Effect of Molecular Assembly on Photocycle of Reconstituted Bacteriorhodopsin: Significant Blue Shift of the Late M Photointermediate in the Liquid Crystalline Phase

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The photocycle of bacteriorhodopsin (bR) reconstituted into dimyristoylphosphatidylcholine vesicles was investigated with transient visible absorption spectroscopy. The measured time-resolved difference spectra indicated that two substates of the M intermediate with almost the same absorption maximum were observed in the gel state, whereas the spectrum of M showed a splitting into an early and a late component shifted by approximately 15 nm in the liquid crystalline phase, suggesting disassembly of bR molecules induces remarkable structural changes around the retinal pocket of the late M intermediate responsible for switching protein conformation from a proton release form to a proton uptake form.

Purple membrane (PM) from *Halobacterium salinarum* is the two-dimensional (2D) crystalline molecular assembly of trimers of a sole membrane protein, bacteriorhodopsin (bR), and several kinds of lipids.[1] The membrane protein functions as a light-driven proton pump, and proton translocation across the membrane is accomplished through several intermediates in a photocycle that is initiated by absorption of visible light by retinal bound to Lys-216 via a Schiff base linkage.[2] Recently we have found that even in the absence of hydrolyzing reagents like hydroxylamine, bR molecules undergo irreversible photobleaching to bacterio-opsin and retinal by continuous irradiation of visible light, when heated at temperatures above 60 °C in the PM[3] and solubilized with mild nonionic detergents.[4] These results strongly suggest that protein–protein interaction in the 2D crystals is essential for recovery of the initial state after the transitions between several photointermediates. In order to address effects of bR molecular assembly on photointermediates, in this study, the photocycle of bR reconstituted into dimyristoylphosphatidylcholine (DMPC) vesicles has been investigated with transient visible absorption spectroscopy in the gel and the liquid crystalline phase, since it is known that the 2D crystalline bR molecules disassemble through the phase transition of DMPC bilayer.[5]

DMPC was purchased from Avanti Polar Lipids, Inc. Triton X-100 and other chemicals were purchased from Wako Pure Chemical Industries, Ltd. All materials were used without further purification. PM from *H. salinarum*, strain R1M1, was isolated and purified by using standard procedure proposed by Oesterhelt and Stoeckenius.[6] The purified PM was suspended in 50 mM phosphate buffer (pH 7.2). Reconstitution of bR into DMPC vesicles was carried out as follows: PM was solubilized with 10 mM Triton X-100 at 25 °C for 3 h in the dark. The solubilized sample was obtained as the supernatant after 1 h ultracentrifuge at 105000 *g*.[5a] The solublized bR, which is highly stable in the dark below 50 °C,[4b] was mixed with DMPC suspension at the molar ratio of 1:150. After the incubation of the bR/DMPC/Triton X-100 suspension for 4 h at 25 °C in the dark, detergents were removed by addition of polystyrene Bio-Beads-2 purchased from Bio-Rad, which resulted in turbid purple suspension. When the precipitate after ultracentrifuge of the suspension at 105000 *g* was resuspended in phosphate buffer, visible absorption was observed at ca. 550 nm, while the supernatant showed no absorption at ca. 550 nm. Static UV–vis absorption spectra were recorded with a Beckmann Coulter DU-7500 spectrophotometer. A JASCO J-820 spectropolarimeter was employed for measurements of circular dichroism (CD) spectra in the visible region. Transient visible absorption difference spectra at the time-resolution of ca. 10 μs was measured with a computer-controlled flash-photolysis apparatus constructed by Kikukawa et al.[7] Absorption maximum of photointermediates was obtained by curve-fitting of difference spectra with skewed Gaussian functions.[8]

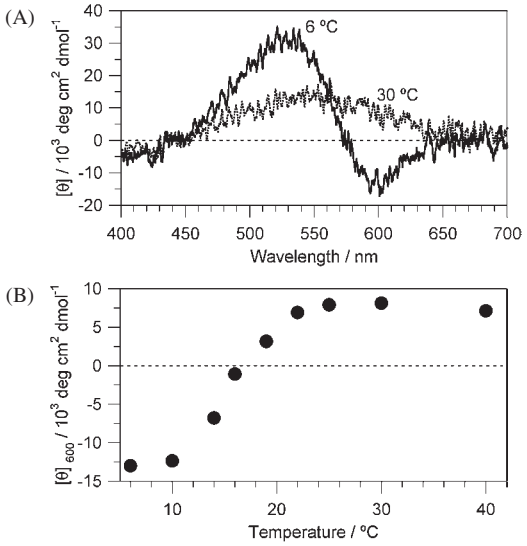

Figure 1. (A) CD spectra and (B) temperature-dependent molar ellipticity changes at 600 nm of reconstituted bR.

