High-Temperature Intermediate State of Bacteriorhodopsin Prior to the Premelting Transition of Purple Membrane Revealed by Reactivity with Hydrolysis Reagent Hydroxylamine

Masashi Sonoyama*,† and Shigeki Mitaku†

Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan

Received: February 18, 2004; In Final Form: September 9, 2004

The structural properties of bacteriorhodopsin (bR) over a wide temperature range of 30−80 °C have been examined through the hydroxylamine reactivity of the Schiff base inside the molecule and through hydrogen−deuterium (H−D) exchange in the peptide groups combined with FT-IR spectroscopy. These experiments were conducted to investigate the origin of recently reported irreversible photobleaching in the temperature region below the two main thermal transition points of the purple membrane (PM). Reactivity measurements of bR with hydroxylamine in the dark revealed a high-temperature intermediate state defined by two transition points at ∼60 and ∼72 °C prior to the premelting of the PM. In the high-temperature intermediate state, remarkable enhanced water accessibility in the internal region of bR molecule was suggested despite no acceleration of H−D exchange in the main chain. A model for the high-temperature intermediate state of bR responsible for irreversible photobleaching was proposed.

Introduction

Bacteriorhodopsin (bR), a membrane protein with a retinal chromophore, functions as a light-driven proton pump on the plasma membrane of *Halobacterium salinarum*.[1] Proton translocation is accomplished via a photocycle including several spectrally distinct intermediates after the absorption of visible light.[2,3] bR molecules spontaneously assemble in two-dimensional (2D) crystal patches called the purple membrane (PM), which is composed of trimers of the molecule and lipids as the structural unit.[4] bR molecules embedded in the PM are thought to be highly stable against heat. The PM has two main thermal transitions at high temperatures: a small reversible premelting transition at ∼80 °C and an irreversible melting transition above 90 °C.[5−7] The former is thought to be a highly reversible process due to disordering of the 2D crystals, whereas the latter corresponds to complete heat denaturation of the bR molecules.[5,8] Nonetheless, destabilization of bR molecules prior to the premelting transition has been revealed by recent extensive denaturation kinetic experiments, both in the dark and under visible light.[9,10] In the temperature range of ∼60−70 °C, bR molecules in the dark undergo reversible structural changes before a relatively slow irreversible thermal bleaching at more elevated temperatures, whereas they are subject to irreversible photobleaching by the continuous irradiation of visible light that originally triggers the photoreaction of native bR molecules. It is noted that the photobleaching[11−14] suggests destabilization of one or more photointermediates due to weakening of the force restoring the ground state from photointermediates in the photocycle. Investigations on photobleaching may lead to an understanding of the restoring force indispensable for completing the photocycle of bR molecules in the native state.

Several structural studies of bR molecules in the temperature region corresponding to the high-temperature intermediate state have already been reported. Our previous studies[9,10] revealed visible circular dichroism (CD) changes from the bilobe type to the positive one, indicating a decrease in the intermolecular interaction inside trimers and a blue shift of the visible absorption due to retinal isomerization. Time-resolved FT-IR spectroscopic studies in a similar temperature range performed by Wang and El-Sayed demonstrated that protein conformational changes from α_{II}-helix to α_{I}-helix begin in the dark,[15] along with an all-trans to 13-cis isomerization of the retinal chromophore, and produce the difference spectra characteristic of the M-photointermediate upon light illumination.[16] Shnyrov and Mateo reported small thermal transitions below the premelting transition at ∼80 °C, based on highly sensitive differential scanning calorimetric (DSC) measurements for PM suspensions.[17] However, little is known about what structural changes in the temperature region are responsible for photobleachig.[9,10]

In the present study, the structural properties of bR molecules within the temperature range of 30−80 °C were investigated by two methods: the reactivity of bR molecules with hydroxylamine, a water-soluble reagent for hydrolysis of the Schiff base inside the molecule, and hydrogen−deuterium (H−D) exchange in the peptide groups[18] combined with FT-IR spectroscopy. Reactivity measurements of bR with hydroxylamine constitute a useful tool for investigating structural changes near the Schiff base.[19−21] The reaction is accelerated significantly when the bR molecules undergo a structural perturbation such as conversion to photointermediates by light irradiation,[19,20] mutation of specific amino acids, or solubilization into the mixed 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/dimyristoylphosphatidylcholine (DMPC) micelles,[21] although native bR molecules in the PM are highly resistant to hydrolysis of the Schiff base in the dark at room temperature.[22,23] Light-induced catalysis during the hydroxylamine

* Corresponding author [telephone +81-(52)789-3708; fax +81-(52)-789-3708; e-mail sonoyama@nuap.nagoya-u.ac.jp].

