Fourier Transform Infrared (FTIR) Spectroscopy

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Advanced article

Article Contents

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Fourier transform infrared (FTIR) spectroscopy is an experimental technique used initially for qualitative and quantitative analysis of organic compounds, providing specific information on molecular structure, chemical bonding and molecular environment. For many years, FTIR has been successfully employed for studying a wide variety of proteins, enzymes, nucleic acids, lipids and glycolipids and photobiological systems. Time-resolved (tr)-FTIR spectroscopy can monitor reactions of the amino acids, the ligands and specific water molecules in the active centre of a protein in the time range from nanoseconds to seconds, thereby providing a detailed understanding of the molecular reaction mechanism.

Introduction

The underlying principle of a Fourier transform infrared (FTIR) spectrometer is the separation of an incoming infrared light beam into two individual beams using an optical beamsplitter, followed by variation of the optical path difference between these two beams using a movable mirror for one beam and a fixed mirror for the other, and by recombination of the two separate beams using an optical combiner so that interference occurs (**Figure 1a**). In this way, an interference signal that depends on the optical path-difference is produced and can be measured using standard infrared detectors. In the simplest case (a monochromatic emission line) the interference signal is a cosine function of the optical path-difference; for polychromatic light sources, a more complicated function is obtained (generally referred to as the interferogram, **Figure 1b**).

It can be shown that this interferogram (detector signal as a function of optical path-difference, mostly in units of centimeters) is the Fourier transform of the original spectrum (signal as a function of inverse wavelengths, mostly in units of cm^{-1}, generally referred to as wavenumbers). To obtain the original spectrum from the measured interferogram, the interferogram is numerically Fourier transformed using the fast Fourier transform (FFT) (**Figure 1b**). Although the experimental basis for FTIR spectroscopy was founded by Michelson in the late nineteenth century, it became widely distributed only in the past decades of the twentieth century with the availability of fast personal computers required for the calculation of the FFT.

FTIR provides several advantages compared to other spectroscopic techniques: (1) simultaneous recording of spectra over a broad spectral range at any desired resolution (multiplex advantage); (2) high optical throughput because a circular aperture is used instead of a narrow slit as in conventional spectrometers (Jacquinot advantage) and (3) very accurate optical calibration by the use of a frequency-stabilised reference laser for determination of the optical path-difference between the two light beams (Connes advantage). Because of these advantages, small spectral changes can be measured accurately by FTIR using the differences between spectra recorded under specific conditions (FTIR difference spectroscopy).

Although FTIR was initially mainly applied to the study of small inorganic molecules, in the past 20 years it was extended to biological molecules, e.g. for static and dynamic studies of protein secondary structure (Surewicz *et al.*, 1993), of enzymes (Alben, 1996), of nucleic acids (Liquier and Taillandier, 1996), of lipids (Lewis and McElhaney, 1996) and glycolipids (Brandenburg and Seydel, 1996). Introducing time-resolved (tr)FTIR difference spectroscopy allows the determination of the molecular reaction mechanism of proteins, especially of bacteriorhodopsin (Gerwert *et al.*, 1990; Gerwert, 1993) and photoreactive reaction centers (Mäntele, 1993; Nabedryk, 1996; Remy and Gerwert, 2003). Several numerical techniques have been applied to the analysis of FTIR spectra, including spectral resolution enhancement by Fourier self-deconvolution,

