the heat radiation from the sample. A further difference is that glass cannot be used because it is not transparent in the infrared. Therefore mirror optics are usually used.

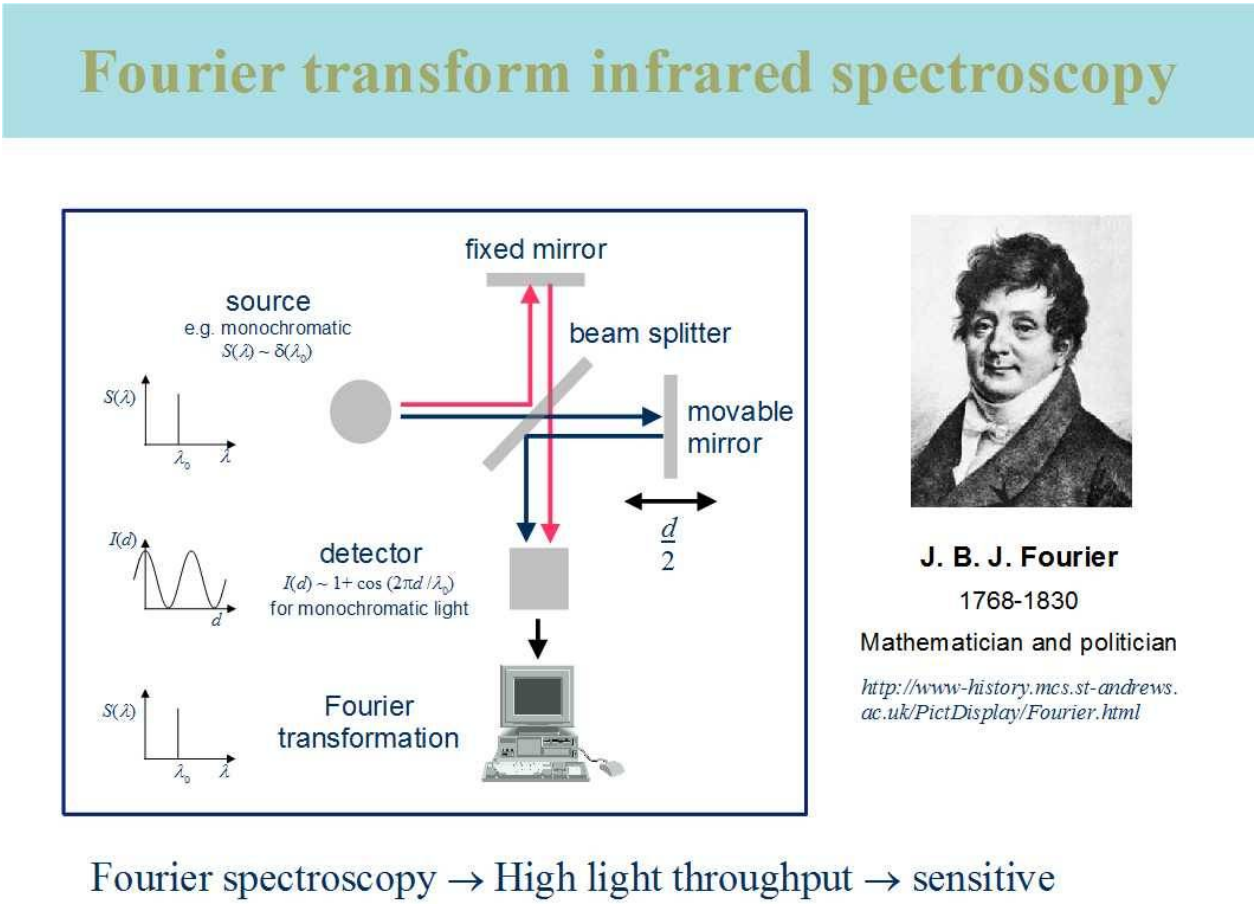
Dispersive IR-spectrometers are currently used only for special applications.

*FOURIER TRANSFORM INFRARED (FTIR) SPECTROMETER*

<u>Advantages</u>

Modern infrared spectrometers are usually FTIR spectrometers. The heart of a Fourier transform infrared spectrometer is the interferometer, like the Michelson interferometer shown here. It has fixed and a movable mirror. Light from the source is split by the beamsplitter, one part of it is reflected to the fixed mirror, on its way back passes the beamsplitter and reaches the detector. Another part passes the beamsplitter on its first encounter, is reflected by the movable mirror and by the beamsplitter before it hits the detector. When the two beams recom-

bine they interfere with one another and there will be constructive or destructive interference depending on the length difference of the two paths. The instrument measures the light intensity in dependence of the position of the movable mirror. This light intensity is the Fourier transform of the spectrum. Another Fourier transform in the computer transforms the measured data back into a spectrum. So we have two Fourier transformations: one performed by the interferometer, one by the computer. The main advantage of the Fourier transform spectrometer is high the light intensity at the detector and in consequence the high signal to noise ratio. Therefore a spectrum can be recorded in as few as 10 ms.




*Fourier transform infrared (FTIR) spectrometer*
*Fourier's picture: http://www-history.mcs.st-andrews.ac.uk/ PictDisplay/Fourier.html*

That the interferometer produces the Fourier transform of the spectrum is best seen, when a monochromatic source is considered. Depending on the position of the movable mirror we will obtain constructive or destructive interference at the detector and the detector signal varies in a cosine function with the mirror position. Now a delta function, describing the monochromatic spectrum and a cosine function are related by the Fourier transformation because the cosine function contains only one frequency. Another Fourier transformation generates again the spectrum.

Samples

Typically a 1 µl drop of an 0.1-1 mM protein solution is used. The optical pathlength of the cuvettes is very small due to the high water absorbance. For $^1H_2O$ is is less than 10 µm. In heavy water - $D_2O$ - the vibrations are slower than in $H_2O$ due to the mass effect and therefore the absorption bands shifted with respect to $H_2O$. In the spectral range of most interest, the absorption of $D_2O$ is therefore weaker than that of $H_2O$ and the pathlength of the cuvettes can be increased by a factor of 5 to 10. This makes it possible to use lower concentrations, but in turn requires larger volumes.

For the cuvettes one cannot use glass windows because glass is not transparent in the infrared spectral range. Instead one often uses flat Calcium fluoride windows.

## Raman Spectroscopy

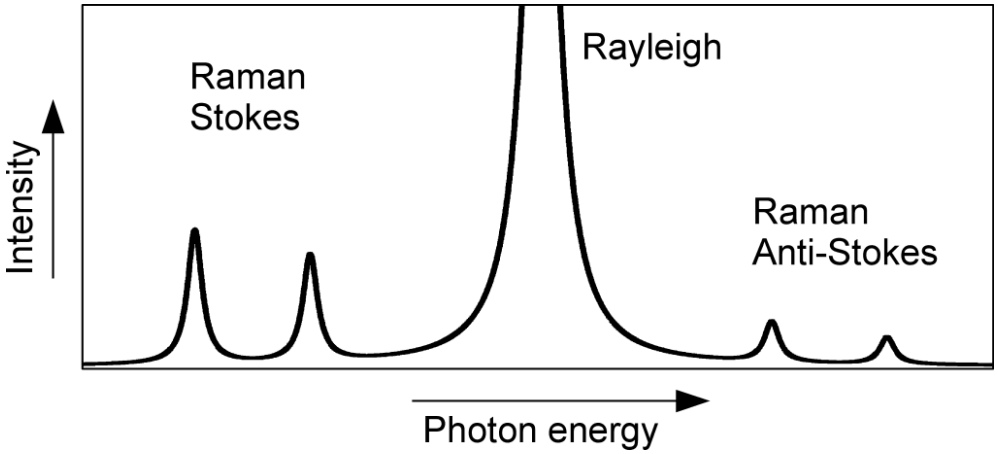
*THE RAMAN EFFECT*

Phenomenon

Raman spectroscopy is a second form of vibrational spectroscopy. In Raman spectroscopy light that is inelastically scattered is detected. This scattering is called Raman scattering and was discovered 1928 by Raman with help of the bright sun in India. The figure on the left shows a Raman spectrum of a simple compound. Most of the light that is scattered is elastically scattered: the scattered photons have the same energy as the incident photons. This is Rayleigh scattering. However, some of the photons have less energy and some have more energy than the incident photons. This is Raman scattering which gives rise to additional lines in the spectrum of scattered radiation. If lines are found at smaller energy than the Rayleigh line, they are called Stokes lines, if they have more energy, they are called anti-Stokes lines. It turns out that the energy difference between the Rayleigh photons and the Raman photons corresponds to the energy needed for vibrational transitions of the scattering molecule.

Rayleigh scattering is unlikely ($10^{-4}$ of the incident radiation) but Raman scattering is even less likely ($10^{-8}$ to $10^{-10}$ of the incident radiation [Grifiths and Haseth] or $10^{-4}$ of Rayleigh scattering [HJH p460]). This is a disadvantage of Raman spectroscopy because only a very small fraction of the incident photons generate a Raman signal which limits the signal to noise ratio.

*Chandrasekhara Venkata Raman*
*http://nobelprize.org/nobel_prizes/physics/laureates/1930/raman-bio.html*

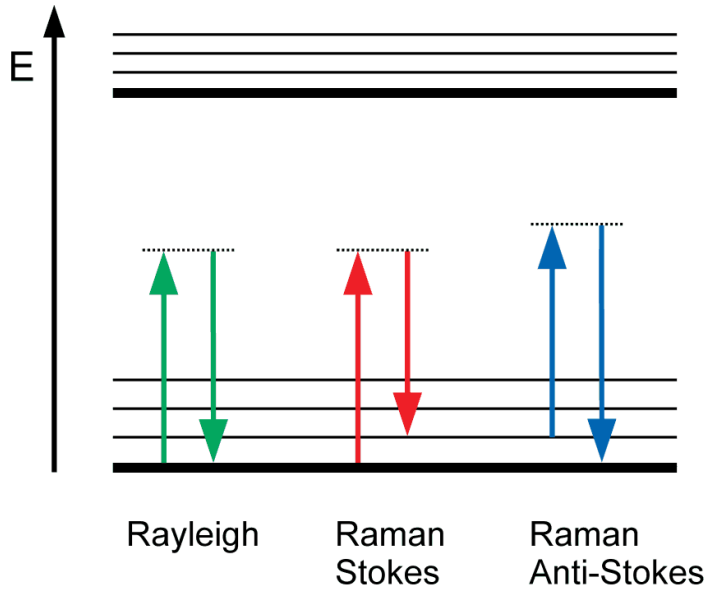



*Hypothetical Rayleigh and Raman scattering from a simple compound. Raman bands (Stokes and anti-Stokes) are shifted with respect to the Rayleigh peak. The two peaks on each side of the Rayleigh peak are the Raman signals of two vibrations with different frequencies (ground state to first vibrationally excited state for Stokes lines and first excited state to ground state for anti-Stokes lines). The signal from Rayleigh scattering is typically more than 10 000 times larger than the Raman signals and therefore extends beyond the plot area.*

*PHYSICAL ORIGIN*

Quantum mechanical view

What is the origin of the Raman bands in the spectrum of the scattered photons? This is easy to understand in the quantum mechanical view which is illustrated in this figure. The figure shows the vibronic states of a molecule for the electronic ground state and for the first electronically excited state as well as the three processes Rayleigh scattering, Stokes Raman scattering, and anti-Stokes Raman scattering.




*Raman scattering: excitation to a virtual energy level and emission of a photon.*

In all of these processes, an incident photon interacts with the molecule and excites it to a virtual state. The photon becomes annihilated in this process (this is not a proper absorption process because energy is not conserved

in this step [L p7]). The virtual state is a non-stationary state of the system. It has therefore a very short life time [Engel: Quantum Chemistry & Spectroscopy] ($10^{-11}$ s [Ga]) and its energy is not well-defined. When it decays, the scattered photon is created.

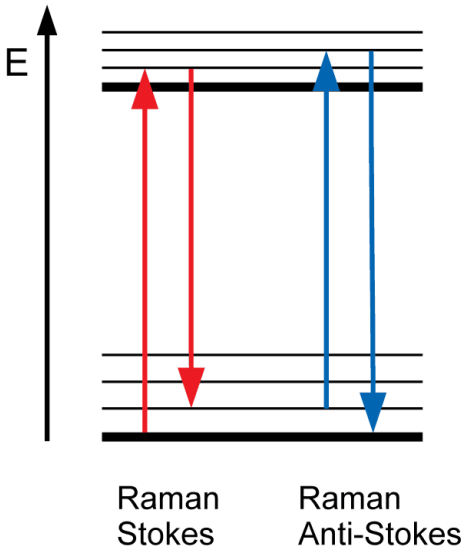
The three processes differ in the energy of the scattered photon. In Rayleigh scattering, indicated by the green arrows, the photon energy of the scattered photon is the same as that of the incident photon. Therefore there is no net energy transfer between radiation and molecule and the final state of the molecule is the same as the initial state.

In Stokes Raman scattering, the energy of the scattered photon is less than that of the incident photon. The energy difference is used to excite the molecule to a higher vibrational state. Only transitions to the next vibrational level are allowed in Raman spectroscopy. This is the same selection rule that applies for the absorption of infrared radiation. Therefore, the difference in energy between the incident photon and the scattered photon matches that of a vibrational transistion $h\nu_{vib}$. As most of the oscillators are in the vibrational ground state at room temperature, the most relevant process for Stokes Raman scattering is from the vibrational ground state to the first excited vibrational state.

In anti-Stokes Raman scattering, the molecule is initially in the first excited vibrational state and after the scattering process in the vibrational ground state. Therefore, the energy of the scattered photon is higher than that of the incident photon. Anti-Stokes scattering is unlikely because only a few oscillators are an excited vibrational state at room temperature which makes the intensity of anti-Stokes lines small.

Resonance Raman effect

Raman scattering is greatly enhanced (factor 100 - 1000) when the incident light energy is close to the energy of an electronic transition. In resonance Raman a photon is first absorbed and then emitted. This is slower than normal Raman scattering but still faster than vibrational relaxation. Emission is therefore from the same vibrational state that was reached upon excitation. Typical concentrations are 0.1 mM.



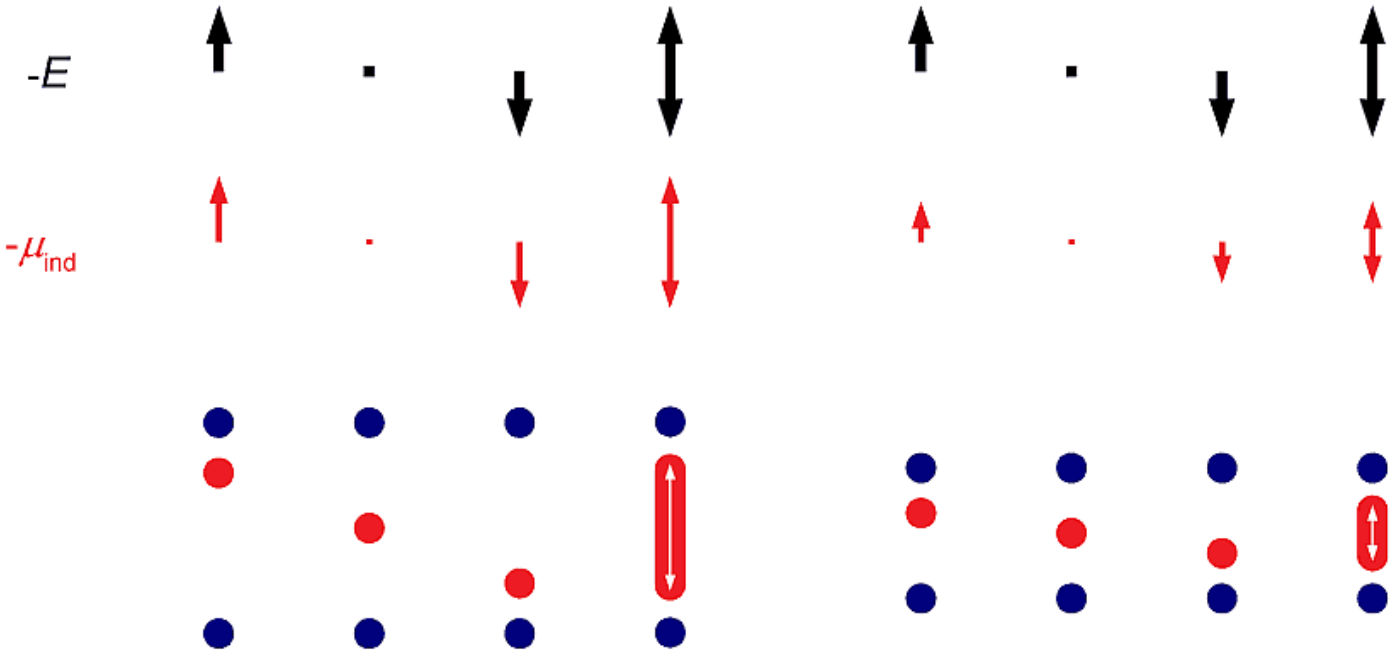

*Resonance Raman scattering: excitation to an excited state energy level and subsequent emission of a photon.*

Classical view

Light that is used in Raman spectroscopy is in the ultraviolet, visible or near infrared spectral range. The frequency of this light $\nu_{inc}$ is too high to interact with the oscillating atoms of a vibrating bond. Instead, the inci-

dent light induces oscillations of the electrons of the molecule. Thinking again in terms of the mass on a spring model, we have identified the driving force, which is the electric field of the radiation, and the oscillating masses, which are the electrons. The oscillating electrons create an oscillating dipole that emits radiation. This emitted radiation is the scattered light.

All this seems to be very similar to the case of UV/visible spectroscopy. However, there is one fundamental difference: the frequency of the electric field is much smaller than the resonance frequency of the electrons. In other words, the radiation energy is not absorbed by the electrons and the electrons do not change their state. Then the electric field is in phase with the amplitude of the electron oscillation (not in phase with the velocity as in the case of resonance).




*Interaction with the oscillating electric field of radiation generates electron oscillations. Blue: nuclei, red: center of charge of the electrons. Left: the bond is extended. The center of the electron cloud is shown for three different time points of the electron oscillation. Because the electron movement is much faster than the vibrations of the nuclei, the bond length does not change during one electron oscillation. Next to the three snapshots of the electron oscillation, the range over which the center of the electron cloud moves is illustrated by the red bar with the white arrow. Right: respective illustrations for the contracted bond.*

The induced dipole moment $\mu_{ind}$, generated by the light-induced electron oscillations, is proportional to the electric field strength $E$ and to a molecular property called *polarizability* $\alpha$.

$$\mu_{ind} = \alpha E$$

A large polarizability has a large induced dipole moment as consequence, which results in a high intensity of the scattered light. Polarizability describes how easily the electrons follow the driving oscillating force. It is related

to the distance over which the electrons can be moved by the oscillating electric field because of the definition of dipole moment

$$\mu = q\ d$$

where $q$ is the charge at both ends of the dipole and $d$ the distance of separation of positive and negative charge. Because both expressions ($\mu_{ind} = \alpha\ E$ and $\mu = q\ d$) are valid for the induced dipole moment it follows that

$$\alpha \propto q\ d_{ind}$$

meaning that the polarizability is proportional to the extent of the induced charge movement. When movement of the electrons is only little restricted, they can move over a large distance which gives a large dipole moment and thus a large polarizability. Thus the polarizability is small when a bond is contracted but larger when it is expanded (Methods in Molecular Biophysics, I.N.Serdyuk, N.R. Zaccai, J. Zaccai, p577-578)[C1990, Fig. 1.32].

To a first approximation (Taylor expansion), the polarizability $\alpha$ of a bond is given by

$$\alpha\ (t) = \alpha_0 + (\partial\alpha/\partial L)\ \Delta L(t)$$

where $\alpha_0$ is a constant, $\Delta L(t)$ the bond distortion from the equilibrium length at a given time $t$, and $\partial\alpha/\partial L$ the derivative of the polarizability with respect to the bond length taken at the equilibrium length. Remember that $\alpha$ oscillates with the vibrational frequency $\nu_{vib}$ of the nuclei.

