Chapter 8

Molecular Reaction Mechanisms of Proteins Monitored by Time-Resolved FTIR and FT-Raman Spectroscopy

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INTRODUCTION

Time-resolved FT-IR difference spectroscopy has been established as a new biophysical tool for the investigation of molecular reaction mechanisms of proteins at the atomic level. Although infrared spectroscopy is a classical method of analytical and structural investigations [1], it can also provide valuable insight into the mechanisms of chemical reactions. Especially timeresolved Fourier-Transform-Infrared-difference-spectroscopy has proved itself as a powerful method for studies of molecular reaction mechanisms of large proteins up to 120000 Dalton and time resolution up to nanoseconds[2].

The infrared spectrum of a protein is dominated by its peptide backbone amide I (C=O) and amide II (C-N, NH) vibrations (Fig. 1). In addition to the strong amide I and amide II bands water also contributes largely to the background absorption (1650 cm^{-1}).

The major problem in measuring reactions consists in selecting the small absorption bands of the molecular groups which undergo reactions from the large background absorption of water and of the quiescent entire protein. The absorbance bands are selected by the performance of difference spectra between spectra of the protein in its ground state and in an activated state. Such measurements require highly sensitive instrumentation. The FTIR method has two advantages over conventional dispersive IR spectroscopy: the multiplex and the Jaquinot advantage. This allows reliable detection of very small absorption changes with a time resolution of a few nanoseconds.

A further important step is the assignment of the bands to individual groups of the protein. This is performed by using isotopically labelling, for example isotopically labelled retinal (5) or isotopically labelled quinone (9) or by using site directed mutated proteins for example asp96asn (26) or Glu204Gln (25) bacteriorhodopsin

Singh; Infrared Analysis of Peptides and Proteins
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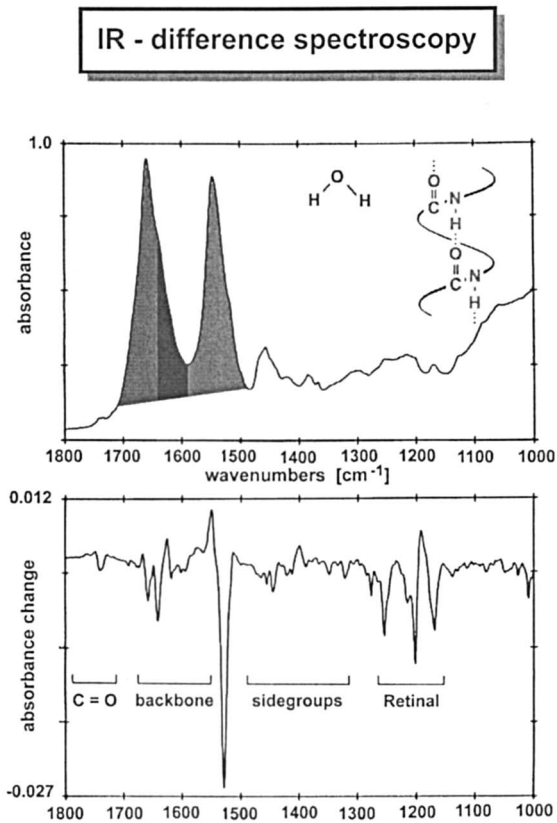

Fig. 1: An IR absorbance spectrum of a hydrated protein (bacteriorhodopsin) and a typical difference spectrum is shown.

mutants. The combination of FTIR with molecularbiology methods allows an unequivocal assignment.