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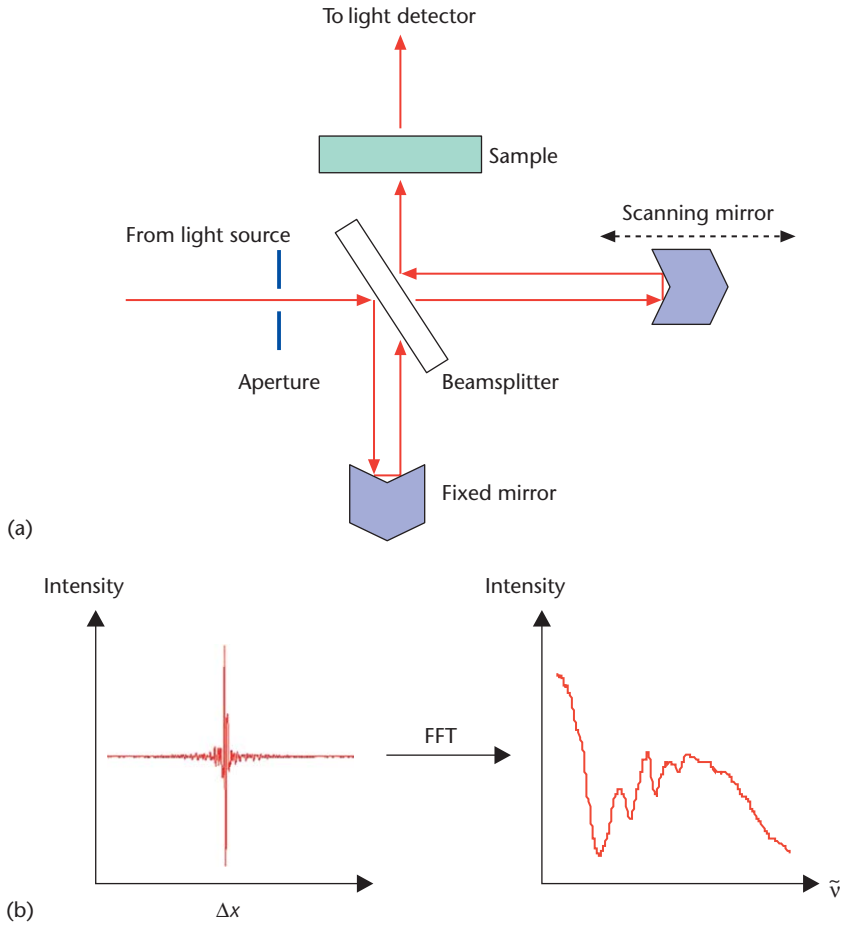


Figure 1 (a) Typical setup of a Fourier transform infrared (FTIR) spectrometer: the light from an infrared light source is sent through an aperture hole to a mirror (the 'beamsplitter') that sends two equivalent beams (one reflected and one transmitted) to a fixed and to a scanning retroreflector mirror, respectively. After acquiring different optical path lengths (in addition to the equal distances of both mirrors from the beamsplitter – the 'zero path difference') these two beams recombine on the beamsplitter and are sent to the sample, and after this to a semiconductor detector. The detector signal as function of the optical path difference between the two mirrors is the interferogram (b). After fast Fourier transform (FFT) calculation the infrared spectrum is obtained. Reproduced from Kötting C and Gerwert K (2007) Protein reactions: resolved with tr-FTIR. *Spectroscopy Europe* **19**(3): S19–S23.

factor analysis and principal components analysis and singular value decomposition.

Use in Investigation of Secondary Protein Structure

FTIR spectroscopy is widely used for the investigation of secondary protein structure, as a technique complementary to other methods such as X-ray structure determination, nuclear magnetic resonance (NMR), and circular dichroism (CD) spectroscopy. The particular advantage of FTIR is the possibility of investigating protein structure and structural changes under conditions not directly and noninvasively accessible by other spectroscopic methods, in a variety of environments such as the aqueous state, in crystals, in organic solutions and even in the presence of other biomolecules. **See also**: Circular Dichroism: Studies of Proteins; Nuclear Magnetic Resonance (NMR) Spectroscopy for Monitoring Molecular Dynamics in Solution; Nuclear Magnetic Resonance (NMR) Spectroscopy of Proteins; Nuclear Magnetic Resonance (NMR) Spectroscopy: Structural Determination of Proteins and Nucleic Acids; Protein Secondary Structures: Prediction; Protein Structure Classification; Protein Structure Prediction

Figure 2 shows a typical absorption spectrum of a protein dissolved in water. The predominant absorptions are those of the amide I band (sum of all C = O backbone stretching vibrations), the amide II band (sum of all NH bending and CN stretching vibrations of the backbone) and the OH-bending vibration of water. The amide I band is especially sensitive to the secondary structure of proteins. On the basis of empirical rules, infrared bands in the 1660–1650 cm^{-1} region are assigned to α helices, in the 1640–1620 cm^{-1} to β sheets, in the 1695–1660 cm^{-1} region to β sheets and β turns and in the 1650–1620 cm^{-1} region to unordered structures. These rules of thumb have been