We return now to the equation $\mu_{ind} = \alpha\ E$ and insert the above expression:

$$\mu_{ind}\ (t) = \alpha_0\ E(t) + (\partial\alpha/\partial L)\ \Delta L(t)\ E(t)$$

This gives two terms. The first one oscillates with the frequency of the incident electric field $\nu_{inc}$. This term gives rise to Rayleigh scattering. The second term gives rise to Raman scattering. It consists of three quantities. The first ($\partial\alpha/\partial L$) is a property of a particular vibration of the considered molecule. It needs to be different from zero in order to observe Raman scattering. If it is large, Raman scattering is more intense. Thus, Raman scattering occurs only when the polarizability changes during the vibration ($\partial\alpha/\partial L \neq 0$). As we will see later, not all vibrations lead to a change in the polarizability. The second term in the above equation has further two time-dependent quantities that oscillate with different frequencies. The bond distortion $\Delta L$ oscillates with the vibrational frequency of the nuclei $\nu_{vib}$, and the electric field $E$ oscillates with the frequency of the incoming radiation $\nu_{inc}$. This modulates the induced dipole moment $\mu_{ind}$ with the frequency of the vibration.

Because the polarizability depends on the bond length, the amplitude of the light-induced electron oscillations depends on the positions of the atoms, which change relatively slowly with the frequency of the nuclear vibration (with vibrations I mean nuclear vibrations, i.e. normal modes, with oscillation the electron oscillations). Each vibration modulates the amplitude of the fast ($\nu_{inc}$) electron oscillations with the low frequency of the nuclear vibration ($\nu_{vib}$). Because the electron oscillations generate the scattered light, also the amplitude of the scattered electromagnetic wave is modulated with the frequency of the nuclear vibration. This modulation is rather slow compared to the driving force of the oscillating electric field. Such a modulated oscillation is equivalent to two oscillations with similar but different frequencies meaning that light with two frequencies is emit-

ted: $\nu_{inc} + \nu_{vib}$, and $\nu_{inc} - \nu_{vib}$ ($\nu_{inc}$: frequency of incident light and $\nu_{vib}$: frequency of vibration). Therefore we observe Raman scattered radiation with higher and lower frequency (energy) than the frequency of Rayleigh scattered radiation. The frequency difference between Rayleigh scattered radiation and each of the Raman scattered waves is equal to the frequency of the nuclear vibrations. In addition, we get Rayleigh scattering with the same frequency as the incident light because the average polarizability during the vibration is different from zero.

An analogy to the generation of Raman scattered light is the tuning of a guitar. If the two strings have nearly the same pitch (frequency) then they are not heard as two tones with different pitch, but rather as one tone the with slowly modulated loudness (also called beat). Which shows that a slowly modulated oscillation is composed of two oscillations with slightly different frequencies. (search for beat frequency, for example: https://www.youtube.com/watch?v=V8W4Djz6jnY)

I will summarize now the selection rules for Raman spectroscopy, which we have already encountered, and discuss an example. One selection rule is familiar from infrared spectroscopy. It says that the harmonic oscillator only jumps to the next vibrational level $\Delta n = \pm 1$.

The second selection rule says that the polarizability has to change with the vibration, otherwise there will be no Raman scattering of this particular vibration. In other words vibrations where the polarizability changes are Raman active, those where it does not change are Raman inactive. For molecules with a symmetry center the selection rules of infrared and Raman spectroscopy are complementary, meaning that vibrations that absorb infrared light (infrared active) do not cause Raman scattering (Raman inactive) and vice versa. Some vibrations can be both infrared and Raman ***inactive*** (Keiter J. Chem. Educ. 1983). Most biomolecules do not have a center of symmetry and their normal modes of vibrations will be both Raman and infrared active. But their infrared and Raman spectra are still complementary to some degree: polar bonds are generally strong infrared absorbers and weak Raman scatterers, whereas apolar bonds are weak infrared absorbers and strong Raman scatterers (http://www.chemvista.org/ramanIR4.html).

Let us consider $CO_2$ as an example:

$\nu_s$ $\nu_{as}$

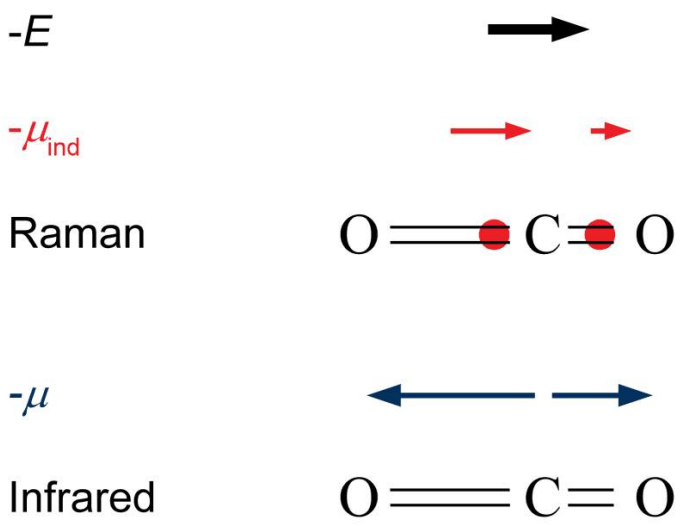
The symmetric stretching vibration is shown at two different time points on the left. When both bonds are contracted, the electric field moves the electrons only over a small distance. The induced dipole moments in both bonds are small and they add to a small total induced dipole moment. In contrast, when both bonds are ex-

tended, the electric field moves the electrons over a large distance and the polarizability is large. Thus the polarizability changes with the vibration and this vibration is Raman active.

The antisymmetric vibration is shown on the right hand side. At both times, one of the bonds is extended, giving a large induced dipole moment within this bond, and the other bond is contracted, giving a small induced dipole moment within this bond. The small and the large induced dipole moment add upp to an intermediate total induced dipole moment, which is the same for both time points shown and in fact does not change during the vibration. Therefore, the polarizability of the whole molecule does not change during the vibration and this vibration is Raman inactive.

So, the symmetric stretching vibration is Raman active, whereas the antisymmetric stretching vibration is Raman inactive. This is the opposite of what we found for the absorption of infrared light. The symmetric vibration was infrared inactive and the antisymmetric vibration infrared active because the dipole moment of the molecule does not change during the symmetric vibration but it does change during the antisymmetric vibration.

The different dipole moments that are relevant for Raman spectroscopy and infrared spectroscopy may be confusing and therefore I compare them in the following using the example of a particular time point during the antisymmetric stretching vibration (see figure below). In Raman spectroscopy, the frequency of the incident light is so high, that it only affects the electrons. They start to oscillate, which makes also the center of charge of the electron cloud oscillate. The figure shows the case when the reverse of the incident electric field points to the right. I have plotted the reverse of the electric field vector because the reverse points in the same direction as the force exerted on the electrons. When the reverse points to the right, the center of charge in each bond also moves to the right. This is illustrated by the red circles. It moves more away from the center of the bond when the bond is elongated than when it is contracted. Therefore, the induced dipole moment is large for the extended bond and small for the contracted bond. Also for the dipole moment, I have chosen to show the reverse direction because the reverse points to the position of the electrons. So far, there was nothing new in the argument. The important point comes now: the induced dipole moments are generated by the electric field of the radiation. Without radiation, there would be no induced dipole moment. The direction of both induced dipole moments is determined by the direction of the electric field vector. Therefore it is the same in both bonds and the induced dipole moments of both bonds add up for the total induced dipole moment.




*Difference between the dipole moments relevant for Raman spectroscopy and for infrared spectroscopy. See text for further details.*

The situation is different for the absorption of infrared light. Here, the relevant dipole moments are the permanent dipole moments of the bonds which are generated by the partial charges on the atoms. These dipole moments are present also in the absence of electromagnetic radiation. In the $CO_2$ molecule, the reverse of the dipole moments of the two bonds always points to the negative charge on the oxygen atoms. Therefore the direction of the dipole moments relevant for the absorption of infrared light is determined by the chemical structure of the molecule and not by the direction of the incident electric field, which was the case for Raman scattering. For $CO_2$, the dipole moments due to the partial charges point in different directions at all times, whereas the induced dipole moments relevant for Raman scattering point in the same direction. The different relative orientation of the dipole moments relevant for Raman scattering and for infrared absorption explains the different behavior of the two stretching vibrations in Raman and in infrared spectroscopy. For the antisymmetric stretching vibration, the total ***induced*** dipole moment does not change during the vibration (the vibration is Raman inactive), but the total dipole moment does change (the vibration is infrared active).

*RAMAN SPECTROMETER*

The excitation light and the scattered light are in the ultraviolett, visible or the near infrared spectral region. Therefore the same optical components can be used as in a UV/vis spectrometer. The light source is a laser, to generate light of high intensity. Rayleigh scattering is eliminated by a very good optical filter and the spectrum of the Raman scattered light decomposed in its spectral components either by a double or triple monochromator or by a Michelson interferometer.

Typical concentrations are 0.1 - 1 mM.

Important: what is plotted in a Raman spectrum is the energy difference between Raman scattered light and Rayleigh scattered light.

Advantages: water is a bad Raman scatterer, measurements in water are therefore easy. optical components for the visible spectral range can be used

the vibrational spectrum from 4000 to 50 $cm^{-1}$ is obtained in one go. In an infrared spectrometer several components have to be exchanged because they absorb in certain spectral regions.

Disadvantages: scattered intensity is often very low

*RAMAN APPLICATIONS*

Since Raman spectroscopy is vibrational spectroscopy it gives the same information as infrared spectroscopy. The differences lie in the vibrations that are detected and in the different experimental requirements and problems. In the following we will return to the discussion of infrared spectroscopy.

## Application 1 - Information on Structure: Secondary Structure Analysis of Proteins

*INTRODUCTION*

There are two main applications of infrared spectroscopy: (i) the analysis of the secondary structure of proteins and (ii) the analysis of the molecular reaction mechanism of proteins.

*ABSORPTION OF THE AMIDE I VIBRATION*

Overview

We will start with secondary structure analysis, which is the most common application of infrared spectroscopy in the biological sciences. Secondary structure analysis is mostly done using the absorption of the amide I vibrations. These are vibrations of the amide groups in the protein backbone. They involve mainly the C=O stretching vibrations. Further contributions come from the CN stretching vibration and the NH bending vibration. All these vibrations are coupled in the amide I normal modes. There are as many amide I normal modes as there are amide groups in the protein backbone. The absorption of the amide I vibrations generates an absorption band in the absorbance spectrum of proteins, which is called amide I band. It is located in the spectral region from 1700 $cm^{-1}$ to 1600 $cm^{-1}$, which is also called the amide I region. But this does not mean that only the amide I vibrations absorb in this spectral region. Side chains and polynucleotides for example absorb also in the amide I range. Nevertheless, in typical spectra of biological cells or tissue, most of the absorption in the amide I region stems from the amide I vibrations. A practical problem is that water absorbs in the amide I range. Water absorption is usually stronger than protein absorption. It has therefore to be subtracted from the sample absorption or to be avoided by drying the sample or by using D2O (absorbs at lower wavenumbers outside the amide I range. This is an example for the mass effect).

There are other infrared bands caused by the absorption of the amide groups of the protein backbone:

| | | |
|---|---|---|
| Amide A and amide B | N-H stretching vibration, | 3300 and 3100 $cm^{-1}$ |
| Amide I | $\nu$(C=O), $\nu$(C-N), $\delta$(N-H) | 1600-1700 $cm^{-1}$ |
| Amide II | $\delta$(N-H), $\nu$(C-N) | 1520-1570 $cm^{-1}$ |

| | | |
|---|---|---|
| Amide III | δ(N-H), ν(C-N) | near 1300 $cm^{-1}$ |

The above are all vibrations in the plane of the peptide group, out of plane vibrations, oop (out of plane), are found below 800 $cm^{-1}$.

The exact position and shape of the amide I band is affected by the secondary structure of proteins. An example is shown on the right. Albumin is predominantly α-helical and avidin has a high content of β-sheet structure. Obviously, the spectra of the two proteins are quite different and this is generally the case for α-helical and β-sheet proteins.

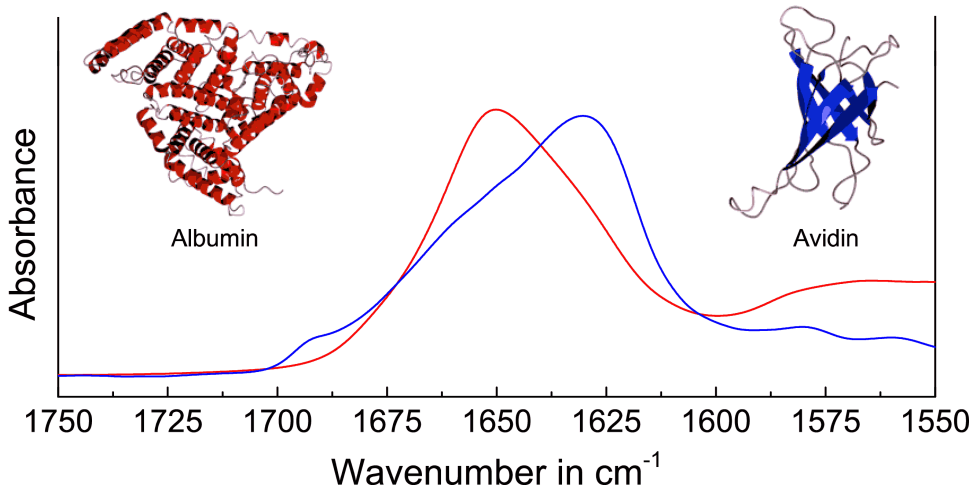



*Figure: Jan Schnatwinkel*

Many studies have correlated specific secondary structures with absorption in specific spectral regions within the amide I range. These regions are listed in the table below but there is no need to remember these numbers. Important is that β-sheets and α-helices can be distinguished because the main β-sheet band is found at lower wavenumbers than the α-helix band. The α-helix band overlaps with that of irregular or random structures.

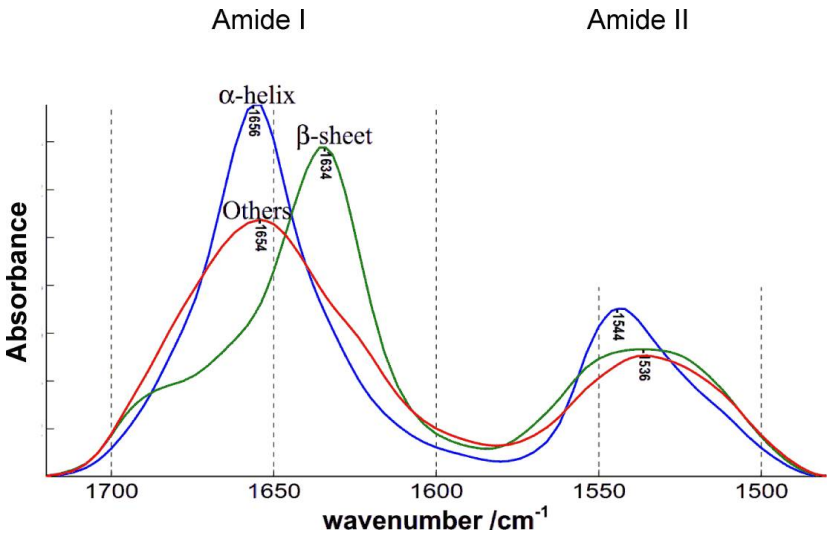
*Average band positions and spectral ranges of different secondary structures listed for ordinary water ($^1H_2O$) and heavy water ($D_2O$ or $^2H_2O$). Heavy water is often used to avoid the strong absorption of ordinary water near 1640 $cm^{-1}$, right in the middle of the amide I range. Table compiled from data in Goormaghtigh et al. (1994), Subcellular Biochemistry 23: 405-450.*

| **Secondary structure** | **Band position in $^1H_2O$ / $cm^{-1}$** | | **Band position in $^2H_2O$ / $cm^{-1}$** | |
|---|---|---|---|---|
| | **average** | **region** | **average** | **region** |
| α-helix | 1654 | 1648-1657 | 1652 | 1642-1660 |
| β-sheet | 1633<br>1684 | 1623-1641<br>1674-1695 | 1630<br>1679 | 1615-1638<br>1672-1694 |
| turns | 1672 | 1662-1686 | 1671 | 1653-1691 |
| irregular | 1654 | 1642-1657 | 1645 | 1639-1654 |

These empirical correlations have been confirmed by statistical analysis of the spectra of proteins with known structure. These can be used to obtain spectra of pure secondary structures shown below. These spectra confirm the empirical correlations, but they do not make the distinction between turn and irregular structures. The main β-sheet absorption is at lower wavenumbers than the absorption of helices and other structures. The β-sheet spectrum has also a high wavenumber shoulder that is often associated with antiparallel β-sheets. Note again that the absorption of α-helices is found in the same spectral region as the absorption of other structures. However, the helix band is more narrow due to a better defined structure. This exemplifies that the band width reflects the conformational heterogeneity of the absorbing structures.

*Spectra of pure secondary structures obtained from statistical analysis of 92 protein spectra. The figure shows the amide I and the amide II range of the spectrum. The amide II range provides also valuable structural information that is complementary to that obtained from the amide I range.*

*Modified from De Meutter & Goormaghtigh Eur. Biophys. J. 2021*




We turn now to the reason for the structural sensitivity of the amide I band. Its shape and position is determined by two main influences:

(i) Hydrogen bonding to the C=O oxygen and to the NH hydrogen decreases the wavenumber and the frequency of the amide I vibration.

(ii) The vibrations on individual amide groups are coupled as will be further explained below. This makes the amide I vibrations of proteins collective vibrations, which means that several amide groups participate in each amide I normal mode.

These two influences make the amide I wavenumber or frequency sensitive to the structure of the protein backbone.

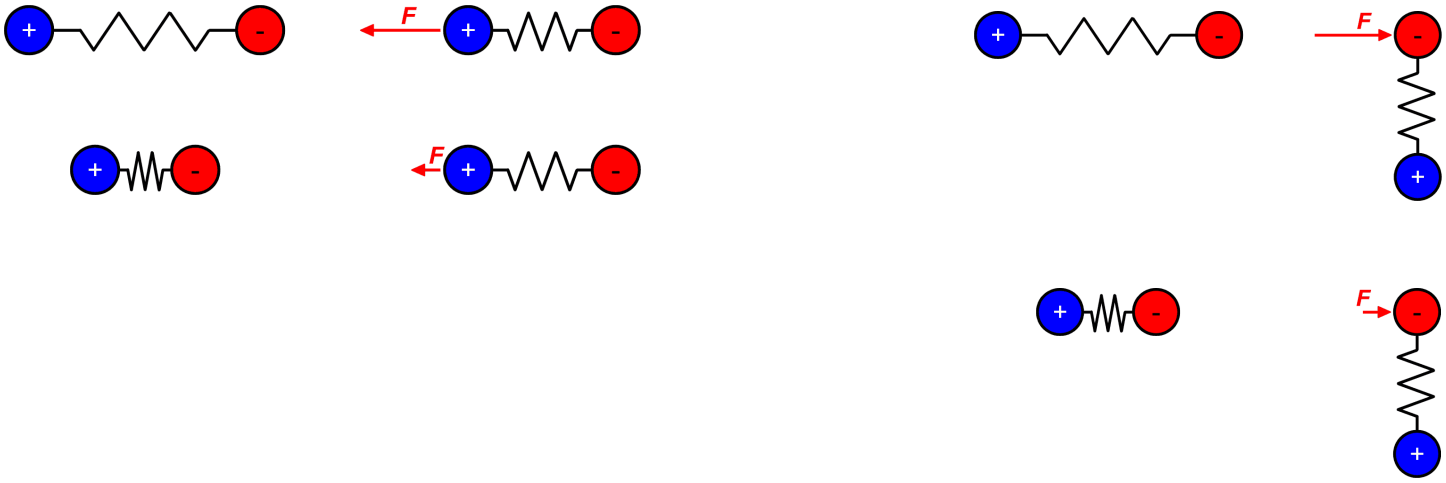
Transition dipole coupling (TDC)

In the following, I will explain the coupling between the amide I oscillations on different amide groups. This coupling has its origin in the electrostatic interactions between the oscillating partial charges on the atoms. Imagine a classical dipole where two partial charges are connected by a spring, which is a simple model for the C=O bond with a positive partial charge on the carbon and a negative charge on the oxygen (see figure below).