The wavelength of UV–vis absorption maximum of reconstituted bR was almost constant at ca. 550 nm in the temperature range studied (data not shown). Disassembly of bR molecules in DMPC vesicles by the phase transition of lipid bilayer was monitored with visible CD spectroscopy. Representative spectra at 6 and 30 °C are shown in Figure 1A. The CD spectrum at 6 °C shows an asymmetric bilobed pattern that is very similar to that of native PM, indicating trimeric assembly of bR molecules in DMPC vesicles in the gel phase. On elevation of temperature to 30 °C, however, the bilobed CD spectrum became positive,

as shown in Figure 1A. The CD pattern demonstrates that bR molecules disassemble into protomer when DMPC bilayer forms the liquid crystalline phase. This is in good agreement with previous studies.[5] Temperature-dependent changes of oligomerization state of bR molecules were evaluated with CD values of the negative minimum at ca. 600 nm, since the negative CD is observable when protein–protein interaction between bR molecules in the 2D crystals is highly retained. As shown in Figure 1B, the CD value at 600 nm starts to increase near the temperature of 10 °C and reached to a plateau at ca. 25 °C, indicating significant transition of oligomerization state of bR molecules in the DMPC vesicles in the temperature range of approximately 10–25 °C.

The measured time-resolved difference spectra indicated remarkable differences near 400 nm between the two phases of DMPC membrane. At 6 °C, two substates of the M intermediate with almost the same absorption maximum were observed in a different time region (data not shown). The spectrally silent transition between the early and the late substates in the M photointermediate is very similar to that previously reported for bR in the native PM.[8,9] On elevation of temperature, however, transient spectra of M gradually showed a remarkable blue-shift as the photocycle proceeds, as shown in the representative time-resolved difference spectra at 27 °C in Figure 2A. The maximum wavelength of the bands from the two M substates as a function of temperature is shown in Figure 2B. The early component gradually indicates smaller blue shift against temperature, whereas the late component dramatically shifts to much shorter wavelengh in the temperature range of 10–25 °C, which resulted in significant splitting of the M band into two components separated by approximately 15 nm in the liquid crystalline phase of DMPC bilayer. For bR molecules in the PM, on the other hand, the absorption maximum of the two substates of M was almost identical in the temperature range studied (data not shown).

The remarkable blue shift of late M intermediate upon disassembly of bR molecules induced by phase transition of DMPC bilayer can be discussed from the viewpoints of its origin and effects on proton pumping mechanism. Varo and Lanyi reported 4-nm shift for late M intermediate of monomeric bR solubilized with Triton X-100.[9] It is well known that the wavelength of visible absorption maximum of bR is strongly dependent on structure around retinal pocket.[10] These results strongly suggest that significant structural changes around the retinal pocket of reconstituted bR in the liquid crystalline phase are induced by disassembly of bR molecules. It is plausible that through the phase transition of DMPC bilayer, changes in secondary structure and local structure of retinal are induced, which are observed for disassembled bR in the PM at alkaline pH and in the solubilized state.[11,12] It should be stressed that the late substate of M is highly perturbed by disassembly of bR molecules, in that it is an essential intermediate responsible for switching to proton uptake from proton release in the photocycle.[2] These experimental results and gradual irreversible photobleaching bR reconstituted in DMPC above ca. 25 °C (unpublished results) may suggest that the perturbed late M intermediate may be a prerequisite for irreversible photobleaching of bR.[3,4]

This work was supported by Grants-in-Aid by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Monbukagakusho).

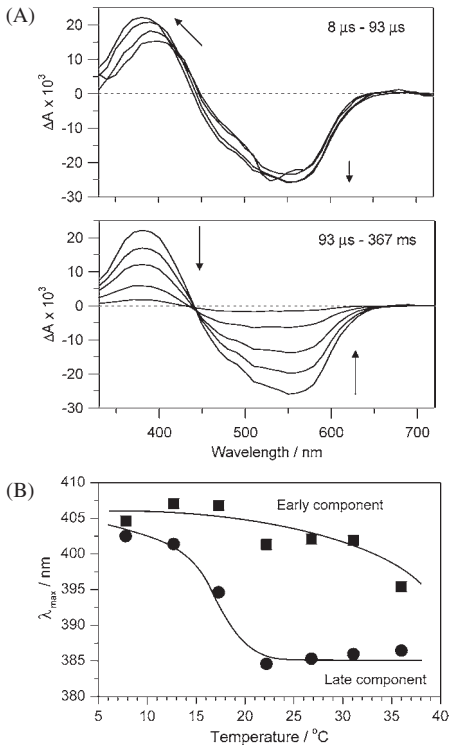

Figure 2. (A) Time-resolved difference spectra of reconstituted bR at 27 °C and (B) its absorption maximum wavelength of the two substates of the M intermediate as a function of temperature.

References and Notes

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Published on the web (Advance View) October 31, 2009; doi:10.1246/cl.2009.1134