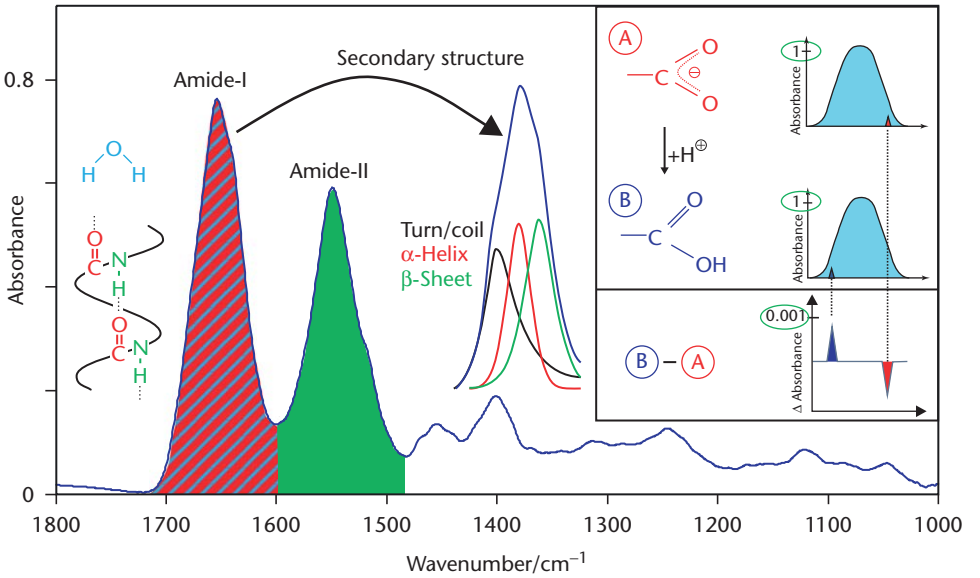


Figure 2 Typical absorbance spectrum of a protein solution (here 10 mM Ras). The main components are indicated by the colours red (C=O stretching vibration, amide I), blue (bending vibration, water) and green (combination of NH bending and CN stretching vibration, amide II). The amide I band is decomposed for the secondary structure. In the inset, two absorption spectra of a protein, which deviate only in the protonation of a carboxyl group, are shown schematically. In the lower part, a difference spectrum of these two states is shown schematically. The background absorptions of the unchanged part of the protein are cancelled out; the absorptions of the reacting group are now resolved. Reproduced from Kötting C and Gerwert K (2007) Protein reactions: resolved with tr-FTIR. *Spectroscopy Europe* **19**(3): S19–S23.

obtained by measuring the amide I bands of polypeptides and proteins whose structures are dominated by one of the typical secondary structures.

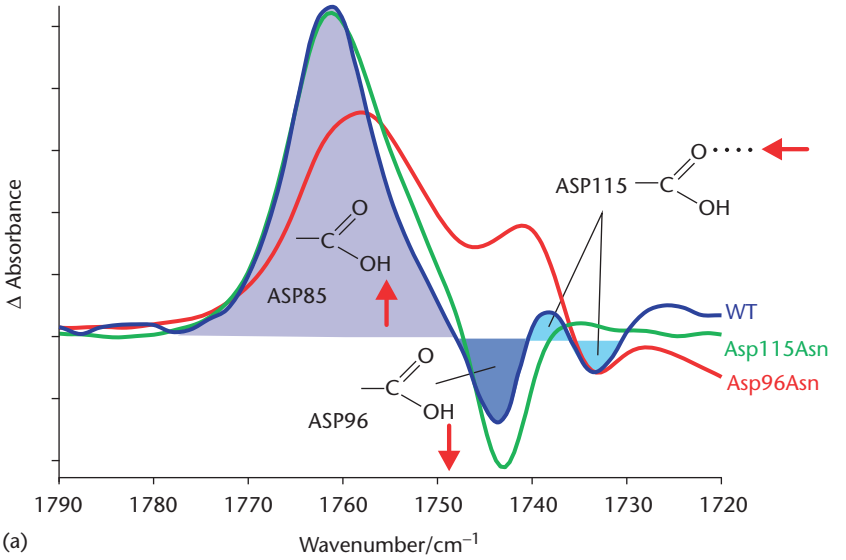
Although a determination of the secondary structure content of a protein from scratch can have large uncertainties, because the fitting of the amide I band is underestimated and has many solutions, comparison with similar proteins or the observation of changes of secondary structure is much more exact (Güldenhaupt *et al.*, 2008). Further, the inclusion of other parts of the spectrum, for example the amide II band can increase the accuracy (Goormaghtigh *et al.*, 2006).

