Investigation of Molecular Mechanisms of Channels, Transporters and Receptors in Membrane

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Membrane proteins are important for homeostasis of living cells, which work as ion channels, transporters, various types of chemical and biophysical sensors, and so on. These proteins are considered as important targets for biophysical studies.

Our main goal is to clarify molecular mechanisms of channels, transporters and receptors in cell membrane mainly by using stimulus-induced difference infrared (IR) spectroscopy, which is sensitive to the structural and environmental changes of organic- and bio-molecules. Recently, Dr. Kimura started to construct a microfluidic device to monitor biological and chemical reactions by infrared and fluorescent microscopic techniques. Dr. Tsukamoto has established a protein expression system with mammalian cell line.

1. Anion-Transport Mechanism of a Chloride Ion Pump *p*HR Studied by Time-Resoved FTIR Spectroscopy

pharaonis Halorhodopsin (*p*HR) is a light-driven inward chloride ion pump protein. The ion transportation is performed through the sequential formation of several intermediates (K, L_1, L_2, N and O) during the photocyclic reaction. The structural details of each intermediate state have been studied by various kinds of physicochemical methods, however it is still open question how the structural changes of protein molecule and water molecules are involved in the translocation of a chloride ion inside protein at physiological temperature. To analyze the structural dynamics, we performed the time-resolved Fourier transform infrared (TR-FTIR) spectroscopy in the whole mid-infrared region under various hydration conditions. Measurements under D_2O reveal the structural information of the water inaccessible backbone of protein itself, and those with H_2O or $H_2{}^{18}O$ give an insight on the dynamics of water molecules inside protein. We concluded that the chloride ion release and uptake occurring in the N and O intermediate states are accompanied by the drastic conformational changes in the water-inaccessible transmembrane-helices and the hydrogen- bonding network rearrangement of the internal water molecules in *p*HR (*manuscripts in preparation*).

2. Time-Resolved Difference FTIR Spectroscopy Triggered by Rapid Buffer-Exchange

Attenuated total reflection (ATR) FTIR spectroscopy is a powerful technique to obtain infrared spectra of membrane proteins immersed in aqueous solution.[1)] By exchanging buffer with and without salts, the difference spectra between the two conditions provide the structural information relating to the interaction between protein and ions. In this year, we executed the construction of the kinetic ATR FTIR spectroscopic system, which can follow the chemical and biological reaction triggered by the rapid buffer-exchange, in collaboration with Mr. K. Okamoto in UNISOKU Co. Ltd.

We have developed the photo-triggering system for time-resolved FTIR measurements, but the application of this system has been limited mainly to the photo-inductive chemical reactions. Rapid buffer exchange (<1 ms) allows us to monitor the early stage of reactions, such as substrate or ion binding. For further improvement of the time-resolution, we will construct the flow-flash system.

Figure 1. Picture of the rapid buffer-exchange system.

3. Development of a Microfluidic Device to Monitor Biological and Chemical Reactions

Real-time observation is one of the powerful techniques to understand the molecular mechanisms of the self-organization

and molecular association. The solution mixing technique realizes many reaction fields for biological and chemical reactions by changing the buffer condition and can be combined with spectroscopic equipment easily. However, conventional mixing techniques limit their targets because of the large consumption of the sample and the stress of the turbulent mixing. We are developing a novel microfluidic device and trying to reduce both the sample consumption to ~1/1000 and the stress by using sheath flow.

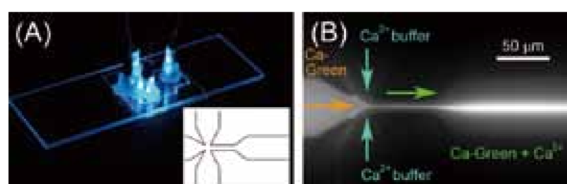

Figure 2. Pictures of the developed microfluidic mixer. (A) Picture of the mixer on the XY-stage of the fluorescence microscope. (inset) Schematic figure of the designed flow channels. (B) Fluorescence image of the Ca-Green mixed with the Ca^{2+} buffer.

