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Time-resolved FTIR spectroscopy for monitoring protein dynamics exemplified by functional studies of Ras protein bound to a lipid bilayer

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ABSTRACT

Time-resolved Fourier transform infrared (FTIR) difference spectroscopy is a valuable tool for monitoring the dynamics of protein reactions and interactions. Absorbance changes can be monitored with time resolutions down to nanoseconds and followed for time periods that range over nine orders of magnitude. Membrane proteins bound to solid supported lipid bilayers can be investigated in near physiological conditions with the attenuated total reflection (ATR) technique. Here, we review the basics of time-resolved FTIR with a focus on Ras, a GTPase that is mutated in 25% of human tumors. We show the first time-resolved measurements of membrane anchored Ras and observed the switching between its activated and its inactivated state. We compared those measurements with measurements of the truncated Ras in solution. We found that both the kinetics and the functional groups involved were very similar. This suggested that the membrane did not have a major influence on the hydrolysis reaction.

1. Outline

This paper combines a broad introduction into time resolved FTIR spectroscopy with new results on membrane bound Ras proteins. Time-resolved FTIR spectroscopy is reviewed in several books [1,2] and journals [3,4]. In the following Section 2, we show the basics and the setup of a FTIR spectrometer, including various sample cells, and the concept of difference spectroscopy. We discuss triggering techniques and both the rapid scan and the step scan technology followed by methods for data evaluation and band assignment. This chapter ends with a discussion on ATR–FTIR spectroscopy. In the next Section 3, we introduce the GTPase Ras and highlight results obtained by time resolved FTIR measurements of Ras in solution. Next (Section 4), we describe the first time resolved experiments of a GTPase in its native environment, anchored at a membrane by ATR–FTIR spectroscopy. These new results and the new experimental capabilities of this novel setup are discussed. Experimental details are given in the final Section 5.

2. Introduction into time-resolved FTIR spectroscopy

2.1. General setup of a time-resolved FTIR spectrometer

A typical setup for a time-resolved FTIR experiment is shown in Fig. 1A. The light source is a globar (SiC heated to 1800 K), which is a black body radiator. Its infrared light passes through an aperture (0.25–12 mm) before entering a Michelson interferometer, which consists of a beamsplitter (KBr for mid-infrared), a fixed mirror, and a movable mirror. Subsequently, the light passes through the sample chamber, which can be equipped, e.g., with a thermostatic transmission cell. This cell can additionally be irradiated with a laser. Finally, the infrared light reaches a liquid nitrogen-cooled MCT (mercury, cadmium and telluride, HgCdTe)-detector.

FTIR spectrometers have crucial advantages over dispersive spectrometers. With a Michelson interferometer (Fig. 1B), all wavelengths can be measured in parallel (multiplex advantage). At the beamsplitter, one half of the infrared light is reflected to a fixed mirror, and the other half is transmitted to a movable mirror. Both beams are reflected back and recombined at the beamsplitter. The positions of the fixed and movable mirrors can be at different distances from the beamsplitter; this leads to a path difference (ΔX). For example, a path difference of zero between two

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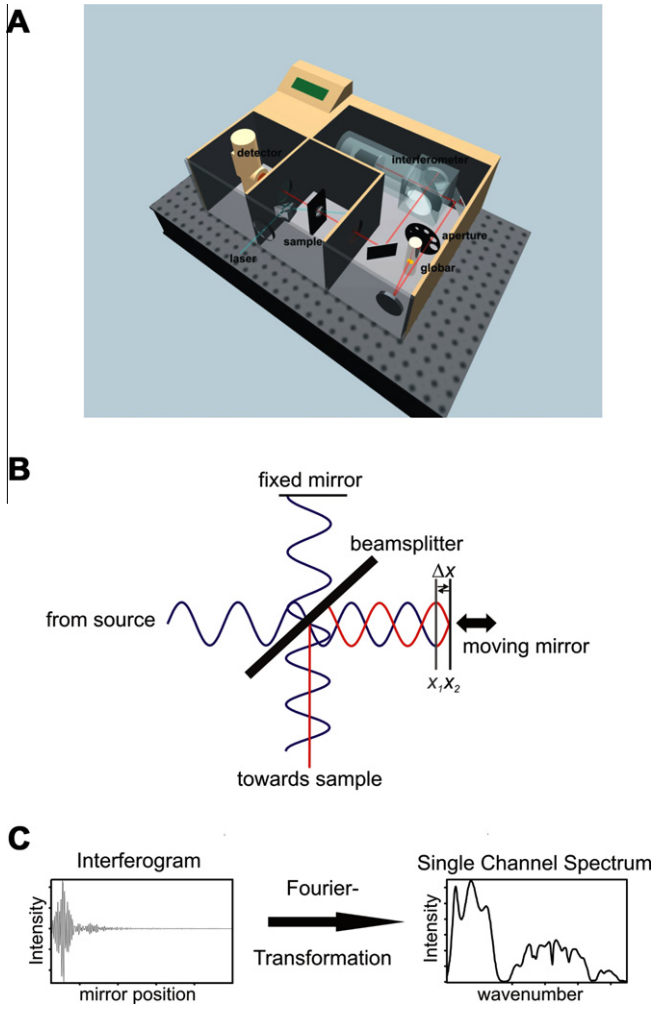