Although the detailed correlations between the spectra and the secondary structures of proteins require a thorough theoretical discussion (Torii and Tasumi, 1996; Khurana and Fink, 2000), it is well established that FTIR is particularly sensitive to the presence of and to changes in β structures (which makes it highly complementary to circular dichroism, which is more sensitive to α helix but less sensitive to β structures, and to Raman spectroscopy). Like circular dichroism, FTIR is commonly used to study thermal unfolding of proteins (Hering and Haris, 2009). The recently developed techniques of time-resolved FTIR spectroscopy permit direct monitoring of the kinetics of β structure formation *in vitro* (Elfrink *et al.*, 2008). **See also**: Protein Folding *In Vivo*; Protein Folding: Overview of Pathways

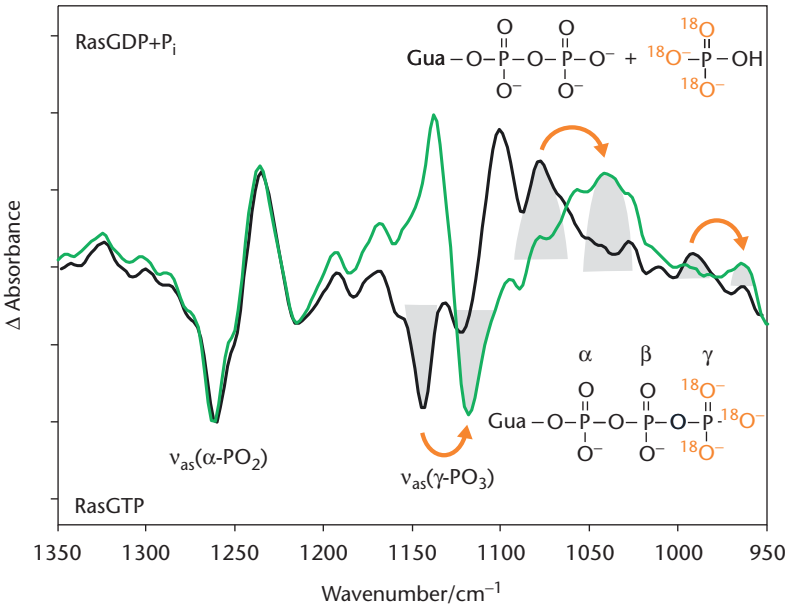
Difference Spectroscopy

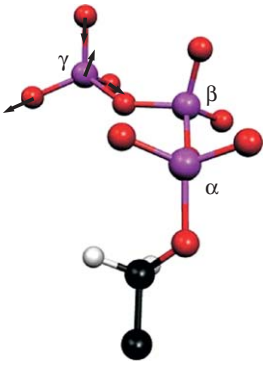
To obtain information on individual groups, we have to look at difference spectra. In the simple case of a reaction A→B (**Figure 2**, inset), one calculates the difference of the absorption spectra of the two states (B − A). The background absorption of the protein is 10^3–10^4 times greater than the absorption of the reacting groups. Thus it is very important to keep the measurement conditions exactly the same throughout. This includes using a temperature-stabilised room and a vibrationally isolated table, and the sample has to remain within the spectrometer all the time. Thus, the reaction has to be started (triggered) within the spectrometer. For photobiologically active proteins this can be done by a laser flash. In other cases photolabile-caged compounds (Cepus *et al.*, 1998), which release a biologically active substance after irradiation, can be employed. A nice example is the study of GTPase (guanosine triphosphatse) reactions (**Figure 3b**; Kötting and Gerwert, 2004; Kötting *et al.*, 2006). Furthermore, micromixing cells allow mixing of two reactants within the submillisecond time regime (Kauffmann *et al.*, 2001).

With the ATR (attenuated total reflection) technique it is possible to immobilise a protein on a surface and study reactions with ligands or other proteins (Ataka and Heberle, 2003). Since the acidity of internal groups is influenced by the local environment, changes in the vicinity of such groups lead to shifts in pK_a. It has been shown that the amplitudes of characteristic vibrations and their centre frequencies vary with the pK_a of the corresponding group, so that changes in the pH of the external solution lead to changes in the ATR FTIR spectrum (Heberle and Zscherp, 1996). Numerical fitting of the pH-dependent spectral amplitudes or frequency shifts to the Henderson–Hasselbach equation provides the pK_a values of the individual groups in proteins.