Let us further assume that one such C=O bond oscillates and a second one is at rest. Then the oscillating charges of C=O bond 1 will exert electrostatic forces on the resting C=O bond 2 that vary with time. In other words, C=O bond 2 "feels" the oscillation of C=O bond 1 and is influenced by it. The oscillating forces cause the charged atoms of resting C=O bond 2 to move so that C=O bond 2 starts to oscillate, while the C=O bond 1 oscillates less, until it stops to oscillate and only C=O bond 2 oscillates. Subsequently, C=O bond 1 starts to oscillate and CO bond 2 oscillates less and so on. This phenomenon reveals that there is energy transfer back and forth between the two oscillators (for a simple mechanical analogue, see the first demonstration of https://www.youtube.com/watch?v=ljaQr6YOVnk. See also the first examples of https://www.youtube.com/watch?v=zlzns5PjmJ4. The two carts can be thought to be connected with springs to each side and to each other. The spring between them represents the coupling between them. The first two movements are the two normal modes of the system of two coupled oscillators, whereas the third shows the case where one of them is at rest initially and energy is transferred between the two oscillators).

We have encountered the phenomenon of energy transfer already when we discussed the vibrating tree trunk. In the tree trunk example, starting the horizontal vibration led with time to a vertical oscillation and vice versa. When the trunk oscillated in the horizontal direction, all oscillation energy resided in the horizontal vibration. Later, when it oscillated in the vertical direction, the oscillation energy was in the vertical vibration. Thus there was energy transfer between the horizontal and the vertical vibrations because the two vibrations were coupled. Because of the energy transfer, none of these two vibrations was a normal mode. Instead, the two normal modes consisted of the two diagonal vibrations. There was no energy transfer between the two diagonal vibrations because when one of them was started, the direction of oscillation did not change.

It turns out that the vibrational coupling between amide I vibrations of different amide groups can be described by coupling between the transition dipole moments of the amide I vibrations of the interacting amide groups. Therefore it is named *transition dipole coupling*. As any interaction between dipoles, the strength of interaction depends on the relative orientations of coupled dipoles and on their distances. Therefore the vibrational spectrum of the amide I vibrations depends on the three-dimensional structure of the protein backbone. This can be easily seen from the simple model of two C=O bonds and for this we discuss the strongest interaction, which is the interaction between the closest atoms of bond 1 and 2 (see figure below). When the C=O bonds align, each one can influence the bond stretching vibration of the other because the interaction forces are parallel to the bonds as explained in the figure legend. When they are perpendicular to each other, then there is very little coupling between the bond stretching vibrations because the force that bond 1 exerts on bond 2 has only a very small component in the direction of bond 2. Only a force component along the bond can influence its stretching vibration.




*Structural dependency of the vibrational coupling. The left and right panels compare two different arrangements of two oscillators. For each arrangement, two time points of the oscillation of the left oscillator are shown. Blue and red balls represent atoms with positive and negative partial charge, respectively. The spring represents the bond between them. Left: the left bond is oscillating and exerts forces on the right bond. For simplicity, we only consider the strongest force (red arrow), which is the force between the two partial charges which are closest (we also ignore forces which the right bond exerts on the left). This force is always attractive but the magnitude of the force varies. It is stronger when the negative charge of the left oscillator is closer to the positive charge of the right oscillator. In addition, the direction of the force is always along the bond and therefore it can influence expansion and compression of that bond. Right: the two oscillators are perpendicular to each other. Now, the strongest force is perpendicular to the right oscillator. It has in this particular geometry no component parallel to the bond and therefore, it has very little influence on the movement of the right oscillator.*

Transition dipole coupling has three effects: (i) it *delocalizes* the amide I vibrations, (ii) it *changes the vibrational frequencies*, and (iii) it affects the *probability of absorbing infrared radiation*. The effect of coupling on the infrared spectrum is strongest when the coupled oscillators vibrate with the same frequency, in other words: when they are in resonance. In a coupled system, we get as many normal modes as we have amide groups. However, not all of them need to be infrared active. We will first discuss delocalization of the amide I vibrations:

Infrared photons are absorbed collectively by a group of coupled amide groups, not by a single group. Thus, the excited vibrational state is delocalized over several amide groups. Delocalized excited states are also observed in other contexts and are termed *exciton*. We will encounter excitons again in UV/visible spectroscopy. The coupling interaction that generates the excitons is also termed *exciton coupling*. In the case we are considering here, exciton coupling is the same as transition dipole coupling. The delocalization of excitation energy means that each amide I normal mode consists of the local amide I oscillations of several amide groups.

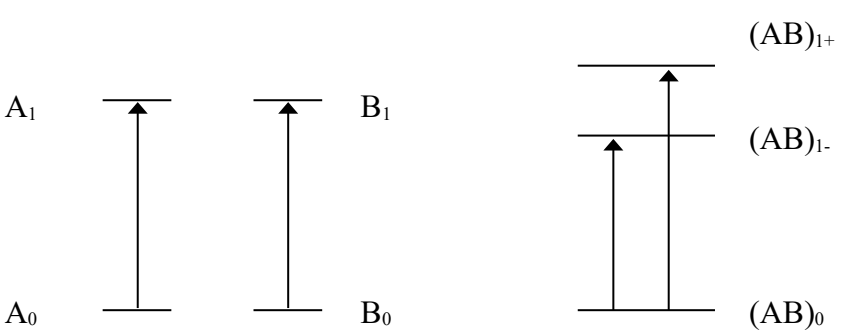
We have encountered the phenomenon of delocalization already before, when we discussed the vibrations of $CO_2$. In $CO_2$, the two CO stretching vibrations couple, which leads to two normal modes with different frequencies. Each of these normal modes consist of both CO stretching vibrations, meaning that the normal modes are

delocalized over the entire molecule. But the nature of the coupling is different for $CO_2$ and the amide vibrations. Whereas in $CO_2$, the two CO vibrations couple mechanically via the movement of the middle atom, the local amide I vibrations couple through space via electrostatic interactions. The coupled amide groups do not need to be connected by chemical bonds, or they can be very far away in the primary sequence.

The second effect of transition dipole coupling is that it changes the vibrational frequencies. Also this is a general consequence of exciton coupling: it leads to a change of the excited state energies. Remember, that the energy of the vibrationally excited states depends on the vibrational frequency. A change of vibrational frequencies can of course be observed in the vibrational spectrum. The change depends on the orientation and distance of the coupled amide groups because this determines the strength of the forces between the oscillators. For two coupled amide groups, two different energies are observed depending on whether the local amide I vibrations oscillate in phase (expand and contract at the same time) or out of phase (one contracts when another expands). The result is a splitting of the excited state energy - a phenomenon called *exciton splitting*.

The figure below illustrates exciton splitting. The left hand side shows the energy levels of two isolated (=uncoupled) amide groups A and B. They are assumed to have the same transition energy, meaning that their amide I vibrations have the same frequency. Only one absorption band will be observed in the vibrational spectrum because the transition energy is the same for both groups. When the two amide I vibrations couple (right hand side), we get two transitions with different transition energies; the excited state energy is split into two energy levels, which are located symmetrically above and below the energy level of the isolated system. The two different transition energies imply that the coupled system has two amide I normal modes with different vibrational frequencies (remember that the transition energy for harmonic oscillators is proportional to the vibrational frequency $\nu$). Therefore we will see two bands in the vibrational spectrum.

Also the phenomenon of energy splitting is familiar from the example of $CO_2$. In $CO_2$, coupling of the two CO stretching vibrations leads to two normal modes with different frequencies.




*Energy levels of two individual amide groups A and B (left) and of two coupled amide groups (right). The interaction results in an exciton splitting of the two excited states $(AB)_{1+}$ and $(AB)_{1-}$ where the excitation energy no longer resides on one amide group. 0 and 1 indicated ground state and first excited state, respectively.*

The third effect of transition dipole coupling concerns the probability of absorbing an infrared photon, in other words, the transition probability from the vibrational ground state to the first excited state of the vibration. The two transitions on the left hand side of the figure above have the same transition probabilities. However, the two transitions of the coupled system on the right hand side may have very different transition probabilities, depending on the relative orientation of the transition dipole moments. Thus, some delocalized vibrations may be much

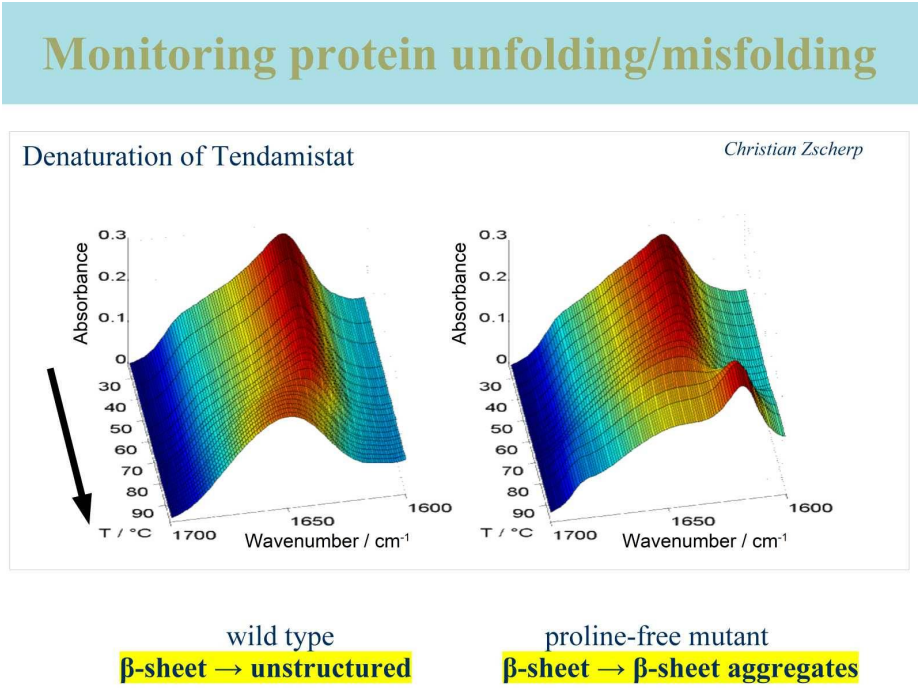
stronger absorbers of infrared light than the uncoupled vibrations, while other delocalized vibrations may absorb much weaker.

We have encountered also this phenomenon already in the discussion of $CO_2$. Each individual CO stretching vibration is associated with a change of dipole moment. But when they couple, only the antisymmetric stretching vibration absorbs infrared light, while the symmetric stretching vibration is infrared inactive.

*THE AMIDE I BAND - PRACTICAL EXAMPLES*

Protein unfolding/misfolding

The sensitivity of the amide I vibration to secondary structure makes it possible to study protein denaturation (unfolding) with infrared spectroscopy. The left hand panel shows the typical spectrum of a small β-sheet protein at low temperatures (top) with a main peak and a shoulder at higher wavenumbers. When the protein unfolds at high temperatures, the spectrum changes to a broad, featureless band which is characteristic of unordered structure.



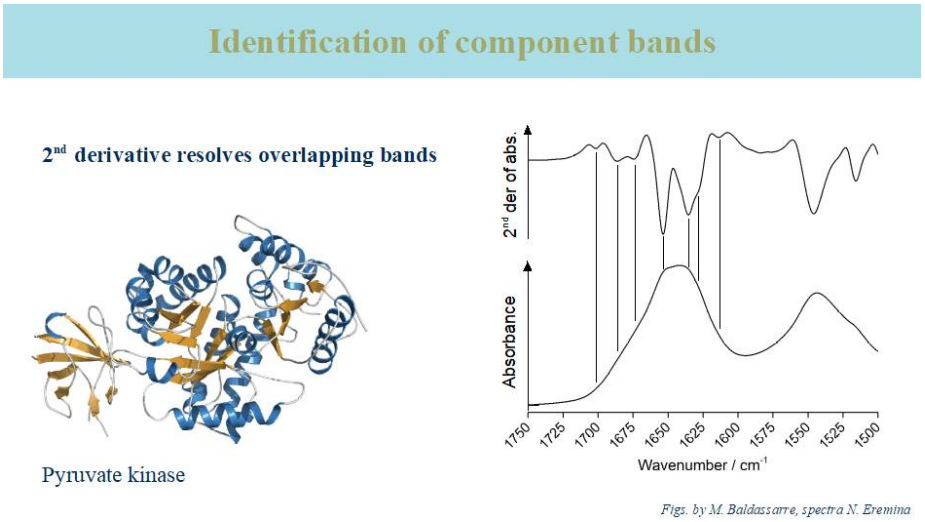

*Zscherp et al. Biochim. Biophys. Acta 2003*

The situation is different when the three Pro residues are replaced by Ala. The starting spectrum is still the same, a typical β-sheet spectrum. However, the final spectrum is very different. It has a very pronounced band at even lower wavenumber than the initial structure and a more pronounced high wavenumber shoulder. These features are again typical for β-sheets. Because of the low wavenumber of the main band, one can conclude that the sheets contain many strands or are very flat or both. This is typical for the intermolecular sheets of protein aggregates. Thus the replacement of the three proline residues leads to a dramatic change of the structure of the denatured state at high temperatures.

The latter example demonstrates that infrared spectroscopy can be used to monitor the aggregation of proteins. This is an active research field because protein aggregation occurs in neurodegenerative diseases and may be the actual cause of these diseases.

The second derivative reveals component bands within the amide I band

The amide I band is usually quite broad and components from different secondary structures are hard to identify. To reveal more information about the underlying component bands, the second derivative of the absorbance spectrum is often analyzed. The second derivative of a single absorption band has a minimum with negative amplitude at the position of the maximum absorbance and positive maxima on both sides of it. The negative band of the second derivative is narrower than the corresponding absorbance band. In a spectrum that consists of several component bands, the second derivative can therefore reveal the positions of the component bands better than the original absorbance spectrum. A disadvantage are positive side lobes that might be confused with real component bands in a complicated spectrum. The figure below shows an example. The component bands are more obvious in the second derivative spectrum than in the absorbance spectrum.

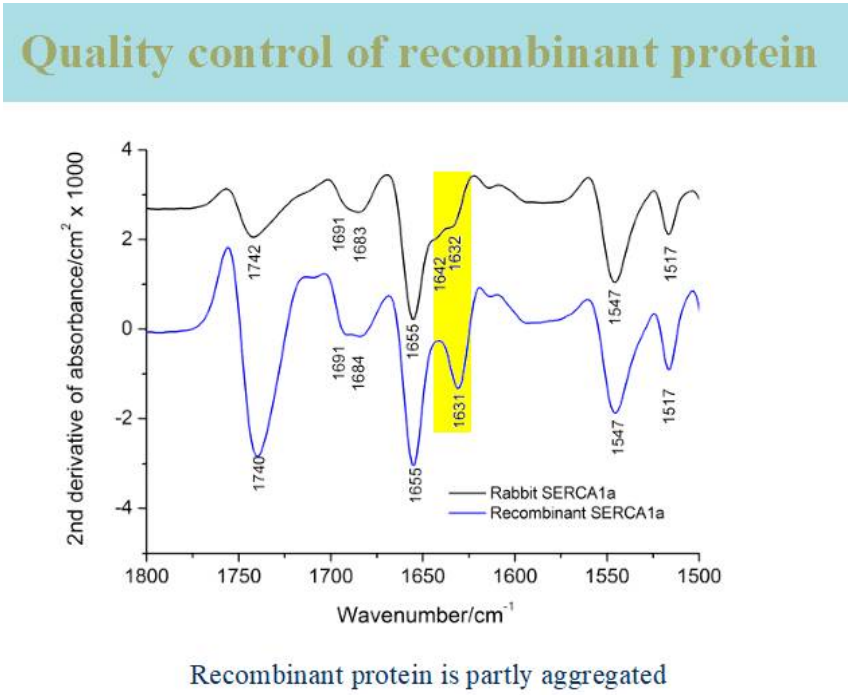



*Infrared absorbance spectrum of pyruvate kinase (bottom right) and spectrum of the second derivative of absorbance (top right). Component bands of the absorbance spectrum show up as minima in the second derivative spectrum. They are indicated by vertical lines.*

Quality control of recombinant protein

The amide I absorption can be used to check the quality of recombinantly expressed protein. The figure on the right shows second derivative spectra of the membrane protein $Ca^{2+}$-ATPase (SERCA1a). The black spectrum is the "gold standard", the spectrum from a rabbit muscle preparation. The blue spectrum is that of a recombinant preparation. The highlighted region indicates that the β-sheet structure of the two proteins is different. This is due to partial aggregation of the recombinant protein. Notable is also the stronger lipid absorption (band at 1740 $cm^{-1}$) of the recombinant preparation, indicating a higher lipid/protein ratio.

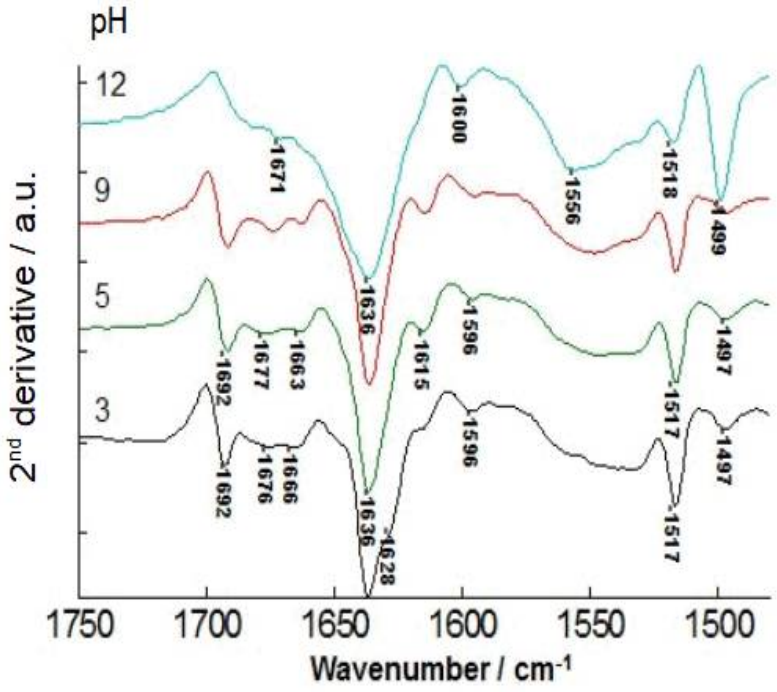
*Chenge Li et al. Analyst 2014*



Stability of protein drugs

Antibodies are increasingly used as protein drugs. To guarantee their intended medical effect, they need to be stable during transport and storage. Structural changes associated with *e.g.* temperature changes, pH changes, or freeze-thaw cycles can be studied in the amide I spectral range. The figure illustrates an example.

*The figure shows second derivative spectra of an antibody at different pH values. The spectrum at pH 12 is clearly different from the spectra at other pH values. Also at pH 3 there is a change at 1628 $cm^{-1}$, likely due to aggregation of the protein. Thus, the structure of this antibody is affected by the pH value.*




*Alejandro Arbesú Valdivia et al., Biologicals 2013*

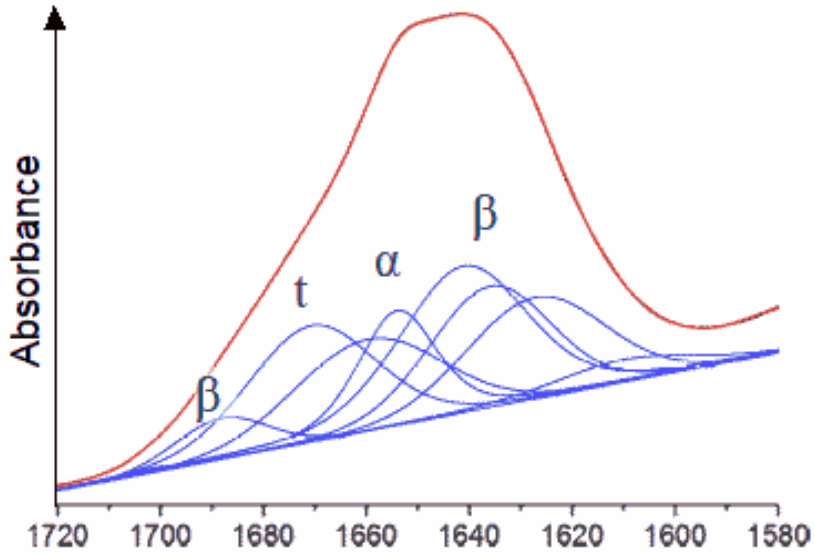
*SECONDARY STRUCTURE DETERMINATION*

The most common approach to analyze the secondary structure of proteins uses the following steps.