In protein chemistry the macromolecule studied most intensely thus far by time-resolved FT-IR difference spectroscopy is the light-driven proton pump bacteriorhodopsin. Many research groups worldwide have contributed to the understanding of the mechanism of this membrane protein [3]. For reviews on FT-IR studies on bacteriorhodopsin, see references [4,5] and [6]. Time-resolved FT-IR spectroscopy has furthermore provided a detailed picture of the light-induced electron transfer mechanism of bacterial photosynthetic reaction centers. For reviews and recent FT-IR work, see references [7,8] and [9]. Beside photobiological proteins, time-resolved FT-IR spectroscopy can also be applied to proteins without an intrinsic chromophore by using photolabile trigger compounds.[10,11] Using "caged" GTP the molecular GTPase mechanism of the oncogenic protein H-ras p21, a molecule that plays a central role in the growth of cancer cells,[12] can be monitored.[13]

Another example is "caged" Ca^{2+}, by which the molecular mechanism of the Ca^{2+}-ATPase is investigated.[14,15] Furthermore "caged" electrons are used to study redox reactions in cytochrome-oxydase.[16,17]

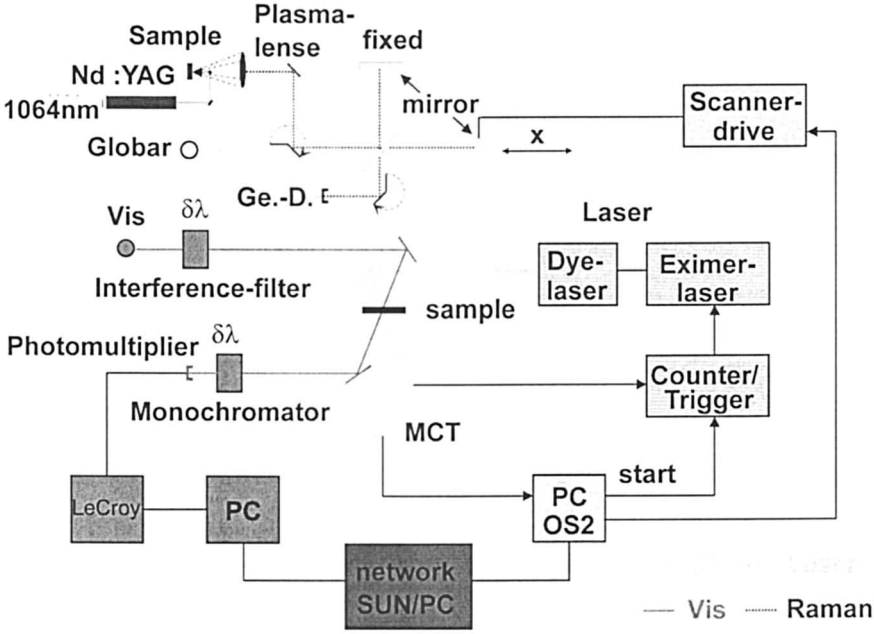

Fig. 2. The experimental setup, consisting of an FT-IR apparatus with globar, beamsplitter, mirrors, controller, detector and preamplifier connected to a 200 kHz and a 200 MHz transient recorder; a photolysis setup with light source, interference filters, monochromator, photomultiplier and transient recorder; and an excimer pumped dye laser system to activate the sample. The VIS and IR data are transfered to a workstation (SUN)/PC network for kinetic analysis.

A typical experimental setup is shown in Fig. 2. This setup allows time resolved FTIR, VIS absorbance change and FT Raman measurements. Various time-resolved FT-IR techniques are developed: the rapid-scan technique, which gives millisecond time resolution[18-20], the stroboscope technique, which gives microsecond time resolution [21,22,] and the step-scan technique, which gives nanoseconds time resolution [23-25]. Here bacteriorhodopsin is presented as an example for the application of time-resolved FTIR spectroscopy to study molecular reaction mechanisms of proteins.

Bacteriorhodopsin

After light-excitation of bacteriorhodopsin's light-adapted ground-state BR_{570} a photocycle starts with the intermediates J_{610}, K_{590}, L_{550}, M_{410}, N_{530} and O_{640} distinguished by the different absorption maxima given by the indices. During the rise of the M-intermediate a proton is released to the extracellular side, and during the M-decay a proton is taken up from the cytoplasmic side. This creates a chemic-osmotic proton gradient across the membrane.