† Present address: Department of Applied Physics, Graduate School of Engineering, Nagoya University, Furo-cho, Nagoya 464-8603, Japan.

10.1021/jp049268t CCC: $27.50
Published on Web 11/20/2004

reaction is generally thought to be caused by an increase in the water accessibility of the Schiff base, although the photointermediate responsible for the light-catalytic hydroxylamine reaction is controversial.[19−21,23] In contrast, combining the method of amide hydrogen exchange from the water with spectroscopic techniques has been useful for examining fluctuations and exposure of the internal region to water due to structural changes in proteins.[18]

An intermediate state defined by two transition points at ∼60 and ∼72 °C was demonstrated by hydroxylamine reactivity measurements in the dark. Arrhenius plots of the reaction showed significant enhancement in hydroxylamine reactivity at the two transition points due to a remarkable increase in the frequency factor, not to a decrease in the activation energy. Examination of H−D exchange of the amide hydrogen with FT-IR has revealed remarkable acceleration in the exchange rate above ∼70 °C. The structural properties of the high-temperature intermediate state are discussed from aspects of water accessibility inside the bR molecules and any relationship to bleaching by heat and/or light irradiation.

Materials and Methods

The PMs of *Halobacterium salinarum*, strain R1M1, were isolated and purified according to the standard method established by Oesterhelt and Stoeckenius.[24] Purified purple membrane was suspended in 25 mM Tris-HCl buffer at pH 7.2. The pH values of the suspensions were adjusted for the temperatures at which the hydroxylamine reaction was carried out. The hydroxylamine solution was titrated to pH 7.2 with aqueous NaOH. Protein concentration was determined from the absorption maximum at 568 nm recorded at room temperature using the extinction coefficient value of 62 700 M^{-1} cm^{-1}.[25]

Reaction kinetics of the Schiff base of bR with hydroxylamine was performed using a Beckmann Coulter DU-7500 photodiode array spectrophotometer in the temperature range of 25−80 °C. Measurements under illumination were conducted using a light irradiation system consisting of a Xe lamp (average light power = 200 mW·cm^{-2}), Y52 color filter, and heat-cut filter for filtering out the light wavelengths shorter than 520 nm and longer than 700 nm. The temperature of the cell holder was controlled using a Julabo F-25 circulating heater. The reaction was initiated by adding 0.1 mL of PM suspension to 2.9 mL of hydroxylamine solution incubated at each temperature. The final concentrations of bR and hydroxylamine were 5 μM and 200 mM, respectively. During kinetic measurements for 60 min, the samples in the cuvette were stirred continuously with a magnetic stirrer. Time constants of the reaction were estimated from the time-dependent absorption changes at 560 nm using Wavemetrics IGOR software.

Temperature dependency of the H−D exchange in the peptide group between 25 and 80 °C was determined using the amide II infrared absorption at ∼1545 cm^{-1} that downshifts to ∼1460 cm^{-1} (amide II′ band) upon deuteration of the peptide hydrogen. The H−D exchange was initiated by mixing 0.1 mL of PM suspension and 2.9 mL of preheated D_2O buffer at each temperature and incubating the mixture for 10 or 120 min, then quickly quenching to room temperature with ice−water. The final concentration of PM was 5 μM. The deuterium-exchanged purple membrane was separated using an ultracentrifuge and resuspended to a concentration of 150 μM, followed by drying overnight on a BaF_2 disk in an atmosphere of 90% relative humidity at 4 °C. The following procedures were performed at room temperature. The sample was sealed with another BaF_2 disk and a 7-μm polyimide spacer film after hydration with 1 μL of D_2O buffer. A Digilab FTS-6000 FT-IR spectrometer equipped with a mercury−cadmium−telluride ($Hg_{1-x}Cd_xTe$) detector was used to obtain infrared spectra of the film samples. Scans (total of 1024) were averaged with a spectral resolution of 2 cm^{-1}.