1. The amide I component bands are identified, for example from the minima in the second derivative spectrum. This provides the approximate band positions of the component bands.

2. Component bands are placed at the identified band positions and the absorbance spectrum is fitted. The band positions may or may not be allowed to vary during the fit. This provides the final positions, widths, areas, and shapes of the component bands.

*The figure shows a fit to the absorbance spectrum of pyruvate kinase (Baldassarre et al. Molecules 2015).*



3. From the final band positions, the bands are assigned to secondary structures using tables like the one shown earlier.

4. The band areas for each secondary structure are summed up and divided by the total area of all bands in the amide I region. This gives the secondary structure contribution of each secondary structure.

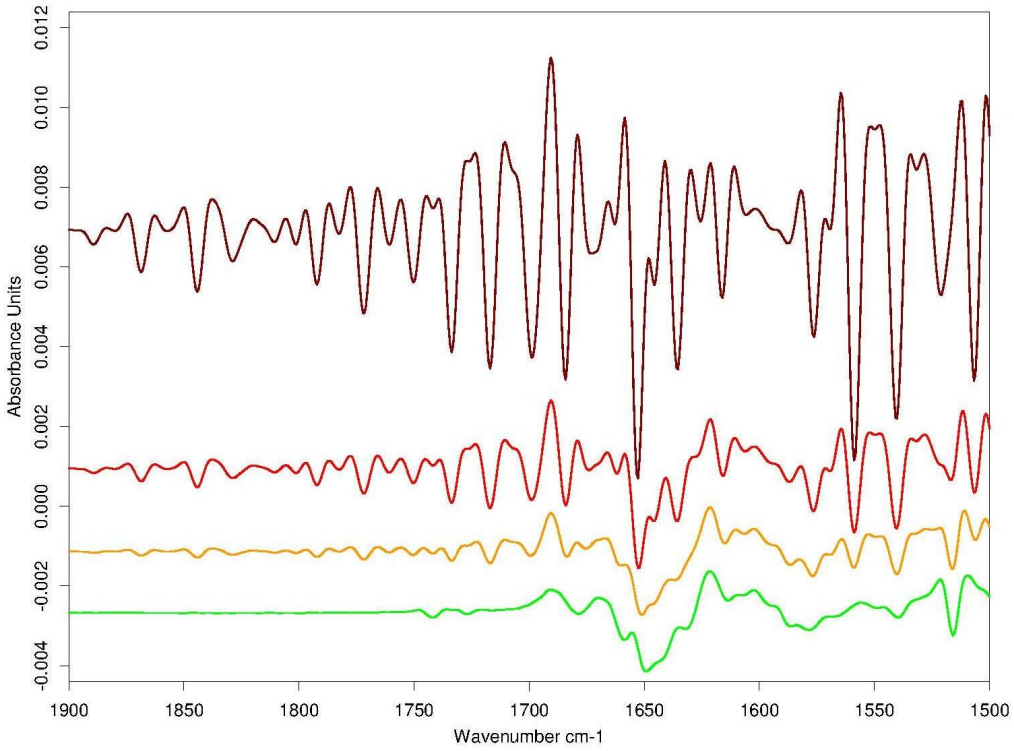
While these four steps seem to be straightforward, there are a couple of requirements, limitations, and problems associated with them. They will be discussed in the following.

*SECONDARY STRUCTURE ANALYSIS: REQUIREMENTS, LIMITATIONS, AND PROBLEMS*

Requirement: good quality spectra

The requirement of good quality data should be self-evident and applies to all research. Should you for example in your future career obtain data that are difficult to explain, then it is often better to spend time on a repeat of the measurement than to spend time on finding an explanation for the strange results. A repeat may simply show that something went wrong in the first experiment.

A major problem for the analysis of the amide I absorption is the presence of water vapor bands. They arise from the humidity of the ambient air inside the infrared spectrometer, not from the liquid water that might be present in the sample. Because water vapor is a gas, absorption of an infrared photon simultaneously causes a vibrational transition and a rotational transition. For each vibrational transition, there are many rotational transitions possible with different transition energies. Therefore water vapor generates a series of sharp absorption bands which are arranged in two branches which are centered around 1600 $cm^{-1}$. (We will not further discuss rotational transitions because they are not observed in the aqueous solutions of common biological samples.) The water vapor bands can easily be mistaken for protein bands (see the figure below) and therefore care should be taken to avoid them. It is possible to subtract water vapor bands from protein spectra to some degree, but it is much better to make a good measurement without water vapor bands in the spectrum. This can be done by flowing dry air or nitrogen gas (this is expensive) through the spectrometer, so that the water vapor content inside is reduced considerably. An additional possibility is to use a sample shuttle. A sample shuttle inside the spectrometer moves the sample in and out of the infrared beam. This makes it possible to measure the sample and the reference spectrum without opening the spectrometer. Otherwise, one would first measure the reference spectrum and then open the sample chamber, put in the sample, and measure its spectrum. This lets water vapor into the instrument, which first needs to be purged away before the sample spectrum can be measured.




*The figure shows four 2nd derivative spectra of the same protein sample with different contributions of water vapor. In the green spectrum, these contributions are very small and the spectrum reveals the component bands of the amide I spectrum between 1700 and 1600 cm-1. In the other spectra however, the sharp features from water vapor can easily be mistaken for protein features and they hide all protein features if there is too much water vapor contribution in the spectrum.*

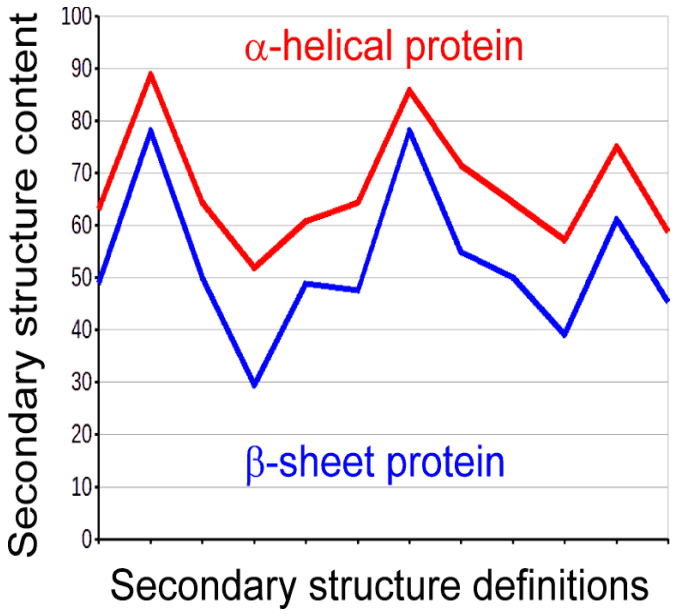
Note there is a small region around 1600 $cm^{-1}$, which is free of water vapor bands. Note also, that there are no bands in the protein spectrum above 1750 $cm^{-1}$. These two characteristics can be used to identify water vapor bands in infrared spectra of proteins: Sharp bands everywhere except around 1600 cm-1 indicated water vapor bands. In contrast, the absence of sharp bands above 1750 cm-1 demonstrates little impact of water vapor on the spectrum.

Limitations: secondary structure definitions

Secondary structure analysis tries to match experimental results with the concept of secondary structure. We all know what secondary structure is intuitively, but what is it really? The definition of secondary structure is not obvious, and several definitions have been proposed. They generate secondary structure contents that can deviate a lot from definition to definition - in some cases by more than a factor of 2. Even using the same definition may yield different secondary structure contents for different experimental structures of the same protein. It was checked, which of these definitions works well together with infrared spectroscopy and it was found that secondary structures assigned by the common DSSP method can be well predicted by infrared spectroscopy.

*The figure shows the secondary structure content of two different proteins obtained by 12 different definitions for secondary structure. The red curve shows the α-helix content of a helical protein and the blue curve the β-sheet content of a protein rich in β-sheets. The content of a specific secondary structure can vary by more than a factor of 2 between different definitions.*

*Data from De Meutter & Goormaghtigh Anal. Chem. 2020*




Limitations: only three types of secondary structure can be distinguished

Only the main secondary structures can be quantified from the amide I band. This was shown in an analysis of spectra from up to 92 proteins with know structure. Several statistical methods were applied and it was concluded, that only three different types of secondary structure can be distinguished (De Meutter & Goormaghtigh Anal. Chem. 2021, Wilcox et al. Biochemistry 2016):

- α-helix or ordered α-helix
- β-sheet or antiparallel β-sheet
- other (= neither α-helix nor β-sheet)

Other structures are hard to predict because they are rare and do not vary much throughout the data set.

Problem: Second derivatives

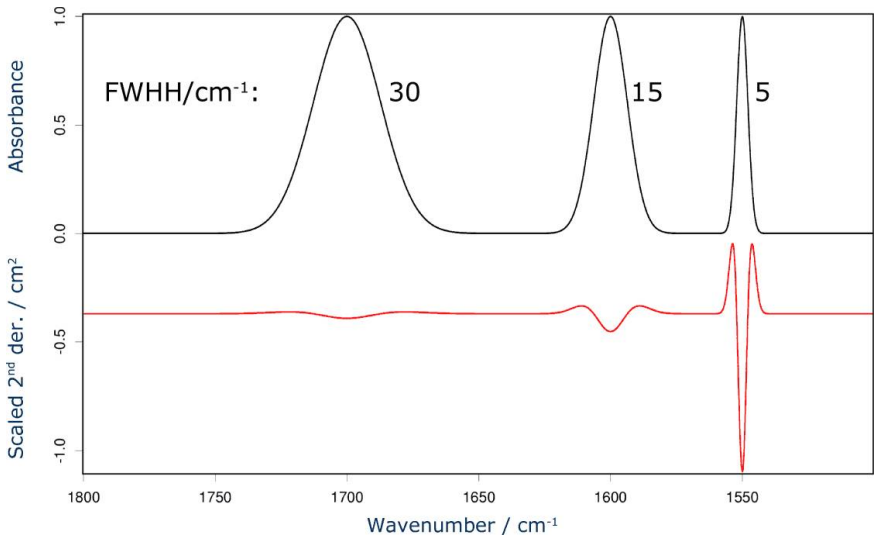
We have already encountered that second derivatives are useful to identify component bands that are otherwise hard to detect in the amide I absorption spectrum. However, one has to be aware of the following property: the second derivative enhances sharp features in the spectrum and suppresses broad features. This is shown in the figure below and has advantages and disadvantages.

1. The sharp bands of noise and water vapor are enhanced in the second derivative spectrum. Therefore, it is very important to start with spectra of good quality.

2. Broad bands from mobile or heterogeneous structures are less obvious in second derivative spectra.

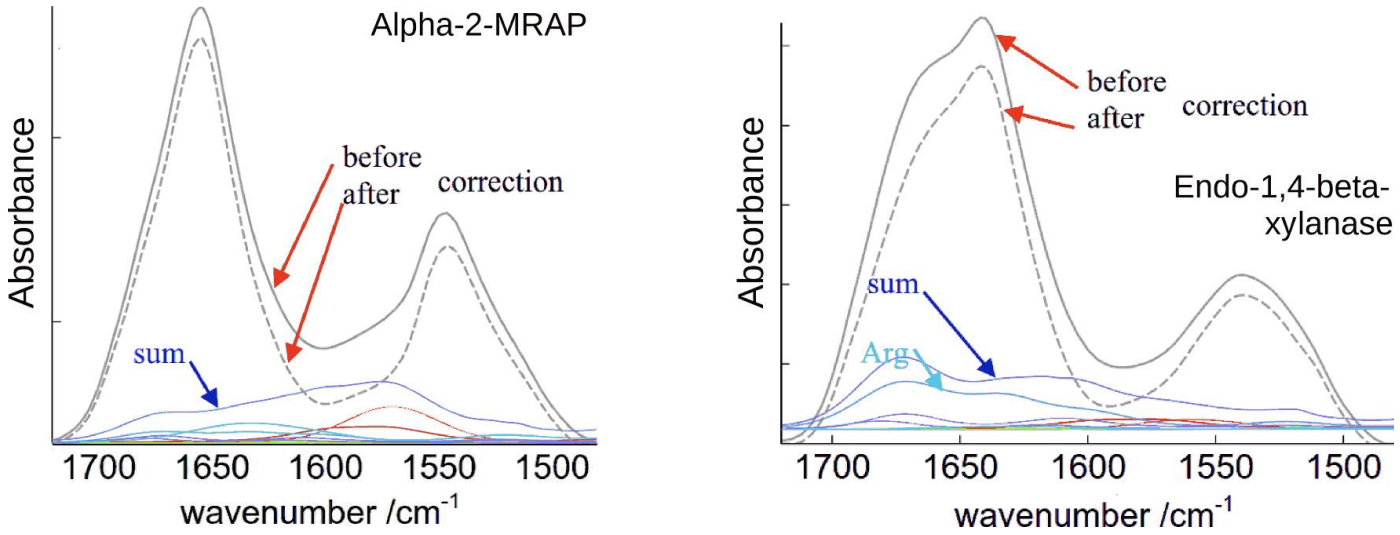
3. The strong absorption of water in the amide I range is also suppressed. Therefore small errors in the subtraction of the water absorption do not show in the second derivative spectrum.

*The black line shows a spectrum of three Gauss lines with different widths. The red line is the second derivative of the spectrum. While the maximum absorbance of the three Gauss lines is the same, the amplitude of the second derivative increases significantly with decreasing band width.*




Problem: Side chain absorption

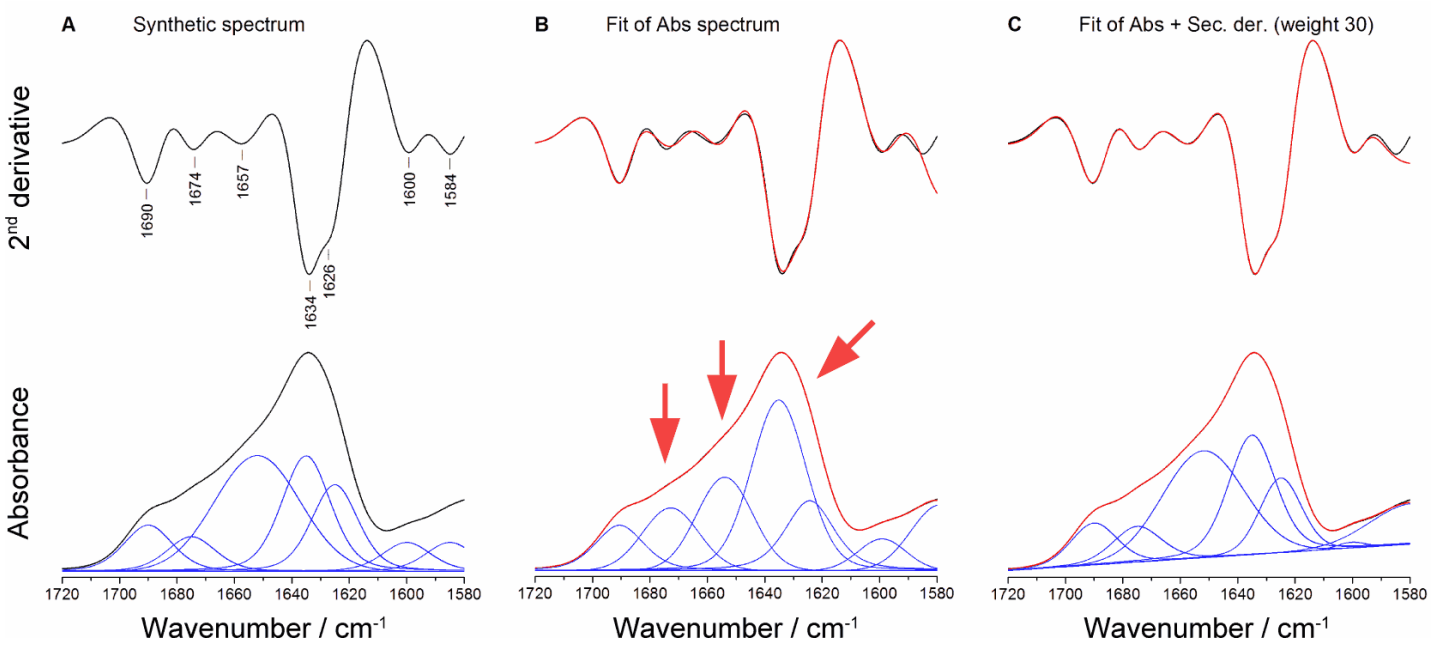
Some side chains absorb in the amide I region and contribute on average 20% to the integrated absorbance. The contribution varies substantially between different proteins, depending on their side chain composition. Also the shape of the side chain spectrum differs for proteins with different side chain compositions. This is shown in the figure below. However, the absorption of side chains does not impose a severe problem for secondary structure analysis and a subtraction of the side chain spectrum has little effect on the obtained result.




*Calculated side chain spectrum of two proteins. The side chain spectrum is the dark blue line that is indicated by the blue "sum" arrow. The spectra labeled with before and after correction show the protein absorbance spectra before and after subtraction of their side chain spectrum. The side chain spectrum is different for the two proteins because Arg contributes more to the right spectrum and Asp and Glu (orange and red curves) less. (Modified from De Meutter & Goormaghtigh Eur. Biophys. J. 2021)*

Problem: The fit may be ill-defined

Now we come to a problem that may have rather severe consequences: A fit to the absorption spectrum is often not very well defined because the spectrum is rather featureless. This is particularly problematic, when many component bands are fitted. The following example shows what can go wrong and that you should not blindly believe a fit however good the result looks.




*The figure shows absorbance (bottom) and second derivative spectra (top). The starting spectrum is shown on the left in panel A. It is an artificial spectrum where the component bands are exactly known. Panel B shows a fit to only the absorbance spectrum. Surprisingly, the original set of component bands is not reproduced by the fit, which demonstrates that the best fit is not well defined. Deviations between the fitted set of component bands (panel B) and the original component bands (panel A) are indicated by arrows. Simultaneous fitting of absorbance and second derivative spectrum, shown in panel C, reproduces the original component bands much better. However, even this procedure is no guarantee to obtain the correct fit.*

The example shows that quite different sets of component bands produce very similar spectra. Small deviations between fit and original spectrum may therefore indicate a rather large mistake in the set of fitted component bands. It is better to consider all available spectral information in the fitting, which means that the second derivative should also be fitted.

Problem: The assignment of component bands to secondary structure is not unique

As already mentioned, the spectral regions of α-helices and other structures overlap. Another ambiguity concerns hydrated helices, the absorption of which overlaps with that of β-sheets. The reason are additional hydrogen bonds between their carbonyl oxygens and water, which lead to a downshift of their amide I band. This is

an example for the effect of hydrogen bonding on the wavenumber of amide I vibrations. As a result of these ambiguities, there may be several possibilities to assign a given component band to a particular secondary structure, which may lead to an erroneous interpretation.

Problem: The band area may be different for different secondary structures

In the final step of the analysis, the band areas of the different component bands are compared in order to calculate the percentage for each secondary structure. This assumes that the band area per residue is the same for all secondary structures, which might not be the case.