The light-induced BR to K transition

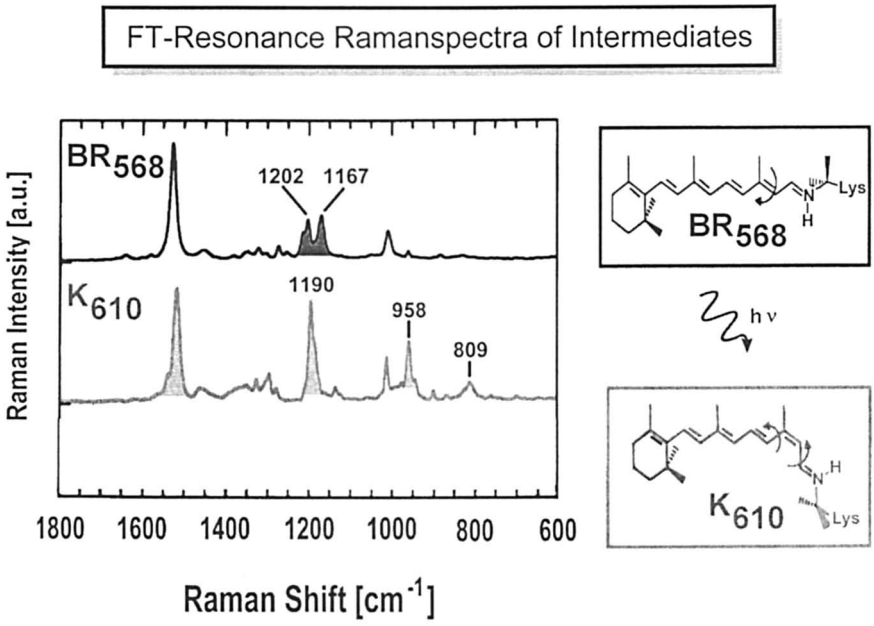

Fig 3 In a) FT-Resonance Raman spectrum of the BR ground state (1202 cm^{-1} C_{14} -C_{15} stretch ; 1167C_{10} - C_{11} stretch) and in b) of the K (1190 cm^{-1}: (C_{14} - C_{15} + C_{10} - C_{11} stretch) intermediate stabilized at 77K is shown.

In Fig.3 a BR and K FT-Resonance-Raman spectrum is shown. Because of the Resonance transition only chromophore bands are observed. The K intermediate is stabilized at 77 K. The band pattern in the 'fingerprint' region between 1300 cm^{-1} and 1100 cm^{-1} is typical of an all-trans (1202 cm^{-1}, 1167 cm^{-1}) configuration in BR and a 13-cis (1190 cm^{-1}) in K [4].The bands at 958 cm^{-1} and 809 cm^{-1} are caused by retinal Hoop (Hydrogen out of plane) vibrations. They are indicative of a strong distortion of the retinal. The BR and K Raman spectra indicate that isomerization of

the chromophore induce tension in the protein that drives the subsequent thermal reactions.

The thermal reactions: K →L→ M→ N→ O→ BR

The absorbance changes accompanying this reaction pathway can be monitored under physiological conditions by the step scan FTIR technique with a time resolution of 30 ns.[25]