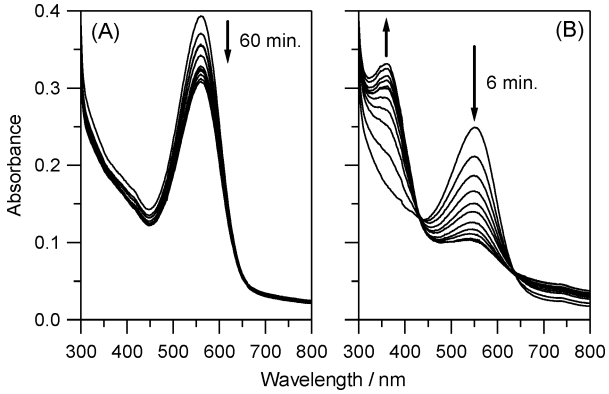

Figure 1. UV−visible absorption spectra of bR after mixing with hydroxylamine at (A) 35 °C and (B) 68 °C. A series of ∼30 absorption spectra were successively recorded after the bR suspension and hydroxylamine solution were mixed.

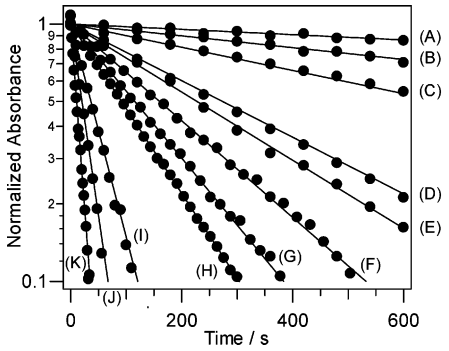

Figure 2. Normalized absorbance changes at ∼560 nm as a function of time after mixing the bR suspension and hydroxylamine solution: (A) 40 °C; (B) 50 °C; (C) 58 °C; (D) 60 °C; (E) 62 °C; (F) 64 °C; (G) 66 °C; (H) 70 °C; (I) 72 °C; (J) 76 °C; (K) 80 °C. The normalized absorbance was calculated by dividing absorbance at each time by the value extrapolated to $t = 0$.

Results

The reaction kinetics measurements of the bR molecules with hydroxylamine over a wide temperature range were conducted mainly in the dark to investigate the structural changes in bR at high temperatures without conversion to the light-adapted form. UV−visible absorption changes as a function of time were measured after the bR suspension and hydroxylamine solution had been mixed at each temperature. Representative absorption spectra at 35 and 68 °C are shown in Figure 1. Absorption near 560 nm diminishes along with an increase in the 360-nm peak, indicating that the Schiff base linkage between the retinal and Lys 216 in the bR molecule is hydrolyzed to retinaloxime and bacterio-opsin. Spectral changes at all temperatures are similar to the results shown in Figure 1. Because only slight spectral changes were observed in the dark near room temperature, as previously reported,[19,23] further analyses were performed at temperatures >35 °C. Time variations in the absorption at 560 nm at representative temperatures are shown in Figure 2. The normalized absorbance was calculated by dividing the absorbance at each time by the value extrapolated to $t = 0$. All of the decay curves could be expressed accurately as a single-exponential function. Figure 3 shows the estimated rate constants

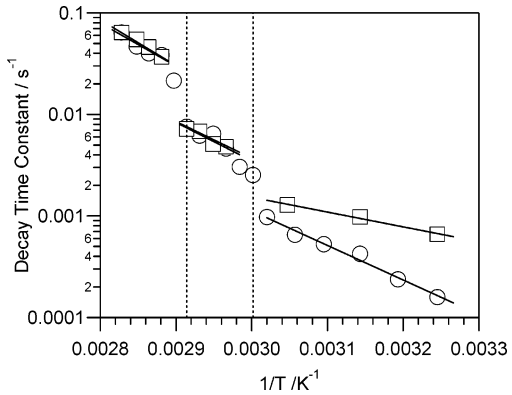

Figure 3. Arrhenius plots of the hydroxylamine reaction of bR (○) in the dark and (□) under continuous light illumination.