*SUMMARY AND EVALUATION*

In summary, we have discussed that

- the quality of spectrum is important. In particular, there should be no signals from water vapor
- there are several definition of secondary structure, which give deviating results
- only three main types of structure can be distinguished (α-helix, β-sheet, other)
- the second derivative suppresses broad bands, which has advantages and disadvantages
- side chains contribute about 20% to the absorbance in the amide I region. However, this does not seem to be a problem for secondary structure analysis
- fitting the absorbance spectrum can be ambiguous
- the assignment of component bands to secondary structures can be ambiguous
- the band area (integrated absorbance) may differ for different types of structure

Nevertheless, secondary structure analysis works quite well in practice, when applied carefully. The average deviation from the "true" secondary structure was reported to be 4-12 percentage points (Byler & Susi 1986, Lee et al. 1990, Dousseau & Pézolet 1990, Baumruk 1996, Baello et al. 2000, Wilcox et al. 2016, De Meutter & Goormaghtigh Anal. Chem. 2021). What this means is the following: when an α-helix content of 30% is predicted and an error of ± 10 percentage points is assumed, the true value is between 20 and 40%, not between 27 and 33%. While most researchers use the band fitting approach, recent studies on the accuracy of the method used statistical methods. So it is not quite clear how good the band fitting approach is.

We will encounter a second spectroscopic method to estimate the secondary structure content: circular dichroism (CD). Generally, infrared spectroscopy and CD give similar results, but CD is better for α-helix prediction, whereas infrared spectroscopy is better for β-sheets (Sarver & Krueger 1991, Pribić et al. 1993, Baumruk 1996, Baello et al. 2000). The combination of both gives the best results (Pribić et al. 1993).

When analyzing the secondary structure of a protein under different conditions, the changes between the secondary structures under the different conditions can probably be determined more accurately than the absolute values of secondary structure content.

On the other hand, unusual structures will probably remain unnoticed and lead to wrong results. For example, not considering the possibility of hydrated helices will attribute all absorption in the lower wavenumber range to β-sheets, which might then be overestimated.

Does all this sound complicated? Maybe yes. On the other hand, it makes secondary structure analysis more interesting because it requires some thinking. There is nothing more boring than a method that can be applied without using one's brain.

*BEYOND SECONDARY STRUCTURE*

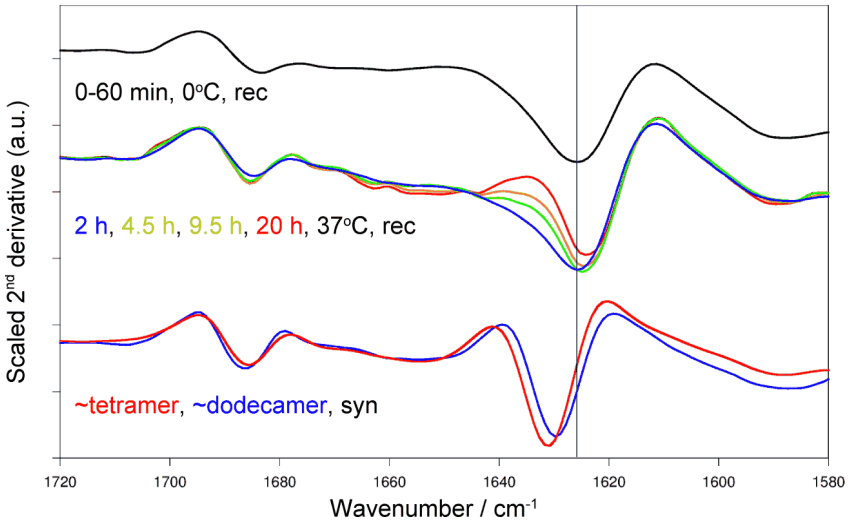
In the beginning I mentioned that the amide I band is shaped by hydrogen bonding and the coupling between local amide I vibrations. The latter makes, that the amide I absorption is sensitive to more structural features than just secondary structure. This is discussed in the following. There is no point for you to be able to reiterate all these structural influences in detail, but you should appreciate the sensitivity of coupled vibrations to a number of structural features.

Influences beyond secondary structure:

1. Formation of α-helix bundles causes an upshift of the amide I band because vibrations in adjacent helices couple with each other. Helix bundles are common structures in membrane proteins.

2. The wavenumber of the main β-sheet band shifts down when more strands are added to the sheet and when it becomes more planar because this enables more and stronger coupling of the amide I vibrations. We have encountered this property already in the discussion of tendamistat denaturation, where it was mentioned that intermolecular β-sheets of protein aggregates absorb at lower wavenumbers than the intramolecular β-sheets of globular proteins because they are larger and/or flatter.

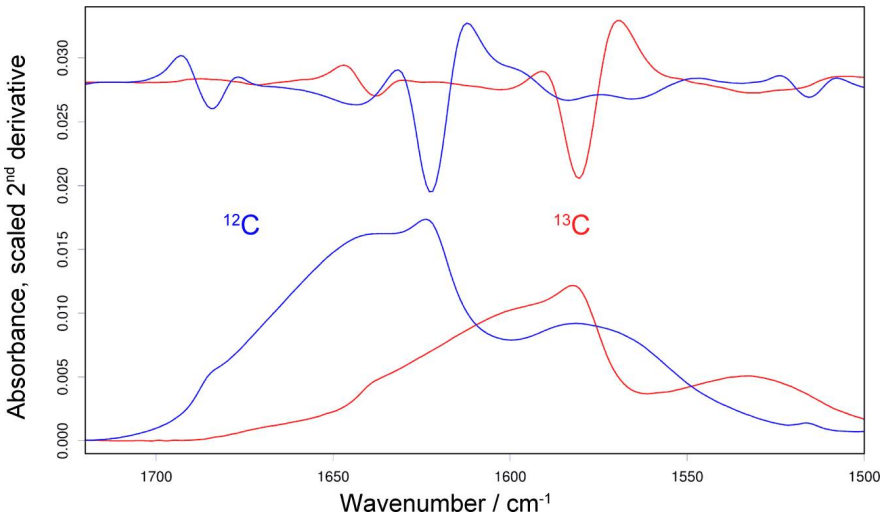
The size effect inferred from spectrum calculations can also be seen in experiments. The example here are our studies of the aggregation of the amyloid-β (Aβ) peptide, which is important in Alzheimer's disease and may be one of its causes. Aβ oligomers of different sizes exhibit their main β-sheet band at different wavenumbers. The larger the oligomer, the lower the band position. In kinetic experiments, the band shifts down with time and becomes narrower. This indicates that the population of β-sheet structures becomes less heterogeneous with time.




*The figure shows second derivative spectra of the Aβ peptide to illustrate the effect of β-sheet size on the spectral position of the main β-sheet band near 1630 $cm^{-1}$. The bottom spectra are for two oligomers prepared in detergent. They are relatively stable over time. The red spectrum is for an oligomer that consists of ~4 peptide molecules, the oligomer of the blue spectrum consists of ~12 peptides. The larger oligomers absorb at lower wavenumber. In a time-resolved experiment in the absence of detergent (top spectrum), the amide I band is initially at even lower wavenumber and shifts further down with time. After 20 h the oligomers consist of ~30 peptide molecules. Also the band becomes narrower with time on its high wavenumber side because there is less structural heterogeneity at later times. Data from Vosough & Barth, Chem. Neurosci. 2021*

3. Stacking of β-sheets, *i.e.* the formation of β-sheet layers, shifts the amide I absorption to higher wavenumbers because vibrations in different sheets are coupled. Such β-sheet layers are formed in amyloid fibers, which are involved in a number of diseases.

Finally, I want to show you that $^{13}$C-labeling is a very powerful approach as a consequence of vibrational coupling and of the mass effect. $^{13}$C-labeling shifts the entire amide I spectrum down by about 40 $cm^{-1}$ because of the heavier mass of the $^{13}$C-isotope compared to the ordinary $^{12}$C-isotope. This is shown in the figure below.

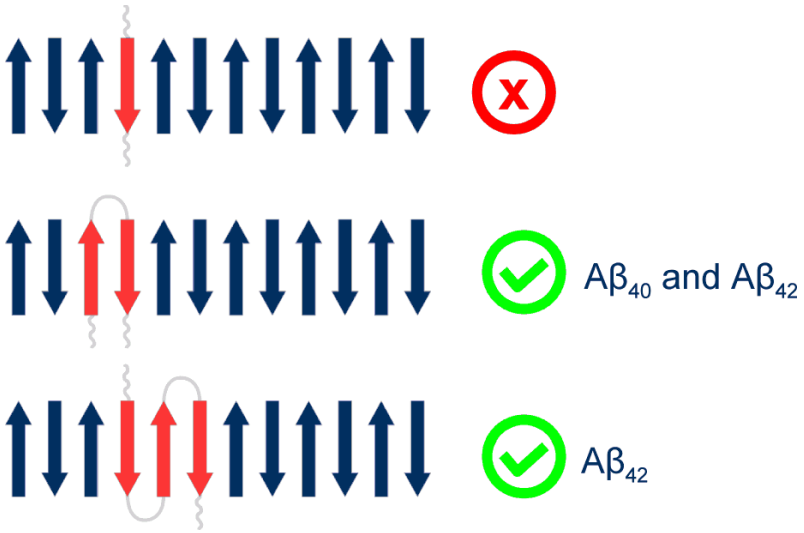



*Absorbance and second derivative spectra of unlabeled ($^{12}$C) and $^{13}$C-labeled Aβ.*

One of the obvious advantages of the isotope shift is the possibility to analyze the structure of both the labeled and the unlabeled protein or peptide in the same experiment. This can be used to study the interaction between two proteins or peptides. When they form a complex, they might undergo conformational changes. When one of them is labeled, one can characterize the conformational change of each interaction partner.

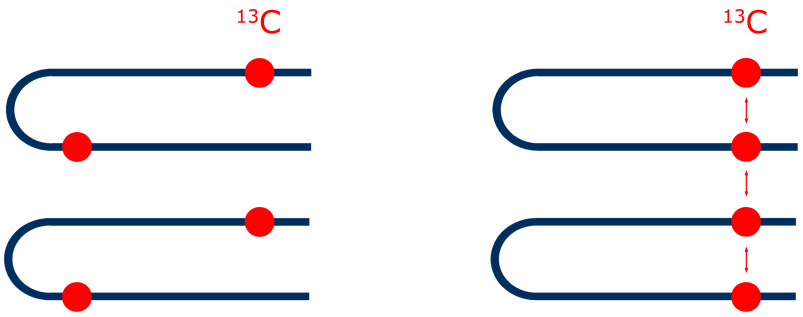
Another advantage is that $^{12}$C- and $^{13}$C-groups do not couple much with each other. As mentioned already, coupling is strongest, when the local amide I vibrations have the same frequency. Because of the mass effect, the $^{13}$C-groups vibrate slower and couple only very little with the $^{12}$C-vibrations. Instead, they disrupt the vibrational coupling between the $^{12}$C-groups. As a consequence, mixing of $^{12}$C- and $^{13}$C-peptides in β-sheets can be detected: it affects the position of both the $^{12}$C- and the $^{13}$C-band. We have used this for example to show that the two main variants of the Aβ peptide with 40 and 42 residues ($A\beta_{40}$ and $A\beta_{42}$, respectively) form common β-sheets where the two variants are randomly or nearly randomly mixed.

When labeled and unlabeled versions of the same peptide are mixed, they form homo-oligomers. At low concentrations of labeled peptide, the labeled peptide will be surrounded by unlabeled peptides. The $^{13}$C-amide groups can then only couple with other $^{13}$C-groups in the same peptide. This effect can be used to analyze the structure of individual peptide molecules within an oligomer and we have found that each Aβ42 and Aβ40 molecule contributes 2 or more adjacent strands to oligomer β-sheets (Baldassarre et al., Chem. Sci. 2017, Baronio et al., PCCP 2019).




*Structural models for the internal structure of individual Aβ molecules in oligomers. β-strands from labeled molecules are shown in red and strands from unlabeled molecules in blue. The single strand model does not fit with the experimental data. The data show instead that each peptide molecule contributes two or more adjacent strands to the β-sheets of the oligomers.*

More detailed structural information can be obtained when specific amide groups are $^{13}$C-labeled. They do not couple, when they are distant in the three-dimensional structure. When they are close however, they couple which leads to a change in the spectrum. In this way, closeness between specific amide groups can be detected, which is analogous to contact information from nuclear magnetic resonance spectroscopy.




*Structural information from site-specific $^{13}C$-labeling. Left: the labeled sites are distant and there is no vibrational coupling between them. Right: the labeled sites are close and there is intra- and intermolecular vibrational coupling between them (indicated by the arrows). This changes the spectrum and therefore, it can be detected whether the $^{13}C$ groups are close or not.*

Summarizing this part, vibrational coupling makes the spectrum sensitive to many structural properties, like the number of strands and their twist in β-sheets. In addition, it makes the spectrum sensitive to the three-dimensional assembly of secondary structure elements. Vibrational coupling can be further exploited when combining it with the mass effect of $^{13}C$-labeling. This provides further information on the structure. It enables to analyze the structure of individual mixture components, to detect strand mixing in β-sheets, and to detect contacts between specific groups.

## Application 2 - Information on protein reaction mechanisms: reaction-induced infrared difference spectroscopy

*OVERVIEW*

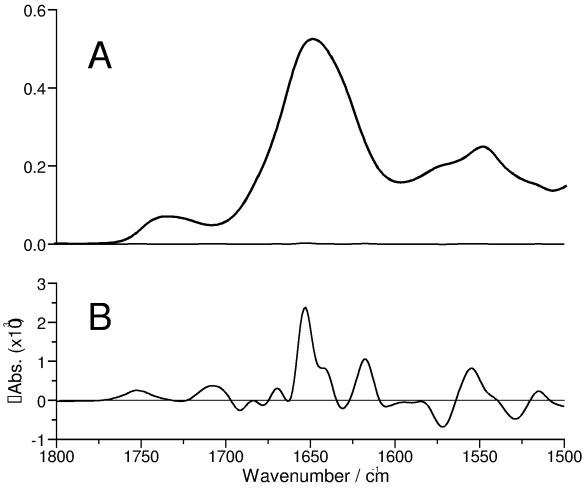
The following section discusses another major application of infrared spectroscopy in protein science: reaction-induced infrared difference spectroscopy.

*AIM*

In reaction-induced infrared difference spectroscopy the aim is to elucidate the molecular mechanism of proteins: Here infrared spectroscopy is at its best, since the method combines the high time-resolution and the high information content. We will see that infrared spectroscopy offers a very detailed and sensitive look into the catalytic heart of working enzymes. The sensitivity is high enough to detect environmental changes around single atoms in a large protein. An example of this are changes in hydrogen bonding. Other molecular events that can be recorded are the protonation of single residues or changes in the redox state of cofactors. Usually a protein reaction of interest is triggered (initiated) in the infrared cuvette and spectra before, during and after the reaction are recorded. From these spectra, difference spectra are calculated that reflect only the changes of infrared absorbance: "active" protein groups will show up, while "passive" groups cancel in the subtraction.

*PROBLEM*

A problem of infrared spectroscopy is the strong absorption of water in an interesting spectral region (amide I range: ~1640 $cm^{-1}$). This requires short pathlengths in $^1H_2O$ (< 10 µm) and $^2H_2O$ (50 µm) and therefore relatively high sample concentrations in order to observe single groups (0.1 to 1 mM protein). Also, adding a liquid to an existing sample is not as comfortable as for example in visible spectroscopy since the infrared cuvettes are usually closed and have to be opened and reassembled for the next measurement. When studying protein reactions, one is often interested in comparing spectra of a protein in different states. This would require a spectrum in state A, dis- and reassembly of the cuvette and recording of a second spectrum in state B. This approach is not sensitive enough in most cases because of changes in the pathlength, air bubbles in the sample, slight changes in concentration etc. The problem is illustrated in the figure below.




*(A) The bold line is the absorption spectrum of a membrane protein ($Ca^{2+}$-ATPase) in $^2H_2O$ (lipid C=O near 1740 $cm^{-1}$, amide I near 1650 $cm^{-1}$, unexchanged amide II and side chains at 1550 $cm^{-1}$). The $Ca^{2+}$-ATPase uses the energy of ATP to pump $Ca^{2+}$ ions against the $Ca^{2+}$ concentration gradient. So, there are many conformational changes when this protein is active. Nevertheless, the thin line are the largest absorbance changes that occur during the catalytic activity of this enzyme. It is a difference spectrum between two protein states. Only when the scale is blown up by a factor of 100 (panel B) it can be seen that the difference spectrum is not just a straight line, but contains a lot of information.*

Such small absorbance changes cannot be detected by comparing the absorbance spectra of different samples. Therefore special techniques are needed. They are based on triggering the protein reaction of interest in the infrared cuvette in order to avoid opening the cuvette and changing the sample. They allow to detect very small absorbance changes, down to environmental changes of single amino acids in large proteins or protein complexes (5000 amino acids). Some of the techniques enable time-resolved studies with a time-resolution of µs with moderate experimental effort. These techniques are the basis of reaction-induced infrared difference spectroscopy and are briefly reviewed in the following.

*DIFFERENCE SPECTROSCOPY AND DIFFERENCE SPECTRA*

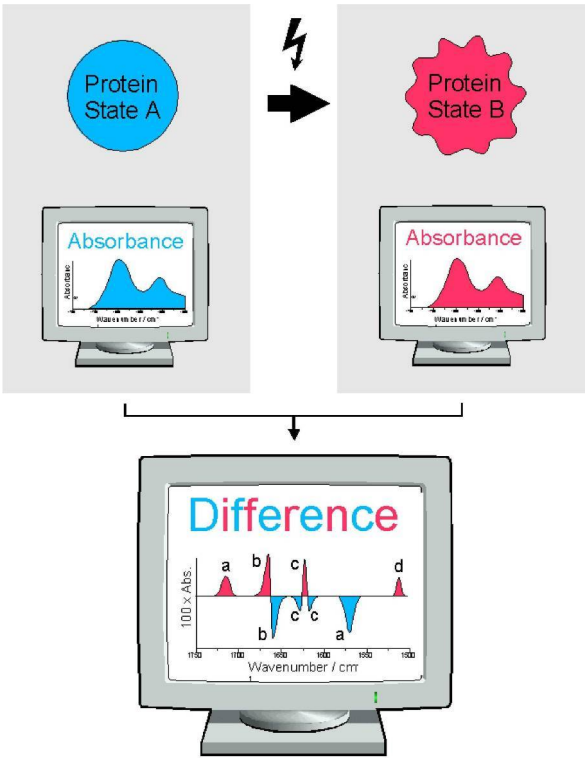
The Fig. below illustrates how a typical reaction-induced difference spectrum is generated. The protein is prepared in the stable state A and the absorbance of this state is measured. Then the reaction is triggered, the protein proceeds to state B and again the absorbance is recorded. State B may also be a sequence of transient states.

In that case the interconversion between the product states $B_1$, $B_2$, etc. can be followed by time-resolved methods.

From the spectrum recorded before the start of the reaction - state A - and the spectra recorded during and after the reaction - state(s) B - difference spectra are calculated. They originate only from those protein residues that are affected by the reaction. All “passive” residues have the same spectrum in both states and their contribution cancels when the difference spectrum is calculated. Therefore, “passive” residues are invisible in the difference spectrum which, therefore, exhibits details of the reaction mechanism on the molecular level despite a large background absorption of the passive groups. As indicated in the idealized difference spectrum in the Fig., the convention in the research field is that negative bands in difference spectra are characteristic of the initial state A, while positive bands reflect the state(s) B during or after the reaction.

Difference bands arise for several reasons and four examples are given in the figure below. Chemical reactions transform molecular groups from the reactant form to the product form, which usually have different infrared absorbance spectra. An example is the protonation of an Asp or Glu residue. In the difference spectrum of the reaction, the absorbance of the disappearing reactant group shows as negative bands, while the absorbance of the product groups give rise to positive bands. The bands of the appearing and disappearing groups may be widely separated in the spectrum. In the figure this is illustrated with the two difference bands marked “a” for the protonation of a carboxylate group. The negative band marked “a” is due to the antisymmetric stretching vibration of the $COO^-$ group that disappears in the course of the reaction and the positive band marked “a” is due to the stretching vibration of the C=O bond of the appearing COOH group. There are further bands due to this reaction but they are outside the spectral range shown.