Microfluidic devices with the flow channels of micrometer scale are made of a single cast of poly(dimethylsiloxane), PDMS, with the photolithography technique. These devices were combined with the fluorescence microscope and the complete mixing with the time resolution of ~20 µs was confirmed by the fluorescent intensity measurements. We are attempting to combine these devices with FTIR or other fluorescence spectroscopy.

The devices were manufactured with the strong supports of Equipment Development Center of IMS (especially Ms. N. Takada and Mr. M. Aoyama).

4. Investigation of Membrane Proteins Which Are Hardly Expressed in *E. coli*

Heterologous expression system using *E. coli* is established and easy way to obtain large amount of purified proteins, but in general, expression of membrane proteins, in particular mammalian ones, is very difficult. In this year, we have tried to establish a protein expression system using cultured mammalian cells for IR spectroscopic analyses of various membrane proteins, including mammalian ones.

In order to study structure-function relationship using purified proteins, it is very important to select target proteins that can be highly expressed in cell lines. Traditionally the screening of membrane proteins is very painful and time-consuming. However, a powerful method named fluorescence-detected size-exclusion chromatography (FSEC) was recently developed (Kawate and Gouaux, 2006). FSEC method evaluates expression level and properties of target proteins using GFP-tag and size-exclusion chromatograph even without purification (Figure 3). We set up an FSEC system and expressed various membrane proteins with GFP tag to select appropriate proteins. Also, we tried to purify screened proteins and apply them to IR spectroscopic analyses.

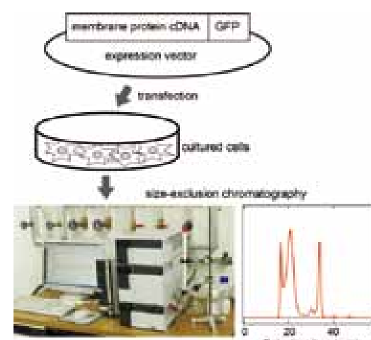

Figure 3. Scheme of the fluorescence-detected size-exclusion chromatography (FSEC).

Channelrhodopsins (collaboration with Prof. Yawo's group in Tohoku Univ.)

Channelrhodopin is a light-gated cation channels, which is originally identified in *Chlamydomonas*. Currently this protein is used as a tool for "optogenetics" which enables excitation of specific neural cells by irradiation. Prof. Yawo's group has developed several interesting channelrhodopsin mutants that show unique electrophysiological properties. Prof. Yawo kindly provided constructs of the channelrhodopsin mutants to us, and we screened the mutants using FSEC method. Several mutants can be expressed efficiently in mammalian cells, and can be purified. Also, we have already succeeded to measure the difference IR spectra before and after photo-activation of the channelrhodopsins. We are now trying to analyze the spectra and extend to time-resolved measurements with 12.5 µs resolution to reveal real-time analyses of open-close mechanism of the channel protein. (collaboration with Prof. Nureki's group in Univ. of Tokyo)

Mammalian potassium channels (collaboration with Prof. Kubo's group in National Institute for Physiological Sciences (NIPS))

Mammalian potassium channels play important roles in various organs, including brain, heart, kidney and retina. Prof. Kubo's group has revealed structure-function relationship of these channels mainly using electrophysiological method. Prof. Kubo kindly provided constructs of various mammalian potassium channels to us. We tested properties of these channels expressed in cultured cells using FSEC, and found several inwardly rectifying potassium channels can be expressed efficiently and show appropriate properties. In addition, we have just successfully purified the channels. Currently we are trying to measure IR spectra of the purified channels.

Reference

1) Y. Furutani, T. Murata and H. Kandori, *J. Am. Chem. Soc.* **133**, 2860–2863 (2011).

Award

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