(a)





(b) $\nu_{as}(\gamma\text{-}PO_3)$

See also: pH and Buffers; Protein–Protein Interactions; Titration and pH Measurement

Band Assignment

In order to derive information on the reaction, the infrared bands have to be assigned to molecular groups of the protein. If structural models of the proteins are available, in addition spatial resolved information is gained. One method is site-specific mutagenesis. Here, the bands of the mutated amino acid are missing compared to the wild-type (**Figure 3a**; Gerwert *et al*., 1989).

Another option is isotopic labelling of an amino acid or a ligand, leading to a shift of the absorption in the spectrum (**Figure 3b**; Gerwert and Siebert, 1986; Allin and Gerwert, 2001). Since a mutation can be invasive, isotopic labelling should be preferred wherever possible. Once a band is assigned, very accurate information on interactions of this group, on protonation states, charge distribution, involvements in the reaction mechanisms and bond orders can be obtained.

Time-resolved FTIR of Proteins

Tr-FTIR difference spectroscopy was established in investigations of the light-driven proton pump bacteriorhodopsin (bR) (Gerwert *et al*., 1989; **Figure 4**). By this model system for light-driven proton transport, the role of the aspartate residue D96 as the internal proton donor of the retinal Schiff base and aspartate D85 as the primary proton acceptor of the Schiff base proton was shown. In later studies, the importance of protein bound internal water molecules for the proton transfer via a Grotthuss-like mechanism was demonstrated (Garczarek and Gerwert, 2006). **See also**: Rhodopsin; Water: Structure and Properties

Theoretical Infrared Spectroscopy

The QM/MM (quantum mechanics/molecular mechanics) approach (Klähn *et al*., 2004; te Heesen *et al*., 2007) can be used to calculate the infrared spectra of proteins. Because proteins are far too big to be completely quantum chemically treated, the main part of the protein and its water surrounding are classically calculated using an empirical force field at a lower computational level, but the active centre or a prosthetic group bound to the protein is treated by high-level quantum mechanical methods, mostly density functional theory (DFT). The QM/MM calculations provide the frequency, the band width and the intensity of infrared absorptions which can be compared with experimentally observed spectra. If the vibrational spectrum is accurately reproduced, the results obtained from the calculation will be reliable, thus more details, for example the geometry, the electron density and the charge distribution of the QM-treated protein part, can be derived from the infrared spectrum. These are the key parameters for determining the molecular mechanism of a protein. **See also**: Molecular Dynamics

Use in Characterisation of Other Biomolecules

The various applications of FTIR to the study of lipids and lipid bilayer membranes can be roughly classified into three groups: (1) detection, assignment and characterisation of lipid phase transitions; (2) characterisation of conformational disorder in liquid crystalline lipids and (3) structure determination of crystalline and quasi-crystalline lipid phases. The use of FTIR has led to the important progress in knowledge of the nature of the lipid assemblies that form in aqueous solution or dispersion, and of the conformations of and interactions between lipid molecules in such assemblies. **See also**: Lipid Bilayers; Membrane Dynamics

Both transmittance and reflectance techniques are used to study lipids and hydrated lipid dispersions; a tabulated listing of the infrared-active groups typical of the more commonly studied lipids is given in a review article by Lewis and McElhaney (1996).

The simplest experimental approach uses unpolarised infrared light to probe the samples, and accounts for the majority of the studies reported so far. For hydrated lipid assemblies, there are different experimental methods to circumvent the strong water absorption in the mid-IR: use of very short path lengths (typically 10–25 μm, which requires highly concentrated lipid samples); use of deuterium oxide (D_2O) as solvent instead of water (which shifts the solvent's absorption bands into another spectral region but can lead to changes in the absorption spectrum of potential hydrogen-bonding acceptor groups owing to the inevitable H/D exchange) and attenuated total reflectance