Alternatively, a vibration may experience a shift in frequency, due to a conformational or environmental change that alters the electron density of the vibrating bonds or the coupling with other vibrations. This band shift leads to a pair of signals, composed of a negative and a positive band which are close together. An example is shown for the two bands marked "b" in the amide I region of polypeptide backbone absorption. Here, the amide I vibration absorbs at lower wavenumber (i.e. has lower vibrational frequency) in the initial state A than in the product state B. Thus there is a negative band at lower wavenumber and a positive band at higher wavenumber. In the case of the amide I vibration of proteins, band shifts can be ascribed to an altered coupling with neighboring amide oscillators due to a change in backbone structure or due to a different degree of hydrogen bonding which changes the electron density in the C=O bond.




A difference band with side lobes of opposite sign is produced when the width of a band changes in the reaction from state A to B. The case where the band width decreases is shown in the Fig. for the bands marked with "c". In this case, the intensity will decrease on the sides of the band but will increase at the center (if the absorption coefficient remains constant) leading to a positive band with negative side lobes. As the band width is a measure of conformational flexibility, the decrease of band width shown indicates a more rigid structure in the product state B.

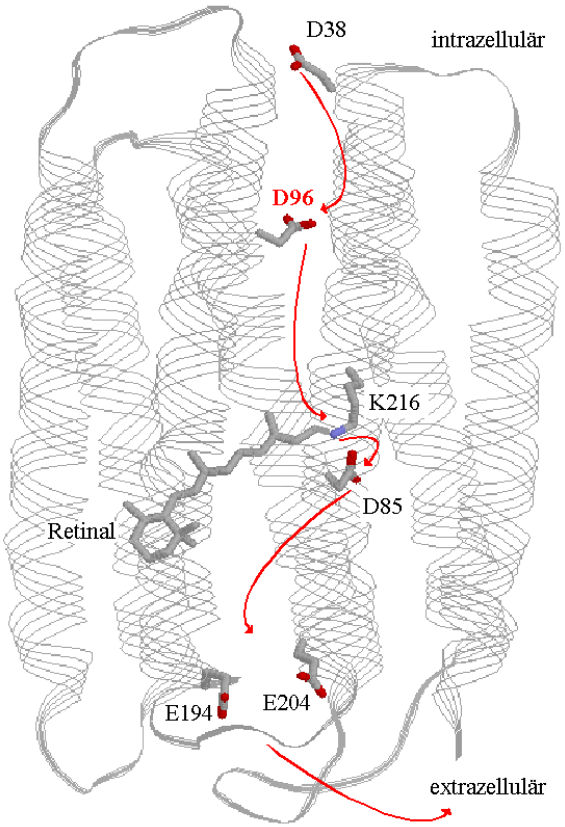
Only one band is observed when the reaction results in a change of the absorption coefficient of a vibrational mode, for example because of a polarity change of the vibrating bond(s). A negative (positive)band in the difference spectrum then indicates a reduced (increased) absorption of the product states B as compared to the initial state A. This case is illustrated with the band marked "d" at a spectral position that is characteristic of Tyr absorption. In the case shown, the increased absorption coefficient of Tyr in state B may be due to an environmental change that leads to an increased polarity in the Tyr ring.

*HOW TO INITIATE REACTIONS IN THE INFRARED SAMPLE*

Light-induced difference spectroscopy

Protein reactions that are initiated by light have been the first to be investigated by reaction induced infrared difference spectroscopy. The protein equilibrates in the dark in the spectrometer, then it is illuminated and the transition from the "dark" state into the light adapted state can be investigated. This can also be done time-resolved by using a short light flash. The number of proteins that can be investigated by this method is of course limited but it is very valuable for photosynthetic proteins and for bacteriorhodopsin.

*[Christian Zscherp]*



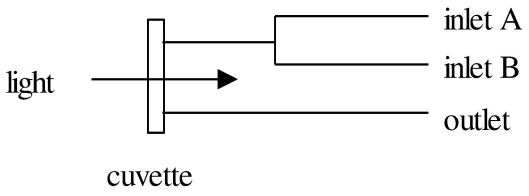

*Proton transport in bacteriorhodopsin. The reaction cycle consists of the following steps:*
*1.) Isomerization of the retinal from all-trans to 13-cis in 0.5ps (BR→ J intermediate).*
*2.) Proton moves from the Schiff base C=(NH)$^+$-Lys216 to Asp85. A proton from Glu194 or Glu204 acidifies the extracellular medium. (L intermediate→ M intermediate)*
*3.) A proton moves from Asp96 via water molecules to the Schiff base, relatively large conformational changes (M→N)*
*4.) Asp96 reprotonates with a proton from amongst others Asp38. Retinal isomerizes back to the all-trans state (N→O).*
*5.) Proton moves from Asp85 via a proton channel to Glu194 or Glu204 (O→BR).*

Bacteriorhodopsin is one of the proteins most intensely studied by infrared spectroscopy. It is a small integral membrane protein which converts light energy into a transmembrane proton gradient. The proton gradient is converted by the ATP-synthase into ATP. The protein contains the chromophore retinal which is also present in our retina. After absorption of a photon, retinal in BR changes its conformation (from all *trans* to 13 *cis*) which starts a cascade of reaction steps which lead to the transport of one proton across the membrane. This involves changes in secondary structure as well as protonation and deprotonation of several groups (including D85, D96, and the Schiff base which links retinal to K216). The photoreaction proceeds via several intermediate states named BR (ground state), J, K, L, M, N, and O. Fortunately, high-resolution structures of the ground state as well as of some intermediate states are available now. However, many fundamental facts concerning the function of bacteriorhodopsin as a proton pump, namely the identification of the critical proton donor and acceptor

groups and the determination of their protonation state in the different intermediates, have been elucidated already several years ago. Since infrared difference spectroscopy is able to directly observe protonation changes of single amino acids, this method played a dominant role in this context.

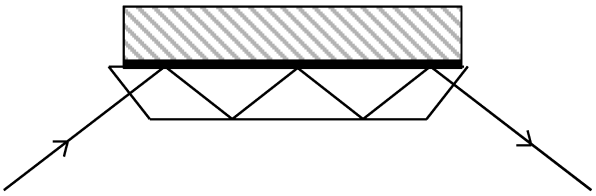
Stopped-flow

Many proteins bind molecules or ions. Upon addition of such compounds, protein reactions may therefore be initiated. One approach to do this is rapid mixing of solutions and this been used for a long time in UV/vis spectroscopy. The principle is shown in the figure: 2 solutions are pressed into a mixing chamber, flow into the cuvette and replace the existing solution. Mixing starts the reaction which can then be followed by spectroscopy. With this technique a time-resolution in the ms time range can be achieved which is limited by the mixing time. The small pathlength of infrared cuvettes makes the application of this technique for infrared spectroscopy very difficult since high pressure is needed which causes leak problems and the high shear forces might denature some proteins. However cuvettes have been developed first for $^{2}H_2O$ and recently for $^{1}H_2O$ which are suitable predominantly for soluble proteins. A disadvantage of the method is the high consumption of material since all tubes have to be filled with concentrated protein solution. Current developments try to minimize the volume needed.



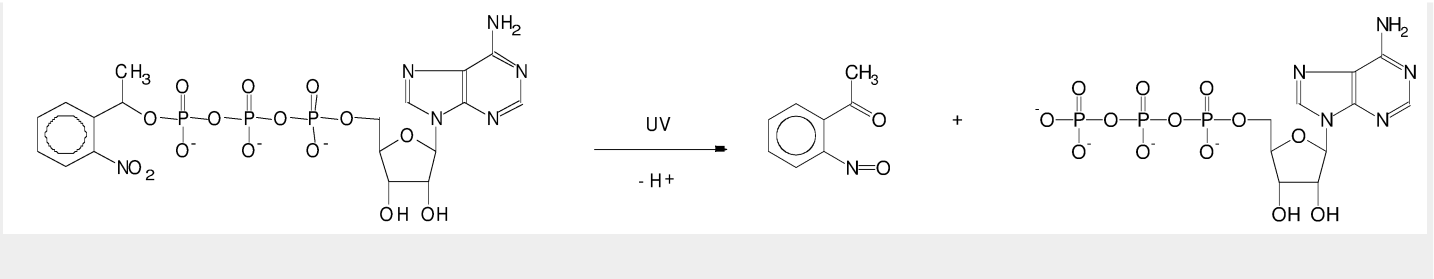

Attenuated total reflection (ATR)

The figure below illustrates the ATR technique. A sample film (black) is prepared on an IR transmittant crystal (white, this is usually only possible for membrane proteins). A buffer is placed on top of the film (hatched). The angle of incidence of the IR radiation is such that the IR light is totally reflected in the crystal (like in a light guide). Upon reflection, light penetrates the sample film and may be absorbed there. Therefore the light that reaches the detector carries the information about the IR spectrum of the sample film. The penetration depth is on the order of the wavelength which means that the optical thickness of the sample is small enough for measurements of aqueous films. If the protein film is thick enough, the additional buffer layer does not influence the measured spectrum because the beam does not penetrate such far into the sample. The advantage of the method is that the buffer often can be exchanged without disturbing the film. This enables many sample manipulations and makes the method quite flexible. The disadvantage is that the preparation of the film is often difficult and sometimes impossible. Sometimes the film detaches when the buffer is exchanged. Kinetic measurements are possible only for slow reactions (slower than minutes).



Photochemically induced concentration jump

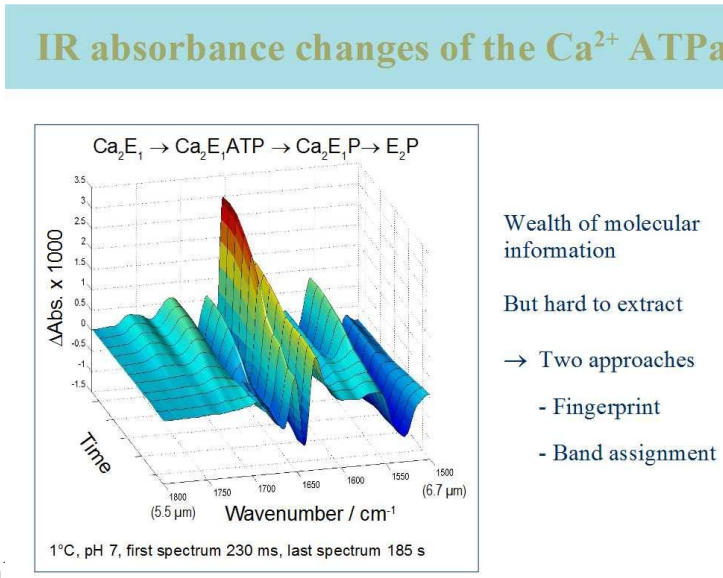
Two methods to add compounds to a protein sample have been mentioned already, the stopped flow and the ATR techniques. A 3rd method is the release of biologically relevant compounds from photolabile derivatives. These so called "caged compounds", are designed to be biologically "silent" in the dark, which means that they do not interact with the protein of interest, but cleave off a biologically active compound upon UV illumination. The archaetype of these compounds is caged ATP. ATP is stored chemical energy and required for many protein reactions. The important terminal phosphate group is protected in caged ATP and therefore caged ATP cannot be used as a substrate by enzymes.




*Photolysis of caged ATP: Upon absorption of UV light caged ATP photolyses into ATP and a side product.*

*INTERPRETATION*

The figure on the left shows the result of a reaction-induced infrared difference spectroscopy experiment. The difference spectra were obtained in a time-resolved experiment. In this example there are three transient states, a first one seen already in the first spectrum, a second that produces minor changes, which are hard to see and a third that produces relatively large changes on a slower time scale. At even longer times, the enzyme returns to the initial state and all difference bands decay to zero.



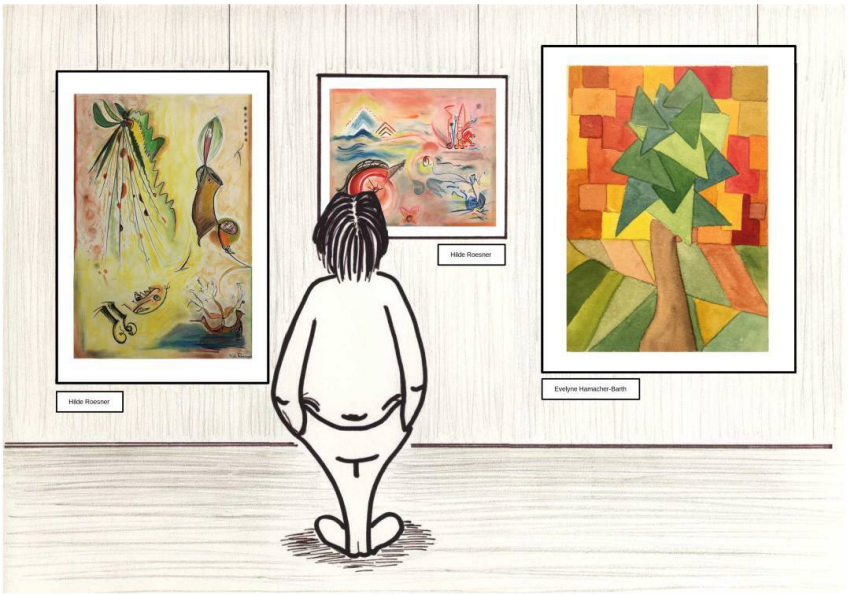

These spectra contain a lot of information, about a task. However, how are we going to interpret these kind of difference spectra? One option is very simple: one can regard the spectra as a fingerprint of the conformational change. This is explained in the next section.

*FINGERPRINT INTERPRETATION OF SPECTRA*

Strategy

Unfortunately, a spectrum at first sight is often not very meaningful. This reminds me of my last visit in a museum of modern arts:

But without a profound arts background I have no other option than to resort to very simple comparisons. For example I can compare the size the paintings and realize that there are two paintings with nearly equal size, whereas the third is a lot smaller. I can also compare the style: two paintings are similar in style, whereas the third one is different. And if I am a very patient observer, I may even notice that with time some of the paintings are exchanged for others.




You may wonder, what has this to do with infrared spectroscopy? These simple comparisons can also be applied to difference spectra. The spectra are then regarded as a fingerprint of the conformational change. The most meaningful spectral region for this approach is the amide I region, which is sensitive to backbone conformational changes. The amide I signals can then be analyzed according to their magnitude, time course and similarity, that is just the same simple comparisons as in the museum. In fact, any spectroscopic signal can be used for a fingerprint approach. Thus this approach has a long tradition for example in the interpretation of fluorescence experiments, where changes in the fluorescence emission were used to define high and low fluorescent states. In comparison, infrared spectroscopy provides more information because only the amide I region contains around 6 different bands which can be positive or negative, whereas fluorescence often analyses just one spectral feature, the intensity of the emitted light.

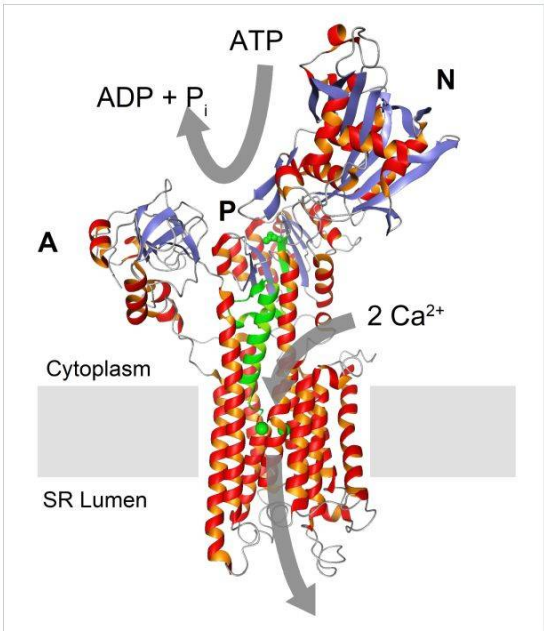
I will illustrate this interpretation approach with one example where the simple museum approach has lead to information on the molecular level. But before, the object of the study will be introduced.

$Ca^{2+}$-ATPase

The nanopump $Ca^{2+}$-ATPase pumps two $Ca^{2+}$ ions at the expense of the hydrolysis of one ATP molecule. The $Ca^{2+}$-ATPase has a maximum extension of about 100 Å and consists of a single subunit composed of 1000 amino acids. It has ten transmembrane helices and three cytoplasmic domains: actuator domain A, phosphorylation domain P, and nucleotide binding domain N. Interestingly, the "hot spots" of this protein, fuel consumption

(at the top the P domain) and pump unit (in the middle of the transmembrane part), are coupled over a large distance - 50 Å. The protein resides in the membrane of the sarcoplasmic reticulum in muscle cells.

## The $Ca^{2+}$ ATPase - a biological nanopump



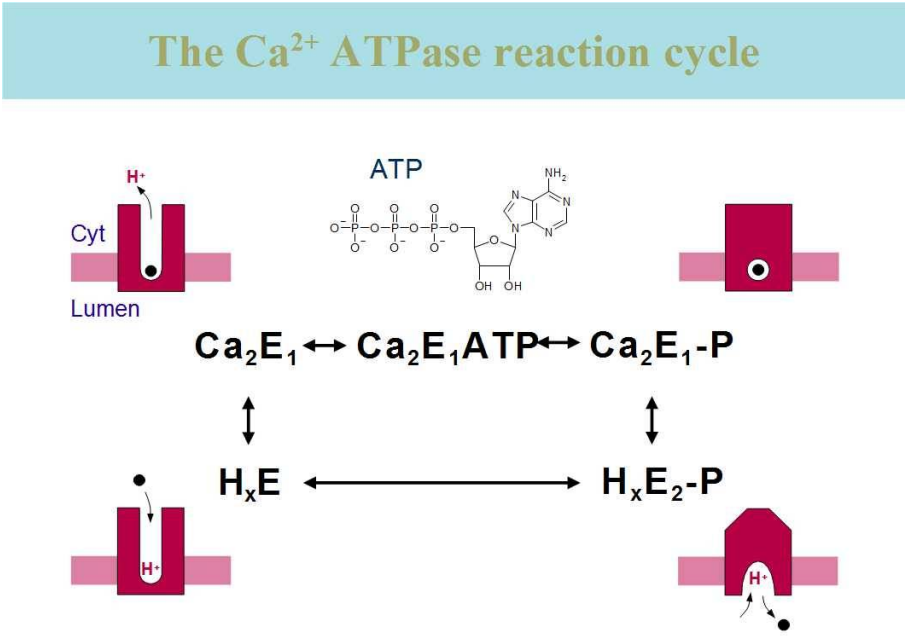

2 $Ca^{2+}$ are actively pumped, driven by the hydrolysis of one ATP molecule

A: actuator domain
P: phosphorylation domain
N: nucleotide binding domain

$Ca^{2+}$ binding sites and ATP hydrolysis site are coupled

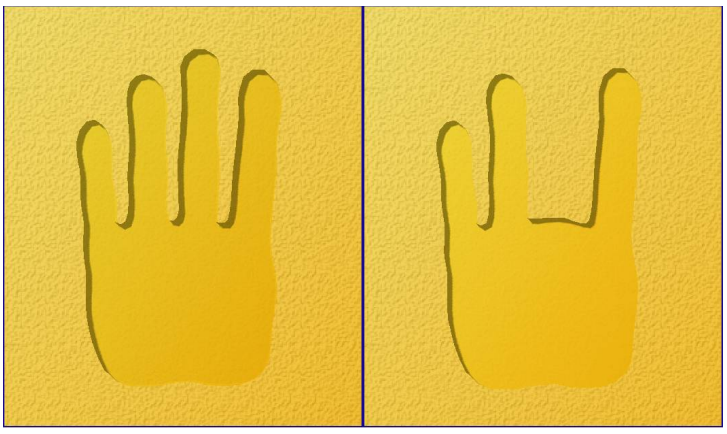
*PDB ID: 1EUL.pdb, Toyoshima et al. Nature 405 (2000) 647-655*

The transport process is driven by ATP hydrolysis and takes place in a reaction cycle that is shown here in a simplified version. $Ca^{2+}$ binds from the cytoplasm of the muscle cells to the high affinity binding sites ($K_d \approx \mu M$) of the ATPase, which releases protons from the empty $Ca^{2+}$ binding sites ($E \rightarrow Ca_2E_1$). This activates the ATPase to use ATP as substrate. ATP transfers its terminal phosphate group to residue Asp351 which leads to the formation of a first phosphoenzyme intermediate ($Ca_2E1$-P), where the 2 $Ca^{2+}$ ions are occluded (= buried, i.e. not accessible from either side of the membrane). In the following step $Ca^{2+}$ is released to the lumen from low affinity binding sites, the Ca2+ binding sites become protonated from the lumenal side, and the phosphoenzyme converts to the $E_2$-P form. Dephosphorylation completes the reaction cycle. In summary, one ATP molecule is hydrolyzed, two $Ca^{2+}$ ions are transported from the cytosol to the lumen and 2-3 protons are transported in the opposite direction.