TABLE 1: Frequency Factor and Activation Energy of Hydroxylamine Reaction with bR in the Dark and under Light Illumination Estimated from the Arrhenius Plot Shown in Figure 1C

temperature range (°C)	frequency factor (s^{-1})		activation energy (kcal·mol^{-1})	
	dark	light	dark	light
<60	1.77×10^7	3.54×10^1	15.5	6.65
64−70	2.38×10^8	7.74×10^8	16.4	17.3
>74	6.19×10^{10}	3.94×10^{11}	19.4	20.7

plotted as a function of the inverse of absolute temperature. Although about half of the absorbance remains after 60 min of reaction at a temperature <40 °C due to slow bleaching, decay time constants were calculated from the data obtained.

The Arrhenius plot (Figure 3) clearly shows three temperature regions with transition points at ∼60 and ∼72 °C. Lower and higher transition temperatures obtained are very similar to reported onset temperatures of irreversible photobleaching by light irradiation and precursory reversible structural changes in the dark and of thermal bleaching in the dark,[9,10] respectively. In all three temperature ranges, strong linear relationships are observed between logarithmic rate constants and the inverse of the absolute temperature. The activation energy, ΔE_a, and frequency factor, *A*, of the reaction were estimated from the Arrhenius plot and are summarized in Table 1. The kinetics parameters at temperatures below ∼60 °C obtained in this study agree well with previous results ($\Delta E_a = 15.5$ kcal/mol, $A = 1.5 \times 10^7$ s^{-1}) reported by Rousso et al.[23] In the two higher temperature regions, the activation energy of the dark reaction is almost the same as that in the lowest temperature range, whereas the frequency factor drastically increases with an increase in temperature. The dramatic enhancement in hydroxylamine reactivity in the dark at the two transition points is, therefore, attributable not to a decrease in the energy barrier but to a significant increase in frequency factor.

The reaction kinetics of bR with hydroxylamine under continuous visible light irradiation also was measured at temperatures from 35 to 70 °C for comparative studies with the dark experiments. Similar spectral changes and absorbance changes at ∼560 nm in the form of a single-exponential decay function were observed at all temperatures (data not shown). The Arrhenius plot of the reaction under illumination is shown in Figure 3 along with results from the reactions performed in the dark. In the Arrhenius plot, a dramatic increase in the reaction rate of bR with hydroxylamine is clearly observed at temperatures similar to those for the dark reaction. Values for estimated activation energy and frequency factor in the three temperature regions are shown in Table 1. The hydroxylamine

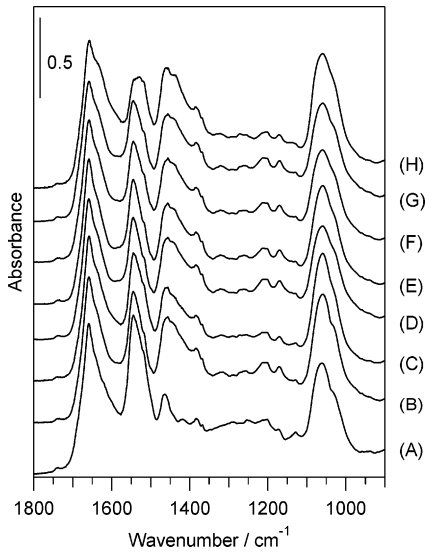

Figure 4. FT-IR spectra of bR: (A) intact; (B) just after hydration with D_2O at 25 °C; after incubation at (C) 35 °C, (D) 50 °C, (E) 60 °C, (F) 66 °C, (G) 70 °C, and (H) 80 °C for 120 min. All spectra were measured at room temperature.

reaction is remarkably light-enhanced below ∼60 °C in comparison with the dark experiments, as previously reported.[19−21,23] This light enhancement effect is attributable to a reduction in the energy barrier. In the two temperature ranges above ∼60 °C, however, no significant light enhancement effect of the hydroxylamine reaction was observed, and the kinetic parameters were very similar to those obtained from the reactions performed in the dark. Extrapolation to the transition temperature of ∼60 °C from the lower temperature produced no significant difference in the reaction rate.