Figure 3 (a) Band assignment by site-directed mutagenesis. In the difference spectrum N–BR of the wild-type (WT) (blue), the positive band shows the protonation of Asp85, the negative band shows the deprotonation of Asp96 and the difference band shows the change in the hydrogen bonding of Asp115. The negative band of Asp96 disappears in the Asp96Asn mutant (red). The difference band of Asp115 disappears in the Asp115Asn mutant (green). (b) Band assignment by isotopic labelling: In the hydrolysis spectrum of Ras, the bands facing downwards are from the Ras·GTP state (see http://mrw.interscience.wiley.com/emrw/9780470015902/els/article/a0003052/current/pdf, http://mrw.interscience.wiley.com/emrw/9780470015902/els/article/a0005534/current/pdf and http://mrw.interscience.wiley.com/emrw/9780470015902/els/article/a0000657/current/pdf; the bands facing upwards are from the Ras·GDP state. Black: unlabelled; green: γ-$^{18}O_3$-GTP. Owing to the labelling, the absorption of the $\nu_{as}(\gamma\text{-}PO_3)$-vibration has shifted, whereas the other bands, for example the absorption of the $\nu_{as}(\alpha\text{-}PO_2)$-vibration are unchanged. The bands of the released P_i facing upwards at 1078 and 990 cm^{-1} are also shifted by the label. Below, the calculated normal modes of the isolated $\nu_{as}(\gamma\text{-}PO_3)$-vibration are shown. Reproduced from Kötting C and Gerwert K (2007) Protein reactions: resolved with tr-FTIR. *Spectroscopy Europe* **19**(3): S19–S23.