Mapping the ATP binding site of the ATPase

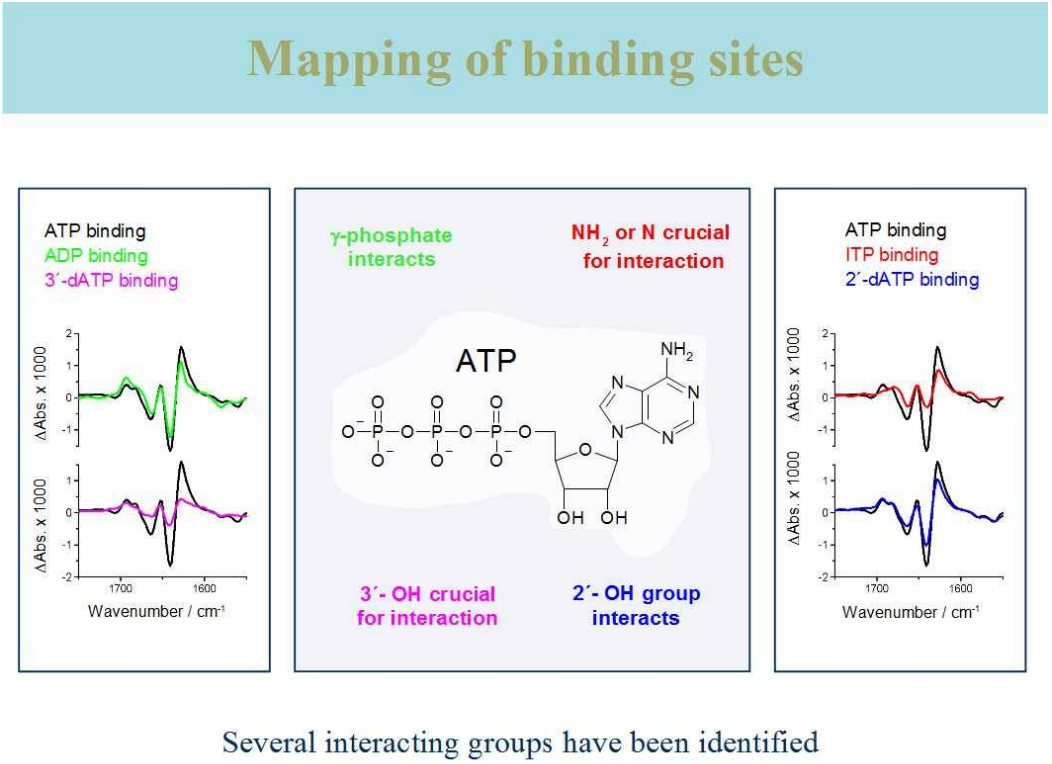
In what follows, I will illustrate the fingerprint approach with an example of our own research where we have used the approach to map the ATP binding site of the ATPase. For that we used derivatives of ATP to identify the important interactions of the ATP molecule with the substrate binding site of the ATPase.



The following simple picture may visualize the approach used: Imagine to press the back of your hand into wet sand, this will generate an imprint as shown if the thumb points upwards. In this picture, the sand represents the protein, the hand is the ligand with the fingers as the functional groups, and the imprint is the conformational change of the protein when the ligand binds. In our example, the four fingers interact with the protein, whereas the thumb does not. If a derivative of the molecule is used without the „thumb" functional group, the imprint will not change and thus you have detected that the thumb does not interact with the protein. If instead a derivative is used without „middle finger", the imprint will

change and this shows that the middle finger interacts with the protein. Screening the imprint with a number of ligand analogues will therefore identify the interacting ligand groups or in other words map structure- interaction-relationships. We can do that with infrared spectroscopy, again using the amide I region which monitors conformational changes of the backbone.

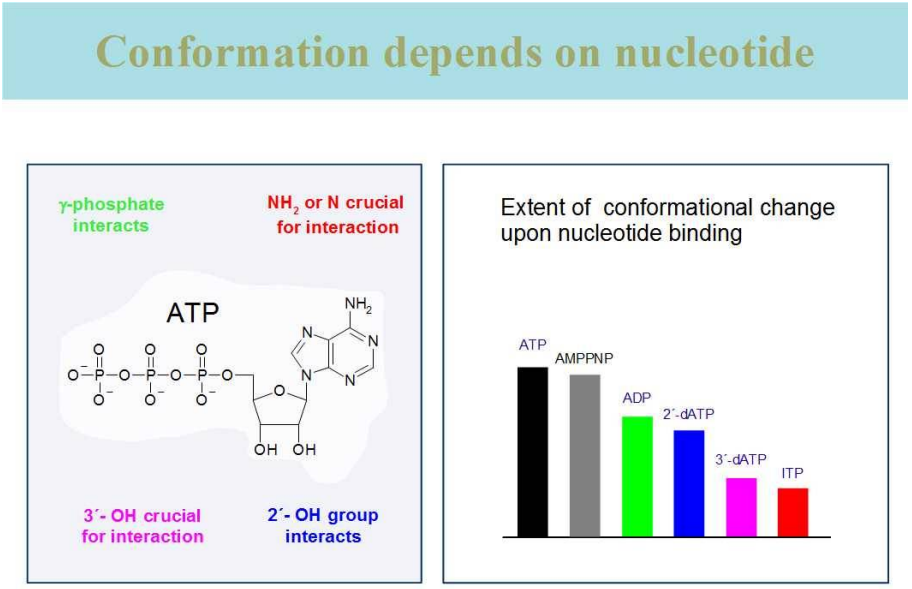
We have mapped the ATP binding site using the derivatives that were modified at the terminal phosphate group, at the amino function of adenine and at the 2 ´and 3´ hydroxyl groups of the ribose moiety. All of them induced conformational changes that were different from those of ATP. The panels on the left and on the right compare the absorbance changes in the amide I range induced by ATP in black and by the ATP derivatives in color.




Several interacting groups have been identified

In all cases, the amide I signals were smaller than with ATP, meaning that also the conformational change was smaller. Some modifications reduce the conformational change only little, whereas other modifications have a strong impact. Thus all of the modified groups seem to be important for binding, although to different degrees. (*Liu & Barth, J. Biol. Chem. 2003*)

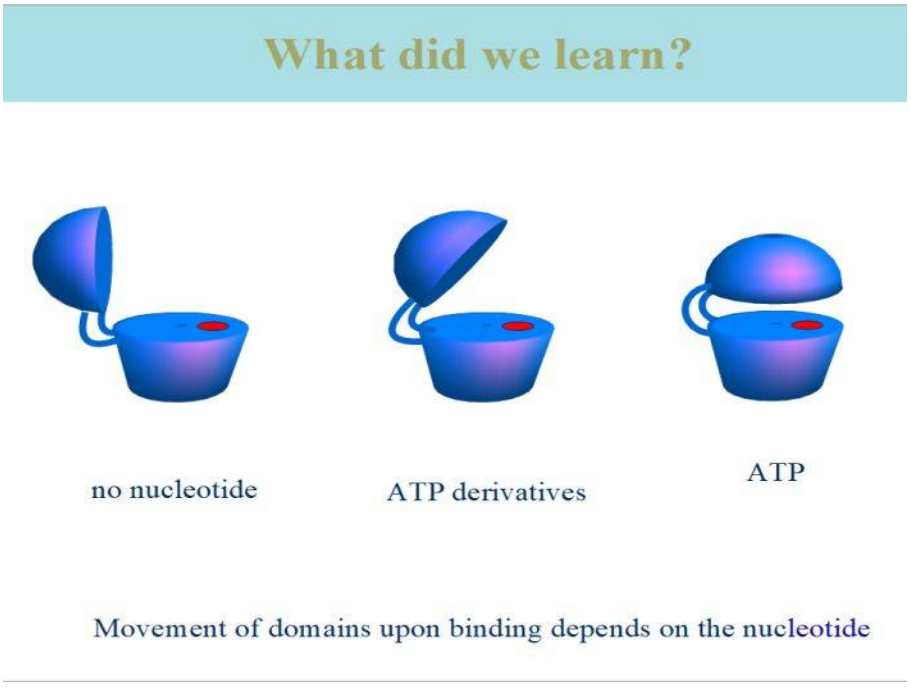
The figure on the right shows the extent of conformational change induced by the different ATP derivatives. The extent of conformational change was simply measured by the size of the signals in the difference spectra. The smallest change is seen for modifications at the 3´-OH group and the region close the amino function of ATP. Thus, interaction between these groups and the ATPase are crucial for the conformational change. A control with AMPPNP showed that this compound gave a very similar spectrum and a very similar extent of




Individual interactions affect the induced fit movement

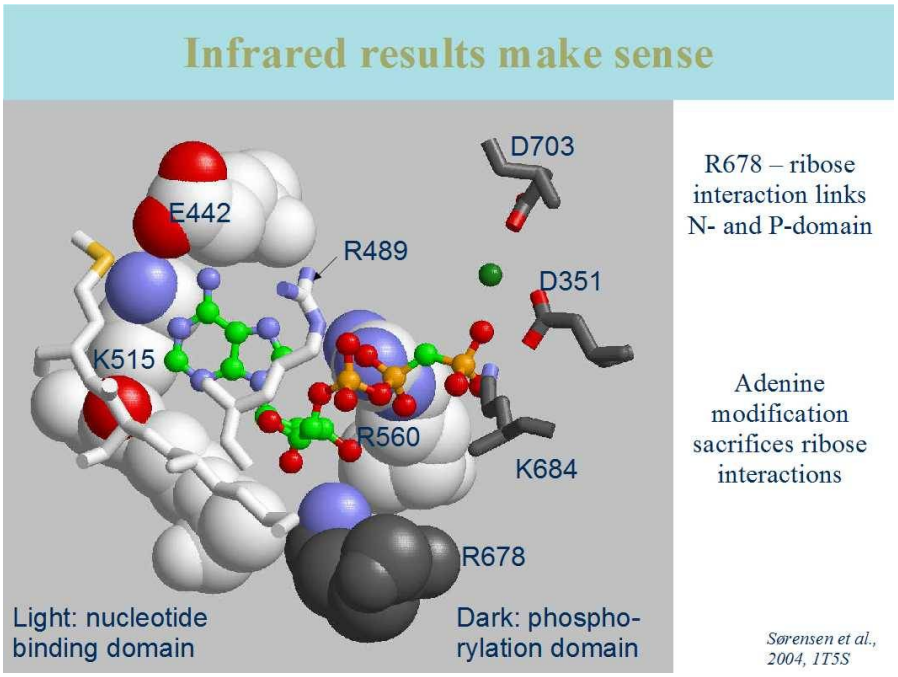
conformational change. It is very interesting that taking away single interactions has such a strong influence on the conformational change induced by nucleotide binding.

*At the time of these measurements it was known that a conformational change is needed to deliver the γ-phosphate of ATP to the phosphorylation site. This is shown in the figure. Without nucleotide the conformation is open, with ATP, the conformation is closed, with the other nucleotides it is somewhere in between. Note that the figure illustrates an average structure, which can be a time-averaged structure if the ATPase alters between open and closed conformations. Obviously, the 3'-OH and the amino group are particularly important for stabilizing the closed conformation.*

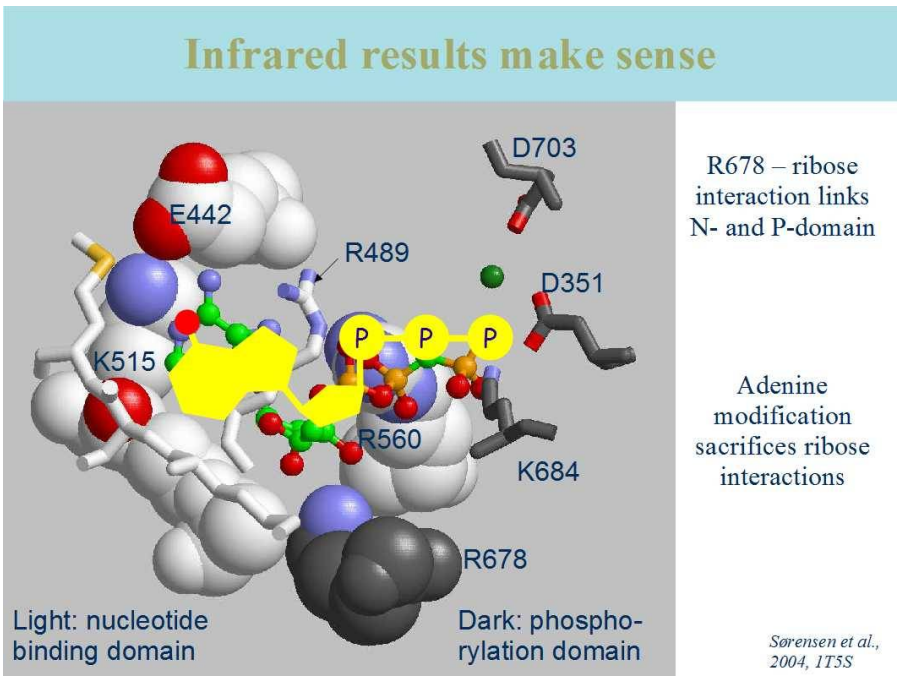



Movement of domains upon binding depends on the nucleotide

*Why is that so? An ATPase structure with a bound nucleotide, published after the infrared experiments, confirms the interactions found and explains why the modified groups are important. Most interactions with the nucleotide are with residues in the nucleotide binding domain. However, the ribose oxygens and the γ-phosphate interact with the phosphorylation domain. They are therefore important to link phosphorylation and nucleotide binding domain and to stabilize the closed conformation.*




*Why then is the amino group important? When we modified this group, we replaced it by a carbonyl group. As shown in the figure, this carbonyl group will not bind in the same way as the amino group because it will be repelled by Glu442. Instead it will interact with Lys515 which tilts the base upwards in the figure and also increases the distance between ribose hydroxyls and the phosphorylation domain.*

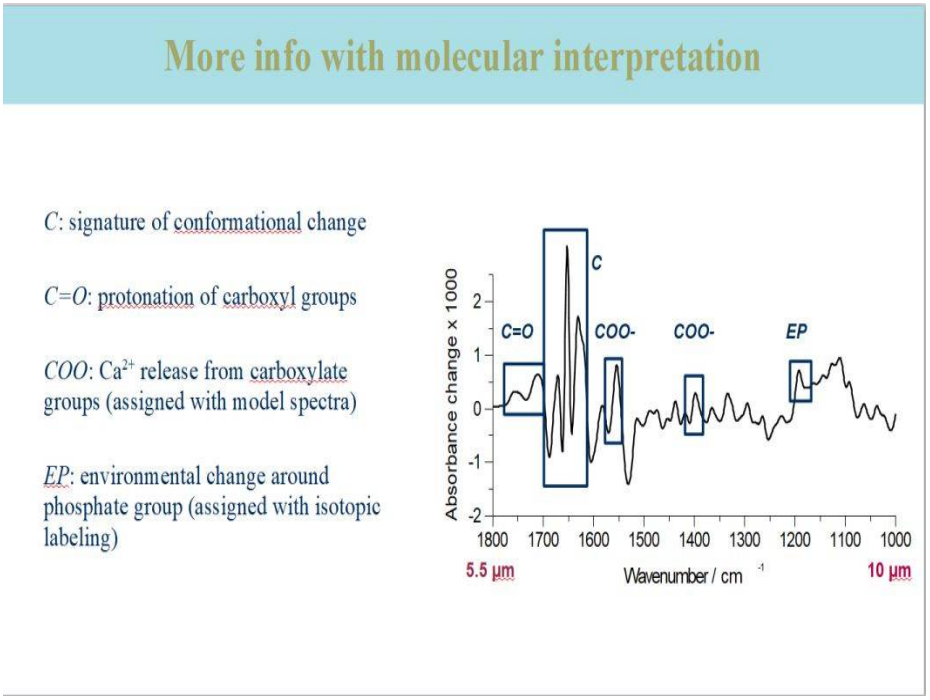



*MOLECULAR INTERPRETATION OF SPECTRA*

The fingerprint approach described above does not use the whole information that is encoded in the spectrum. The ultimate aim of spectrum interpretation is to assign all the bands in a difference spectrum to specific molecular groups. This will then yield information on the environment and interactions of the respective groups. If this is done for time-resolved experiments, we will get a sort of movie of the molecular events that take place

during the protein reaction. Again, I will illustrate the strategies for a molecular interpretation mainly by examples from our research.

*The spectrum on the right shows the difference spectrum between two ATPase states ($Ca_2E1$ and E2-P). Several regions are marked in this spectrum because they give information on different functional groups. The largest bands are observed in the amide I region and they reveal the conformational changes of the protein. We have used this region above for the fingerprint approach. In other regions, we can see protonated carboxyl groups (indicated C=O), unprotonated carboxyl groups (indicated $COO^-$) and phosphates (indicated EP). We will discuss the former and the latter region.*

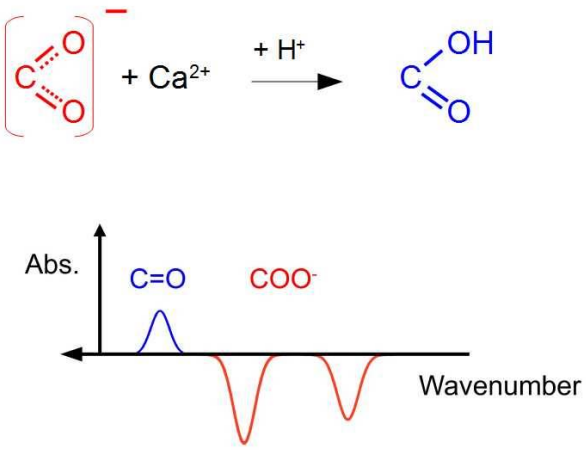



Protonation of carboxyl groups in the $Ca^{2+}$ binding site

In most spectral regions, there are multiple assignments possible. Only above 1700 $cm^{-1}$ bands can be assigned with certainty to protonated carboxyl groups and we will now look into this region. $Ca^{2+}$ release from the $Ca^{2+}$ ATPase is accompanied by the protonation of carboxylate groups of Asp and/or Glu residues. Four of these residues are found in the $Ca^{2+}$ binding sites, but it is also known that not all of them get protonated.

