Minireview

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

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Time-resolved FTIR difference spectroscopy can provide a valuable insight into the molecular reaction mechanisms of proteins, especially membrane proteins. Isotopic labeling and site-directed mutagenesis allows an unequivocal assignment of IR absorption bands. Studies are presented which give insight into the proton pump mechanisms of proteins, especially bacteriorhodopsin. H-bonded network proton transfer via internal water molecules seems to be a general feature in proteins, also found in cytochrome c oxidase. Using caged GTP the intrinsic and GAP catalyzed GTPase activity of H-ras p21 is studied. Furthermore, protein folding reactions can be recorded with ns time-resolution.

Key words: Bacteriorhodopsin / Caged GTP / FTIR / H-ras p21 / Proton transfer.

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 (Gerwert et al., 1992).

The infrared spectrum of a protein is dominated by its peptide backbone amide I (C=O) and amide II (C–N, NH) vibrations in Figure 1 at 1660 cm⁻¹ and 1550 cm⁻¹. In addition to the strong amide I and amide II bands water also contributes largely to the background absorption (1650 cm⁻¹) (Colthup et al., 1990). 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.

A further important step is the assignment of the bands to individual groups of the protein. This is performed by using isotopic labeling or by using site-directed mutated proteins. Also individual residues and the peptide chain can be site-detected isotopically labelled via genetic techniques (Ludlam et al., 1995). This combination of FTIR with molecular biology 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 (Grigorieff et al., 1996). For reviews on FT-IR studies on bacteriorhodopsin, see Braiman et al. (1988); Gerwert (1992) and Siebert (1993). 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 Robert et al. (1989), Mäntele (1993) and Brudler et al. (1994). Besides photobiological proteins, time-resolved FT-IR spectroscopy can also be

Fig. 1. An IR Absorbance Spectrum of a Hydrated Protein (Bacteriorhodopsin) and a Typical Difference Spectrum.
applied to proteins without an intrinsic chromophore by using photolabile trigger compounds (McGray et al., 1989; Cepus et al., 1998). Using ‘caged’ GTP the molecular GT-Pase mechanism of the oncogenic protein H-ras p21, a molecule that plays a central role in the growth of cancer cells (Wittinghofer and Pai, 1991), can be monitored (Cepus et al., 1998). Another example is ‘caged’ Ca²⁺, by which the molecular mechanism of the Ca²⁺-ATPase is investigated (Barth et al., 1991; Trouillier et al., 1996). Furthermore ‘caged’ electrons are used to study redox reactions in cytochrome oxidases (Lübben et al., 1996, 1999).

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Fig. 2 Schematic Representation of the FTIR Experimental Setup.
The FT-IR apparatus is equipped 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 transferred to a workstation (SUN)/PC network for kinetic analysis.

Fig. 3 IR Absorbance Changes during One Photocycle of Bacteriorhodopsin.
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.
A typical experimental setup is shown in Figure 2. This setup allows time-resolved FTIR, VIS absorbance change and FT Raman measurements. Various time-resolved FT-IR techniques have been developed: the rapid-scan technique, which gives millisecond time resolution (Braiman et al., 1987; Gerwert, 1988), the stroboscope technique, which gives microsecond time resolution (Gerwert et al., 1990; Braiman et al., 1991; Souvignier et al., 1992) and the step-scan technique, which gives nanosecond time resolution (Uhmann et al., 1991; Palmer et al., 1991; Rammelsberg et al., 1997). Here bacteriorhodopsin is presented as an example.

**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 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 (Rammelsberg et al., 1997).

In Figure 3 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.

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 and is not time resolved here. Its disappearance at about 200 µs indicates the deprotonation of the Schiff base. This loss of charge of the Schiff base greatly reduces the IR-absorbance of the chromophore. [The Schiff base connects the retinal to Lys216 of the protein (Figure 4).] The deprotonation kinetics of the Schiff base agree nicely with the protonation kinetics of the counterion Asp85, which can be followed at 1761 cm$^{-1}$ (Gerwert et al., 1989) (Figure 4). Recently, for the proton release pathway to the protein surface a protonated H-bonded network has been identified (Rammelsberg et al., 1998). 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 (Gerwert et al., 1989; le Coutre et al., 1995). The final disappearance at 1190 cm$^{-1}$ shows the chromophore’s relaxation to the all-trans BR-ground state configuration.

**H-ras p21**

The small GTP-binding protein H-ras p21 plays a central role in signaling leading to cell-growth (Wittinghofer and Pai, 1991). P21 appears to act as a switch: in the GTP bound form a signal is given to an effector molecule which is part of a cascade leading to cell proliferation and differ-
The intrinsic GTPase activity is accelerated by GAP (GTPase activating protein). The transition from the active GTP bound to the inactive GDP bound form is accompanied by a conformational change. In order to specify in more detail the structural changes occurring in the GTP to GDP reaction, we have started complementary to the X-ray studies investigations by time-resolved FTIR difference spectroscopy.

The reaction is studied using caged GTP complexed to p21 instead of GTP to prevent hydrolysis. Using an intensive UV flash (308 nm, 100 mJ) the caged group is photolyzed, GTP released and GTPase activity is precisely started. In Figure 5 difference spectra between p21-GTP and finally p21-GDP are shown. The region between 1300 and 900 cm$^{-1}$ is dominated by phosphate bands. Using isotopically labeled caged GTP the phosphate vibrations are assigned, for example the band at 1144 cm$^{-1}$ is assigned to the $\gamma$-Phosphate vibration (Cepus et al., 1998). Its absorbance change can be used as marker hand for the GTPase kinetics.

The results on p21 show that molecular reaction mechanisms of proteins without intrinsic chromophore can now be investigated with time-resolved FTIR. In future studies, in addition to the intrinsic mechanism of p21 the GAP catalyzed GTPase mechanism and the interaction with the downstream effector Raf will be examined.

The presented results demonstrate that time resolved FTIR difference spectroscopy in combination with molecular biological methods allows a detailed analysis of the molecular reaction mechanism of proteins. The use of caged compounds provides a powerful tool also for non-photobiological proteins.

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