Investigation of Molecular Mechanisms of Transporters and Receptors in Membrane by Using Stimulus-Induced Difference FT-IR Spectroscopy

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The cell, the elementary unit of life, uptakes nutrient and exhausts waste. Its ion balance between inside and outside of cell membrane is adjusted strictly. These works are performed by membrane proteins such as channels and transporters. There are some other membrane proteins such as cell sensor proteins which detect various environmental changes. A main goal of my group is to clarify molecular mechanisms of transporters and receptors in cell membrane mainly by using stimulus-induced difference infrared spectroscopy which is sensitive to structural and environmental changes of protein molecules.

I have moved to IMS in this March and have been setting up my lab. In this review article, I'd like to present my recent studies done in my previous work place, Nagoya Institute of Technology, and progress in a recent research by using a new FT-IR spectrometer installed in IMS.

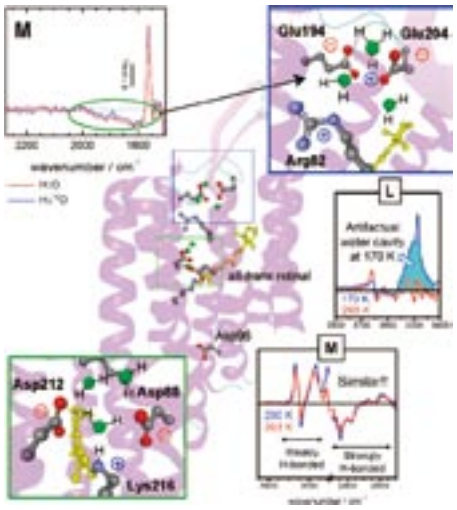

Figure 1. X-ray crystal structure of bacteriorhodopsin and O–H stretching vibrations observed in its photocycle. Several water molecules exist in the protein cavities and play an important roll for pumping protons.

1. Time-Resolved FT-IR Spectroscopy Detecting O–H and O–D Stretching Vibrations of Internal Water Molecules in a Light-Driven Proton Pump Protein, Bacteriorhodopsin

Bacteriorhodopsin (bR) is one of well known light-driven proton pump protein, which has an all-*trans* retinal covalently linked to Lys216 via a protonated Schiff base (SB). Upon light absorption, photo-isomerization occurs from the all-*trans* to 13-*cis* form in less than one picosecond, followed by a cyclic reaction that comprises a series of intermediates, called as the K, L, M, N, and O states, back to the bR ground state (BR). During the bR photocycle the first proton transfer reaction takes place in the L to M transition. A proton is transferred from the protonated SB to Asp85, together with the apparently simultaneous release of a proton from the so-called proton release group (PRG), which is composed of water molecules, Glu194 and Glu204, to the extracellular side. Further proton transfers take place in the later intermediates (accompanied by other protein and retinal structural changes), leading to a net proton transport from the cytoplasmic to the extracellular side, and the recovery of the bR ground state.

By use of time-resolved FT-IR spectroscopy with bR film samples moderately hydrated with water, H_2O, or isotope labeled water, $H_2{}^{18}O$, infrared difference spectra in O–H

stretching region were successfully collected and some of the bands were assigned to the internal water molecules (Figure 1). Especially, we firstly gave experimental evidence that the continuum band in the 2000–1800 cm^{-1} region was originated from the protonated water cluster which probably locates in the extracellular side.[1)] It was also elucidated that water structure formed in the L intermediate at room temperature is different from that trapped in low temperature.

Now, I try to extend observable spectral range to the region around peak of water absorption, 3400 cm^{-1} in H_2O and 2400 cm^{-1} in D_2O. By use of D_2O and optimizing hydration level, accurate light-induced difference spectra were collected (Figure 2). In the X–H and X–D stretching region, structural changes in the hydrophobic and hydrophilic part were separately observed, respectively. By analyzing these spectral changes, real-time hydrogen-bonding changes of the internal water molecules and protein moiety of bR will be elucidated, leading to better understand of the light-driven proton pumping mechanism of bR.