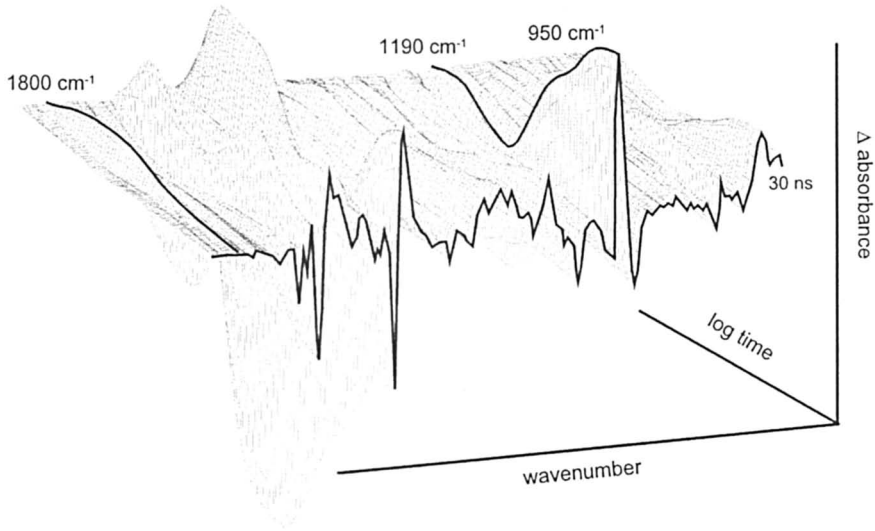

Fig. 4. A three dimensional representation of the IR absorbance changes between 1800 cm^{-1} and 1000 cm^{-1} with 30 ns time-resolution and 3 cm^{-1} spectral resolution accompanying bacteriorhodopsin's photocycle as revealed by a global-fit analysis. The time axis has a logarithmic scale in order to show the complete bacteriorhodopsin photocycle from 30 ns up to 10 ms in one representation.

In fig. 4 the IR absorbance changes between 1800 cm^{-1} and 1000 cm^{-1} during the photocycle are shown in a three-dimensional representation. Beyond a background absorbance of up to one absorbance unit, changes on the order of 10^{-3} to 10^{-4} are monitored with 3 cm^{-1} spectral resolution and 30 ns time-resolution.

In Fig. 5 a typical kinetic, the absorbance change at 1190 cm^{-1}, is shown.

The appearance of the C-C stretching vibration band at 1190 cm^{-1} indicates the all-trans to 13-cis isomerization of retinal. It takes place within 450 fs (compare also the Raman-bands in Fig 3a and b) and is not time resolved here. Its disapperance at

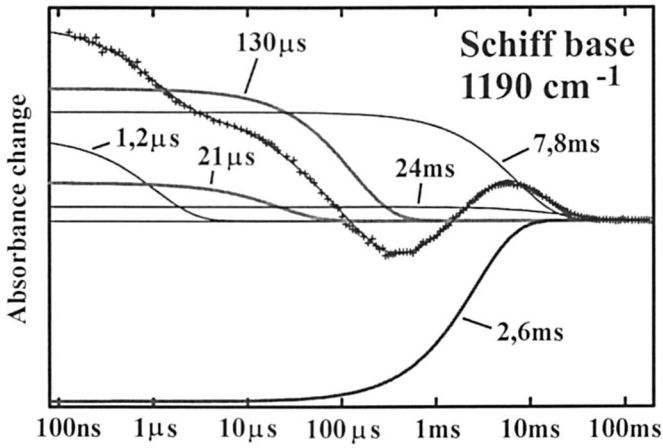

Fig. 5: The absorbance change at 1190 cm^{-1} from fig. 4 is shown in dependance of time. The results of a multi-exponential analysis which yields the rate constants and the respective amplitudes are also presented.

about 200 µs indicates the deprotonation of the Schiff base. This loss of charge of the Schiffbase greatly reduces the IR-absorbance of the chromophore. (The Schiff base connects the retinal to Lys216 of the protein (fig. 6).) The deprotonation kinetics of the Schiff base agree nicely with the protonation kinetics of the counterion Asp85, which can be followed at 1761 cm^{-1} [20] (fig. 4). Recently, for the protonrelease pathway to the protein surface a protonated hydrogen bonded network in a Grothuß like proton transfer mechanism is identified (fig6)[25]. The reappearance of the band at 1190 cm^{-1} in the millisecond time domain indicates the reprotonation of the Schiff base. It is reprotonated by Asp96 [20,27]. The final disappearance at 1190 cm^{-1} shows the chromophore's relaxation to the all-trans BR-ground state configuration.