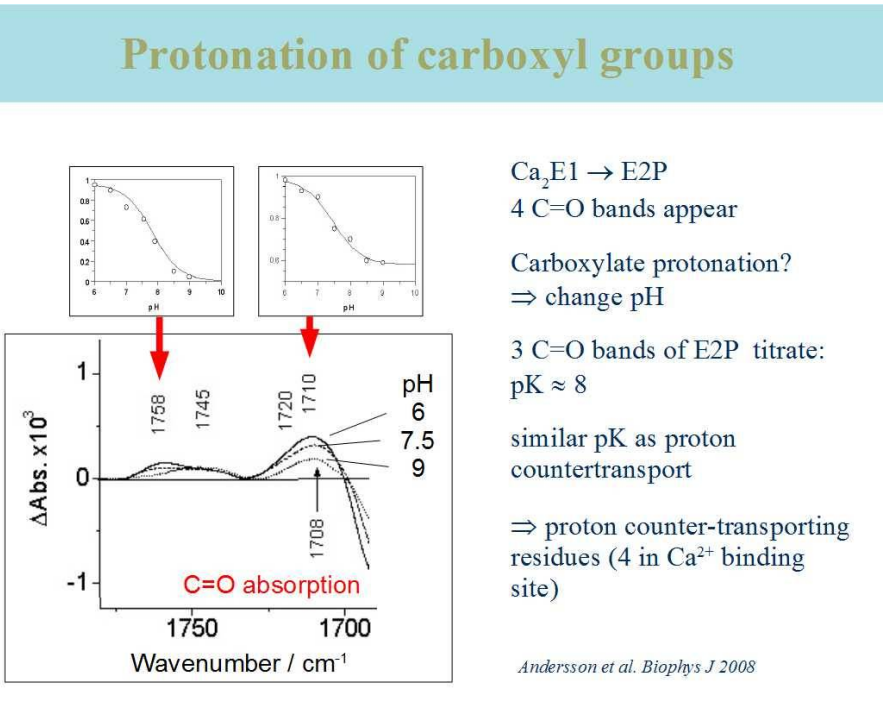
## What happens?

The figure shows what spectral changes we expect when carboxylate groups become protonated. In the infrared difference spectrum, this leads to two negative bands due to the antisymmetric and the symmetric stretching C-O vibrations of the ionized group. The appearing carbonyl group (C=O) of the protonated carboxyl group gives rise to a positive band above 1700 $cm^{-1}$. There will be other positive and negative bands due to the protonation reaction, but they are outside the shown spectral region.




In order to be sure that the signals above 1700 $cm^{-1}$ really originate from the protonation of residues involved in proton countertransport, measurements of the $Ca_2E_1 \rightarrow E_2P$ reaction were performed at different pH values. The motivation for this is the fact that proton countertransport ceases at high pH, obviously because the residues no longer become protonated at high pH. The ability to protonate is described by the p$K_a$ value of a molecule or molecular group. When the pH is equal to the p$K_a$ value, 50% of the molecular groups are protonated, 50% unprotonated. The lower the pH, the more groups are protonated.

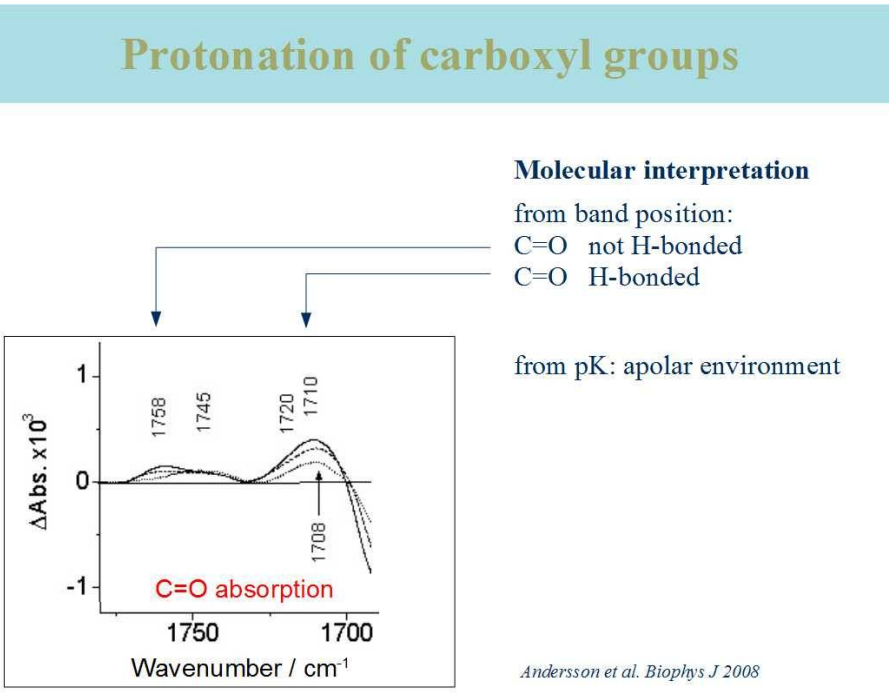
*The obtained spectra are shown in the figure. Our experiments indicated that 4 bands (labeled in the figure) appear when E2P is formed. That there is a band at 1720 cm$^{-1}$ may not be obvious in this plot, but can be seen when the high pH spectra are subtracted from the low pH spectra.3 of them diminish at high pH. When the band intensity is plotted against the pH value, the p$K_a$ of the corresponding groups can be determined. It is similar to the p$K_a$ of proton countertransport, i.e. the infrared bands disappear in the same pH range in which also proton countertransport ceases. This has established that the 3 signals are really due to the protonation of residues involved in proton countertransport.*




Having assigned some of the signals to proton-countertransporting residues, we can use the spectra to study their molecular environment.

From the band positions it can be concluded that those groups absorbing at 1720 and 1710 cm$^{-1}$ are hydrogen bonded at the C=O group whereas that absorbing at 1758 cm$^{-1}$ is not.

Although some of the carboxyl groups are hydrogen bonded, they all reside in an overall apolar environment. This can be concluded from the high pKa values of around 8. In aqueous solution, the pKa of carboxyl groups is much lower: around 4.

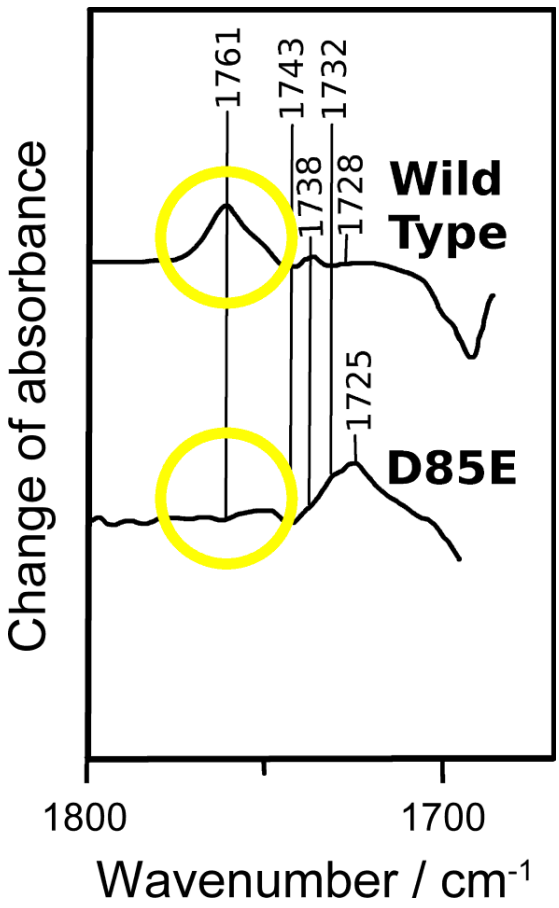


This example demonstrates that the information obtained from infrared spectroscopy can be very detailed. In order to find out exactly which residues become protonated, mutants have to be used, where individual Asp or Glu residues are replaced by non-protonatable residues like Asn or Gln. This was not possible for the $Ca^{2+}$ ATPase, because it turned out to be very difficult to produce enough recombinant ATPase with single amino acid replacements and because of lack of funding.

Therefore we turn to bacteriorhodopsin, where mutants were extensively used to elucidate the reaction mechanism of the protein.

Use of mutants to identify the protonated residues in bacteriorhodopsin

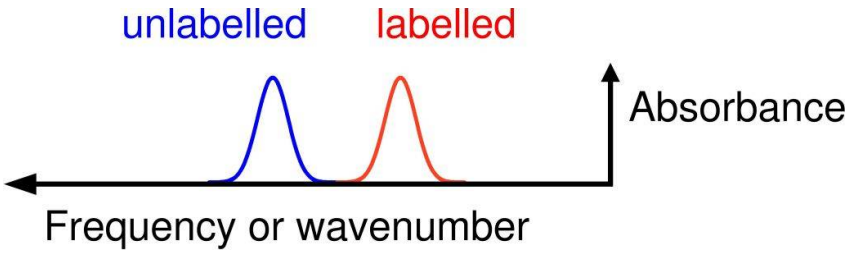
An example were the use of point mutations has considerably contributed to our understanding of difference spectra and of the molecular mechanism of a particular protein is bacteriorhodopsin. The figure shows two spectra of the BR to M reaction. The positive band at 1761 $cm^{-1}$ is observed for the wild-type protein, but not for the protein variant where Asp85 was replaced by Glu. Therefore the band is assigned to Asp85. The band is in the spectral region of protonated carboxyl groups and tells us therefore that Asp85 is protonated in the M intermediate. The positive band appears in the BR to M reaction, which means that Asp85 becomes protonated in the reaction. Using many more single amino acid replacements, infrared spectroscopy has identified all groups that are involved in proton pumping by bacteriorhodopsin [*Spectra from Braiman et al. BC 27, 8516, redrawn and modified by C. Baronio*]. In combination with time-resolved experiments this has revealed the individual steps of proton transfer and their sequence. Note that such information could not have been obtained by X-ray crystallography, because hydrogen atoms are very hard to detect with this method.




After this excursion to bacteriorhodopsin, we will now return to the $Ca^{2+}$-ATPase.

Use of isotopes

The use of mutants is one strategy to assign bands in infrared spectra. Another strategy is to use isotopes. In our work on the $Ca^{2+}$ ATPase, we have used isotopic labeling to assign the bands of the phosphate group. This technique offers fascinating possibilities. It has enabled us to selectively observe one important group in a large protein, which is only transiently present. In other words it enabled us to detect 2 out of 50000 protein vibrations. This experiment is a bit complicated and I will show only a simpler variant of it.




Why is isotopic labeling a powerful technique to assign bands in infrared spectra? As a mass increase lowers the vibrational frequency of an oscillator, isotopic labeling shifts bands in infrared spectra. So when we compare the spectra with and without labeling, we can identify the contribution of the labeled group to the spectrum.

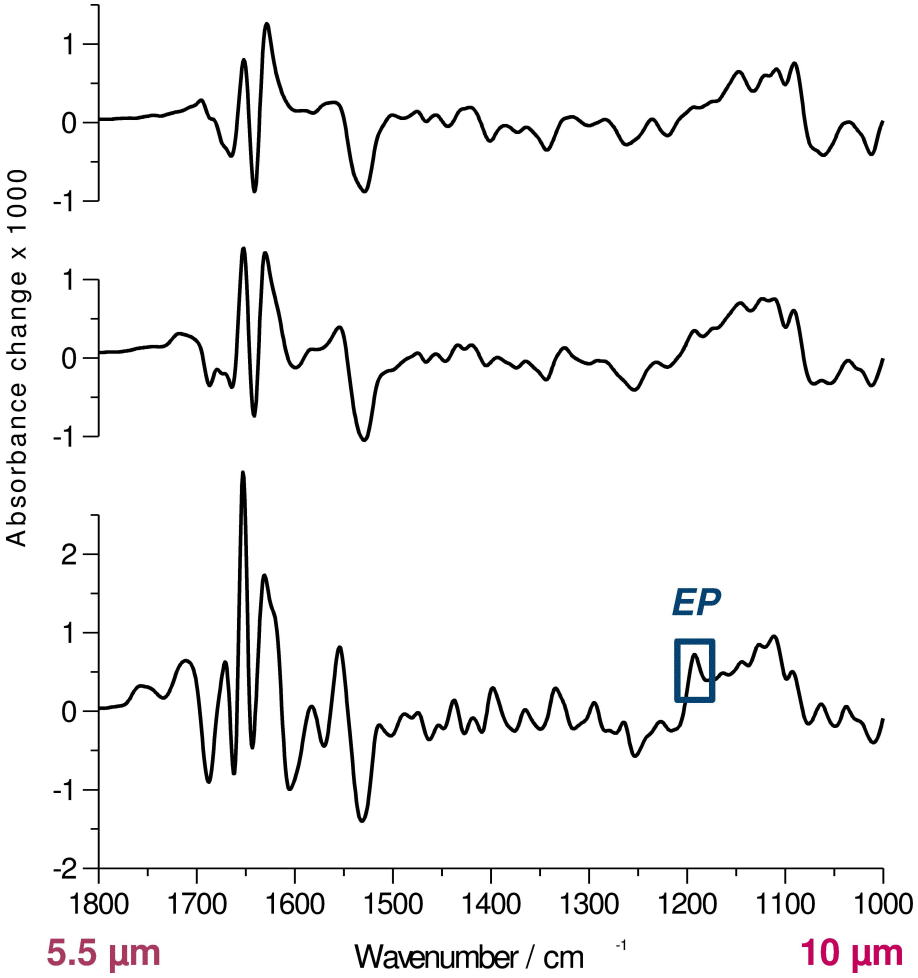
There are several labeling strategies. The ideal experiment would be to label a particular amino acid or amino acid side chain. This is a better experiment than using point substitutions of amino acids, because these may affect the reaction mechanism and may even inactivate the protein. However, site-directed isotopic labeling is difficult to achieve.

It is easier to label all residues that consist of a particular amino acid. For this, the protein is produced recombinantly and the host organism is fed with the labeled amino acid or with a precursor of it.

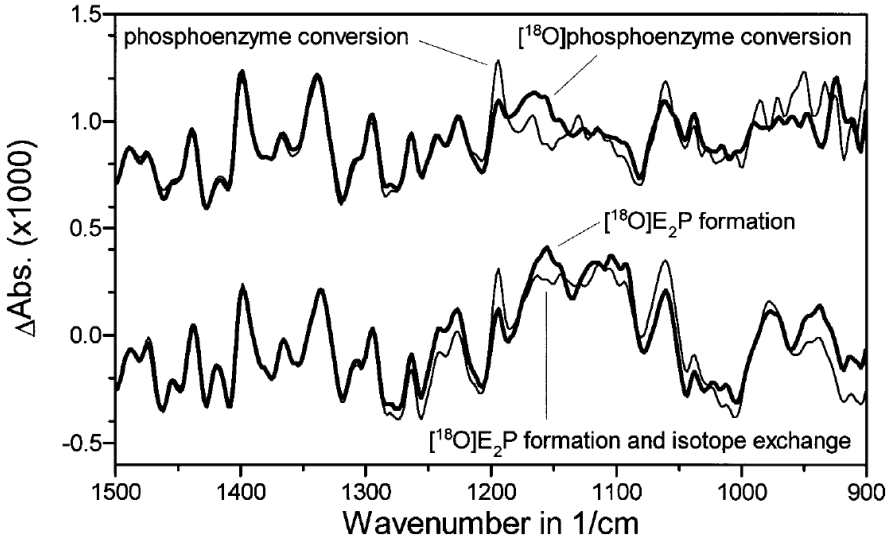
It is also helpful for the interpretation to label particular atoms of a protein, for example by feeding the host organism with labeled nutrients or by $^{1}H/^{2}H$ exchange. The latter is simple to achieve by replacing the solvent $^{1}H_2O$ with the solvent $^{2}H_2O$ ($D_2O$).

So there are many different ways to use isotope labeling in order to interpret infrared spectra. The example that I want to discuss here is isotopic labeling of the substrate. We have used this in our work on the $Ca^{2+}$ ATPase to assign the bands of the phosphate group. This strategy offers fascinating possibilities. It has enabled us to selectively observe one important group in a large protein, which is only transiently present. In other words it enabled us to detect 2 vibrations out of a total of 50000 protein vibrations. This experiment is a bit complicated and I will show only a simpler variant of it.

*We have used labeling to assign one of the bands in our spectra to the phosphate group of the ATPase phosphoenzyme E2-P. The band is marked in the figure.*



This figure shows the result of the isotope labeling experiment. The thin line spectrum on the top is similar to the spectrum shown above. The thick line spectrum on the top is a repeat of the experiment were [$^{18}O_3$]caged ATP was used. When this caged ATP was photolysed, it transferred its labeled gamma-phosphate to the ATPase which produced a labeled phosphoenzyme. In the thick line spectrum, the sharp band at 1194 $cm^{-1}$ is clearly missing. Therefore this band can be assigned to the unlabeled phosphate group of the phosphoenzyme E2-P.




In other experiments, we have identified a second phosphate band at 1137 $cm^{-1}$.

What is the benefit of identifying the phosphate bands in the spectrum? With this information, the phosphate environment in the catalytic site of the ATPase can be modeled with quantum chemical methods. This gives then information on the bond lengths and bond strengths of the phosphate group. We found that the enzyme environment makes one of the terminal P-O bonds shorter. This indicates that the respective phosphate oxygen interacts weaker with the protein environment than with water in an aqueous environment. All other phosphate bonds are unaffected. This is particularly important for the bridging P-O bond (between phosphate and protein). This P-O bond is cleaved in the next step of the reaction cycle. Since we find the same bond length for this bond in the enzyme environment as in water, we conclude that the enzyme environment does not weaken the bond in the intermediate state that is adopted before bond cleavage. Such information cannot be obtained from X-ray crystallography because: (i) The resolution of most structures is not good enough to detect bond lengths changes. (ii) The phosphoenzyme E2-P is an intermediate in the ATPase reaction cycle. It cannot be crystallized. Instead phosphate analogues like $BeF_3^-$ are used to crystallize this state which implies that no information on phosphate bonds can be obtained.

Summary

In summary, infrared difference spectra of protein reactions contain a wealth of information. To reveal this information, the bands in the difference spectra need to be assigned to the protein groups that cause them. This is not always easy, but several strategies exist. We have discussed a few important ones above. Those and an additional one are listed in this slide:

- various strategies for isotopic labeling as just discussed
- the use of mutants, as in the example of bacteriorhodopsin

- modification of the substrate as for example used for the fingerprint approach. In spite of the simplicity of the fingerprint approach, it can give molecular information

- the use of model compounds, which I have not discussed here. For example, one can record the infrared spectra of ATP, ADP and phosphate to calculate a difference spectrum of the ATP hydrolysis reaction.

## Conclusion

Finally, I list some advantages and disadvantages of vibrational spectroscopy (infrared and Raman spectroscopy). Advantages are:

(i) The vibrational spectrum of a molecule encodes a lot of molecular information. Unfortunately it is quite hard to extract this information from the spectrum. A number of methods are employed to do this and yield information that is part of the jigsaw puzzle that builds up our knowledge on proteins. In favorable cases, bond lengths and bond geometries can be determined. Vibrational spectroscopy is one of the few methods to characterize the protonation state of functional groups, in particular in time-resolved experiments.

(ii) Proteins can be investigated that are too large for NMR or too difficult to crystallize for X-ray crystallography. Membrane proteins can also be investigated. These are difficult to crystallize and usually too large for NMR.

(iii) The sample preparation for standard measurements is simple.

(iv) The time for recording an infrared spectrum is short (less than a minute).

(v) The time resolution of infrared spectroscopy is high, up to μs with commercial FTIR spectrometers.

(vi) It gives good value for money and simple spectrometers are not very expensive

Disadvantages are:

(i) For ordinary infrared and Raman spectroscopy, the signals are weak. This requires high concentrations of the molecule of interest, which is not always possible to achieve for example because some proteins aggregate. Note however, that the amount of sample for infrared spectroscopy is small (several μg) because the sample volume is small (a few μL). On the other hand, there are specialized techniques with very high sensitivity like resonance Raman spectroscopy and several techniques that can measure the vibrational spectrum of nanoscale objects.

(ii) Water absorbs strongly in a relevant spectral region of the infrared spectrum of proteins. This requires short pathlengths of the cuvettes which make mixing experiments difficult. It also requires high concentrations of the molecule of interest. In contrast, water is not a problem for Raman spectroscopy.

(iii) Calculation of the absorption spectrum is difficult for larger molecules like proteins. This makes it difficult to compare experimental results with calculated spectra from model structures.

All in all, vibrational spectroscopy has made significant contributions to the understanding of proteins and other biomolecules. It is also very valuable for characterizing biological cells and tissue, a topic that I did not focus

on here. Several recent technical developments have increased the sensitivity of detection and therefore vibrational spectroscopy will continue to be an important tool in the life sciences.