Fig. 1. (A) Schematic of an FTIR spectrometer on a vibration isolation table [73]. (B) Schematic representation of a Michelson interferometer. An electromagnetic wave is split at the beamsplitter; one half is reflected to a fixed mirror, the other half is transmitted to a movable mirror. Both parts are recombined at the beamsplitter. For mirror position X_1, the path difference, ΔX, is zero and the wave interferes constructively; for mirror position X_2, the path difference, ΔX, is half a wavelength, and thus, the inference is destructive. (C) The result of the measurement is an interferogram, where the intensity is plotted against the mirror position. After Fourier transformation, the intensity I is obtained as a function of the wavelength (single channel spectrum) [74].

monochromatic waves (movable mirror at position X_1) leads to constructive interference; a path difference of half a wavelength (movable mirror at position X_2) leads to extinction. By varying the path difference (by moving the movable mirror), the interference pattern of polychromatic light after recombination leads to an interferogram. The interferogram is a plot of the intensity at the detector against the mirror position. After Fourier transformation, the intensity I as a function of the wavelength is obtained (single channel transmittance spectrum, Fig. 1C). An absorbance spectrum A is obtained by comparing two single channel transmittance spectra, one with a sample (I) and one without a sample (I_0), according to the following equation:

$$A = -\log\frac{I}{I_0}.$$

With modern FTIR spectrometers, a complete spectrum can be obtained within 10 ms. Further advantages of FTIR spectrometers are the absence of dispersive elements (slits combined with prisms or gratings that attenuate the signal intensity; the Jaquinot advantage), and the high accuracy of the wavelength (Connes advantage).

Some sample cells are shown in Fig. 2. The most common cell is a simple transmission cell with IR-transparent windows (e.g., CaF_2) as shown in Fig. 2A. Due to the high absorptivity of water in the mid-infrared spectral region, meaningful spectra of hydrated proteins can only be obtained by transmission measurements through very thin (2–10 μm) films. This involves placing a drop of protein in suspension or solution onto an IR transparent window and then carefully concentrating it under a nitrogen stream or under vacuum. Alternatively, a suspension of a membrane–protein can be centrifuged and the pellet can then be squeezed between two IR transparent windows. A typical measurement requires about 100–200 μg protein. The concentration of the protein in the film is 2–10 mM. The sample chamber is closed with a second IR-window, which is separated from the first by a mylar-spacer that is a few μm thick.

Alternatively, ATR cells (Fig. 2B) can be used instead of transmission cells [5,6] (For details see Section 2.7). Both transmission cells and ATR-cells can be used as flowcells (Fig. 2C). Here, the sample can be exchanged with another sample by means of a tubing system. This can increase the quality of the difference spectra enormously, because the whole setup (sample thickness, window position, etc.) can be maintained exactly the same for different samples.

Silicon is transparent in the mid-infrared range. Therefore, micromachined silicon components offer great potential for FTIR spectroscopy, particularly for studying microsecond mixing experiments [7]. In these devices, a protein solution is placed between two streams of mixing buffer to form a laminar flow pattern. Because the protein layer is thin, diffusing reactant molecules (e.g., ligands) stream from the buffer solution into the protein solution within microseconds. The induced reaction can be monitored with high time resolution by scanning along an observation channel downstream of the mixing area with the focused beam of a FTIR microscope.

Fig. 3 shows the absorbance spectrum of a protein. A small protein of 20 kD has about 10^4 absorbance bands in the infrared region. Thus, from the absorption spectrum alone, one cannot obtain information on individual bands; it is only possible to determine global features of the protein. The spectrum is dominated by amide I (C=O stretch) and amide II (NH bend coupled with C–N stretch) bands, which are present in every amino acid. From this backbone absorption, information on the secondary structure can be gained [8,9]. Water absorptions (O–H bend) are found in the same region as amide I.

For a FTIR difference spectrum of reaction A → B, one calculates the absorbance spectrum of B minus the absorbance spectrum of A. Thus, the vibrations from groups that did not change during the reaction will cancel each other out, and the remaining bands represent only the changes in absorbance that occurred during the reaction (Fig. 3, inset). Now, individual residues can be resolved. It is important that the same conditions are accurately maintained during the reactions; otherwise the background, with a $\sim 10^3$ times stronger absorbance, will obscure the difference spectrum. Highly sensitive instrumentation is required to monitor these very small changes. FTIR spectroscopy is able to reliably detect small changes, due to the multiplex and the Jacquinot advantages, which increase the signal to noise ratio.

2.2. Trigger techniques for time-resolved FTIR

The changes in absorbance due to a reaction are several orders smaller than the background absorbance of the protein; therefore, the difference technique requires a sharp initiation (triggering) of the protein reaction, and this activation must be achieved without