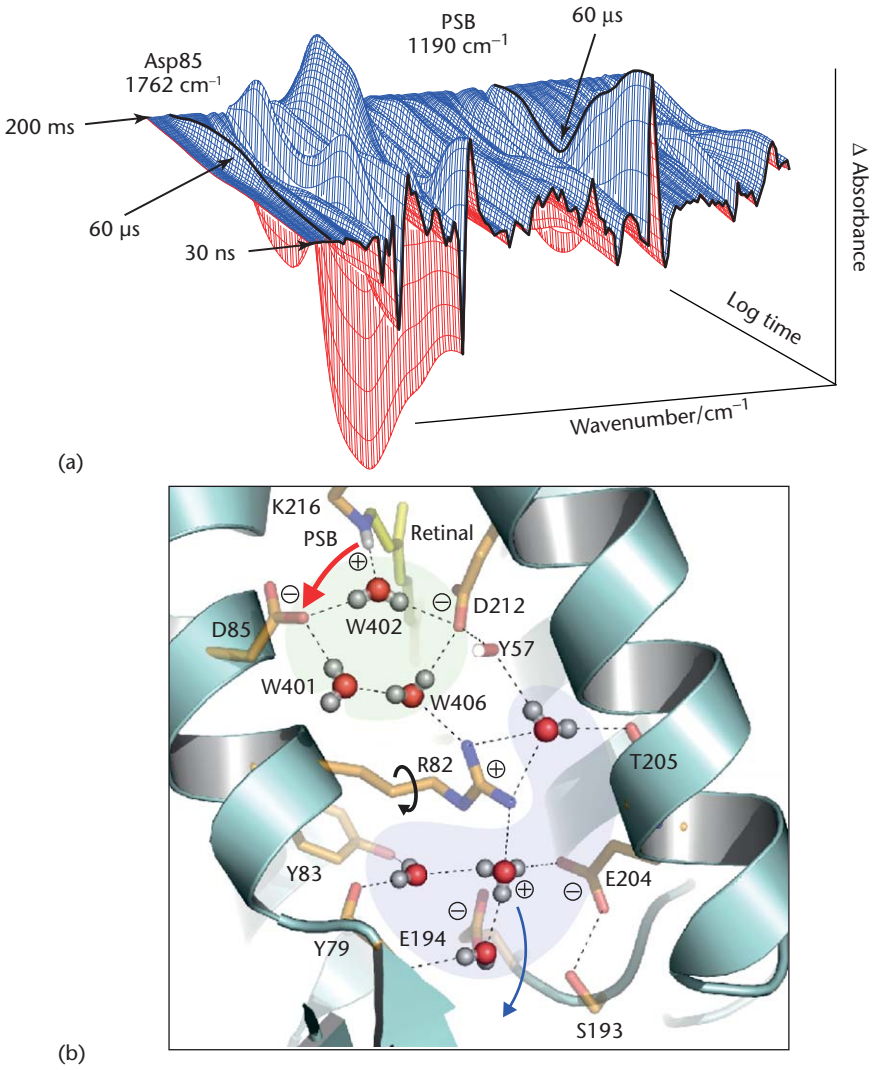


Figure 4 (a) Time-dependent absorbance changes during the bR photocycle from 30 ns to 200 ms at 4 cm^{-1} resolution. The absorbance of protonated Asp85 (1762 cm^{-1}) and protonated Schiff base (PSB) (1190 cm^{-1}) are indicated. In the L→M reaction (completed after 60 µs), a proton is transferred from the PSB to Asp85, as seen in the decay at 1190 cm^{-1} and the increase at 1762 cm^{-1}. (b) Owing to light-induced isomerisation of the chromophor retinal, the strong H-bond of water 402 is broken and approximately half of the energy is stored in the protein. After isomerisation, the free OH-group of the dangling water 401 is H-bonded and can no longer stabilise the charge of Asp85. The proton is transferred from the PSB to Asp85. Owing to the neutralisation of Asp85, a downward movement of Arg82 is induced. This movement of the positive charge destabilises the protonated water complex near the protein surface. The protonated water cluster (blue) stores a proton, probably in an asymmetric Eigen-complex ($H^+(H_2O)_3$). This destabilises the second hydration shell. In contrast to the random Grotthuss-proton transfer in water, in the protein the water complex is deprotonated by a directed movement of Arg82. The proton is stabilised in the second hydration shell by amino acids instead of water molecules. Reproduced from Kötting C and Gerwert K (2007) Protein reactions: resolved with tr-FTIR. *Spectroscopy Europe* **19**(3): S19–S23.

(ATR) methods in which the sample is placed on the surface of a special crystal. Polarised infrared radiation is useful for determining dichroic ratios or linear dichroism, parameters from which orientational information can be deduced (e.g. the macroscopic orientation of the transition moments of infrared-active groups in lipid assemblies). In addition, reflectance techniques can be employed to study lipid monolayer or bilayers at the air–water or other interfaces (with the advantage that lipid monolayers are macroscopically well aligned).

Using different FTIR experimental methods, significant progress has been achieved concerning glycolipids such as diacyl sugars, cerebrosides, gangliosides, lipopolysaccharides and mucopolysaccharides (Brandenburg and Seydel, 1996): this includes the determination of chemical structures (and polymorphism), the interactions of glycolipids with various agents (e.g. intoxicants, anaesthetics and antibiotics) and membrane–sugar interactions. **See also**: Glycolipids: Distribution and Biological Function

Use in Medicinal Biology

It is now well established that biochemical changes of tissues and body fluids can be monitored using FTIR spectroscopy (Diem *et al*., 2008; Dieter Naumann *et al*., 2009). Although the infrared spectra of such samples are very complex owing to the large number of constituents, these spectra contain much information about the biochemical state of the sample. For useful information to be obtained, a large (statistically relevant) number of spectra obtained using different samples must be available, and multivariate analysis techniques have to be used for interpretation of the spectra.

FTIR is used in medicinal biology for infrared clinical chemistry (quantitative analysis of blood or other fluid analytes), infrared pathology (assessment of a tissue biopsy) and infrared *in vivo* analysis (noninvasive procedures). For example with FTIR one can obtain simultaneous reagent-free determinations of at least six serum analytes: albumin, cholesterol, glucose, protein, triglycerides and urea (within clinically accepted standard errors). There is an increasing number of applications of FTIR in medicinal biology, such as the diagnosis of amyloid diseases (in particular Alzheimer disease), the rapid quantitative determination of collagen after myocardial infarction, the diagnosis of arthritis using human synovial fluid samples and the real-time monitoring of tissue oxygenation (e.g. after plastic surgery).

By means of infrared microscopes spatial resolved information with a resolution of a few micrometer can be obtained. Using Focal planar array (FPA) detectors, the biochemical composition of each point of a tissue slices can be investigated within minutes. By statistical methods (e.g. hierarchical cluster analysis) false colour images can be obtained. A pathologist can annotate the result to train a neuronal network. In the end an automatic classification of various tissues is possible. FTIR spectroscopy is, for example used in the diagnosis of skin cancer because one can clearly separate spectral signatures of normal areas of epidermis, of follicle and dermis, and of basal cell carcinoma tumour. **See also**: Alzheimer Disease; Amyloidosis

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