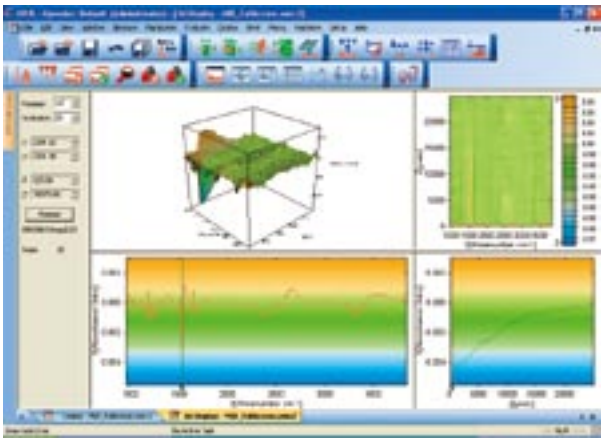

Figure 2. Time-resolved FT-IR spectra of bacteriorhodopsin in the whole mid-infrared region. By use of D_2O and optimizing hydration level, plausible spectral changes were observed.

2. Perfusion-Induced Difference FT-IR Spectroscopy Investigating Ion Binding Sites of Membrane Proteins

Membrane proteins are important for homeostasis of living cells, which work as ion channel, ion pump, various types of chemical and biophysical sensors, *etc*. These proteins are considered as one of important targets for biophysical studies. However, their molecular mechanisms have not been studied well, because X-ray crystallography and NMR spectroscopy are hard to access them in general. Recently, it has been demonstrated that stimulus-induced attenuated total reflection (ATR) FTIR spectroscopy is another promising technique for investigating molecular mechanism of membrane proteins.

In 2009 before coming to IMS, I have demonstrated molecular mechanism of the proton releasing group (PRG) in *pharaonis* phoborhodopsin (*p*pR) by means of Cl^--induced and light-induced difference ATR-FTIR spectroscopy in aqueous condition.[2)] *Pharaonis* phoborhodopsin (*p*pR, also called *pharaonis* sensory rhodopsin II; *p*SRII) is a photoreceptor for negative phototaxis in *Natronomonas pharaonis*, and in the absence of transducer protein, *p*HtrII, *p*pR can pump protons like BR. Fast, BR-like proton release was observed during the lifetime of the M intermediate (ppR_M) at acidic pH, but it was diminished in the absence of Cl^-. It was suggested that Cl^- binding controls the pKa of PRG in *p*pR and ppR_M.

As shown in Figure 3, Cl^--induced difference ATR-FTIR spectra clearly demonstrated that Cl^--binding to *p*pR accompanies protonation of a carboxylic acid (C=O stretch at 1724 cm^{-1}). The amino acid was identified as Asp193, because the corresponding band is shifted to 1705 cm^{-1} in the D193E mutant protein. It means that the PRG of *p*pR includes Asp193, whose pKa change is partly controlled by Cl^-.

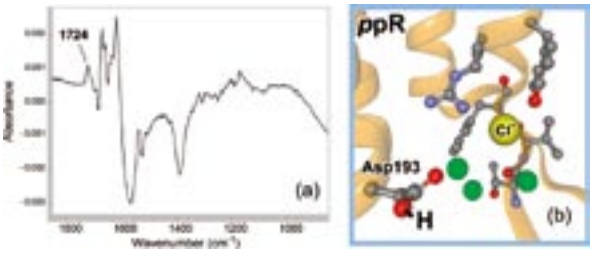

Figure 3. (a) Chloride-ion binding induced difference infrared spectra of a membrane protein (*pharaonis* phoborhodopsin; light sensor in an archaebacterium). The 1724-cm^{-1} band was assigned to the protonated carboxylate group of Asp193. (b) The existence of hydrogen at Asp193, which has not been detected by X-ray crystallography, was revealed by infrared spectroscopy.

Application of ATR-FTIR to other membrane proteins, such as an ion channel (KcsA) and a transporter protein (V-ATPase), started before coming to IMS. It has been succeeded to measure the difference infrared spectra between the conditions of several types of ions and pH. Based on these spectra, the molecular mechanism of recognition and transportation of ions will be discussed in the near future.

At IMS, I will develop new methods of stimulus-induced ATR-FTIR spectroscopy by constructing stopped-flow system or using caged compounds for improving time-resolution. Reduction of buffer waste is also important for using isotope labeled water or expensive reagents, which will be achieved by designing a micro-flow chamber.

References

1) V. A. Lórenz-Fonfría, Y. Furutani and H. Kandori, *Biochemistry* **47**, 4071–4081 (2008).

2) Y. Kitade, Y. Furutani, N. Kamo and H. Kandori, *Biochemistry* **48**, 1596–1603 (2009).