Based on the FTIR experiments a detailed model of the lightdriven proton pump bacteriorhodopsin is elucidated and presented in fig. 6.

The presented results should demonstrate that time resolved FTIR difference spectroscopy in combination with molecular biological methods allows a detailled analysis of the molecular reaction mechanism of proteins. In future work the use of caged compounds will provide a powerfull tool also for non-photobiological proteins.

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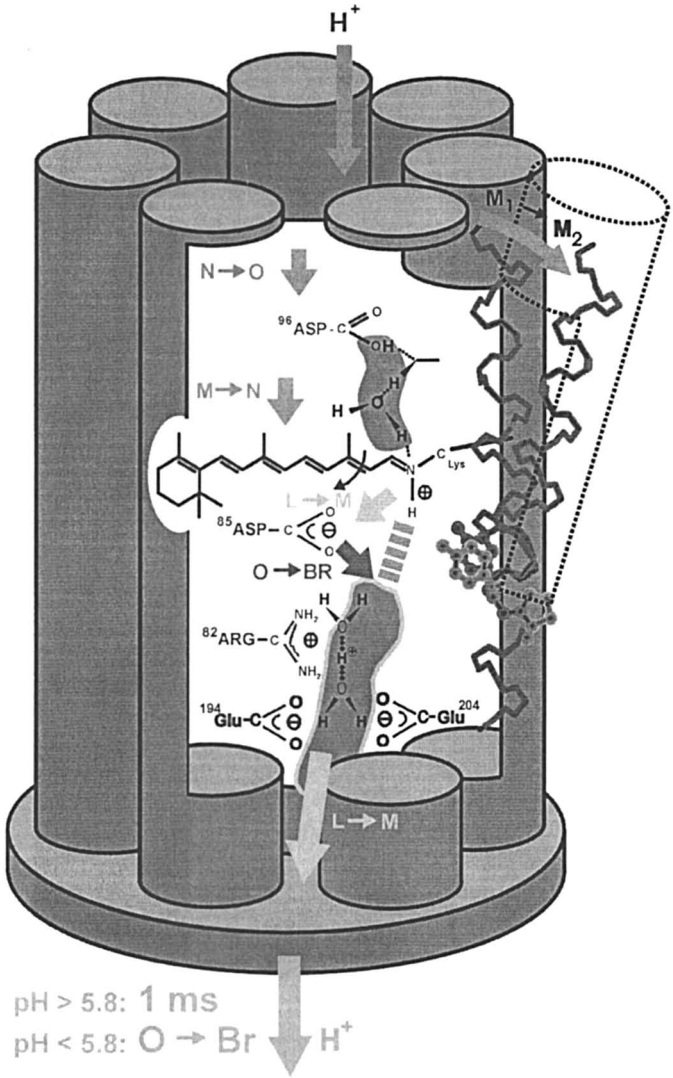

Fig. 6. The current model of the proton pump mechanism of bacteriorhodopsin, (for references, see text): After the light-induced all-trans to 13-cis retinal isomerization in the BR to K transition, the Schiff base proton is transfered to Asp85 in the L to M transition. Simultaneously a proton is released from a protonated H-bonded network spanned by internal water molecules to the extracellular site. This network is controlled by Arg 82, Glu204 and Glu194. Asp85 reprotonates the network in the O to BR reaction. The Schiff base is oriented in the M_1 to M_2 transition from the proton release site to the proton uptake site by a small backbone movement of helix F and determines thereby the vectoriality of proton pump. A larger backbone movement is observed in the M to N transition as compared to the M_1 to M_2 transition. Asp96 reprotonates the Schiff base in the M to N transition. Asp96 itself is reprotonated from the cytoplasmic site in the N to O transition.

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