H−D exchange in the peptide groups was examined by FT-IR spectroscopy. Figure 4 shows the IR spectra of bR film samples prepared at room temperature after incubation for 120 min in D_2O buffer at each temperature. The intensity of the amide II band at 1544 cm^{-1} was comparable to that of the amide I band at 1661 cm^{-1} for the intact sample, as reported by Rothschild et al.[26] and Cladera et al.[27] Upon hydration of the intact sample by deuterium oxide at room temperature, the amide II band remains intense, although it weakens somewhat upon a slight increase in the amide II′ band at 1456 cm^{-1} due to the deuterium-exchanged peptide group, indicating that bR molecules in the PM are highly resistant to H−D exchange and that only small sections of the peptide groups are deuterium-exchanged. This is in good agreement with results reported previously.[26−28] No large spectral changes were observed at temperatures up to 70 °C. When incubated at elevated temperatures, however, the amide II band at 1548 cm^{-1} shows a remarkable decrease in intensity, whereas the amide II′ band at 1456 cm^{-1} becomes more intense, indicating an acceleration in H−D exchange in the peptide groups above ∼70 °C. To investigate the H−D exchange of bR more quantitatively, the ratio of the intensities of the amide II band to the amide I band was calculated for samples incubated for 10 and 120 min in deuterated media at each temperature. Results are shown in Figure 5. The peak ratio is almost constant below ∼70 °C at the level of the sample hydrated by D_2O at room temperature, indicating deuterium exchange of the peptide hydrogens occurs to some extent at room temperature but that the bR molecules are strongly resistant to H−D exchange upon heating within a range from room temperature to ∼70 °C. Nonetheless, experimental results for the 120-min incubation in the temperature

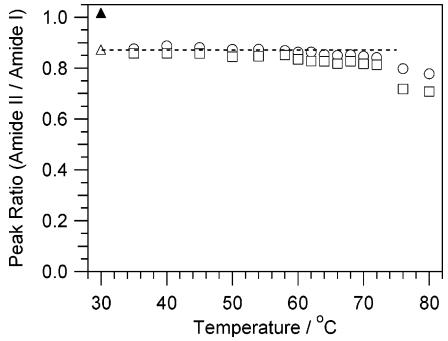

Figure 5. Relative absorbance of the amide II band to the amide I band as a function of temperature. Incubation time: (○) 10 min and (□) 120 min. Results for the intact sample (▲) and the sample hydrated with D_2O buffer just before measurements at 25 °C (△) are also shown.

range of 60−70 °C suggest a minute acceleration of H−D exchange in the peptide groups. However, upon incubation above 70 °C, the band ratio decreases dramatically, indicating much faster H−D exchange in this temperature region, although even after incubation at 80 °C for 120 min, the amide II band remains intense.

Discussion

Kinetics analysis for the hydroxylamine reaction in the dark revealed significant transitions at ∼60 and ∼72 °C prior to the premelting transition at ∼78 °C, although bR molecules embedded in PM are generally thought to have two thermal transitions, a reversible premelting transition at ∼78 °C (T_m) and an irreversible melting transition with a T_m of ∼96 °C.[5−8] The temperature of the first transition is close to the onset temperature of photobleaching by continuous visible light irradiation and its precursory structural changes in the dark.[9,10] In addition, a very small thermal transition at the temperature (62 °C) of the first of five successive transitions beneath the overall endotherm was reported on the basis of highly sensitive DSC measurements with an annealing procedure by Shnyrov and Mateo.[17] Therefore, the experimental results for hydroxylamine reactivity obtained in this study are likely to be based on changes in the structure and stability of the bR molecules during the thermal transition at ∼60 °C. Comparison of the kinetic parameters for the dark hydroxylamine reaction in the lowest and the middle temperature regions (Table 1) shows that the 60 °C transition has a significantly enhanced frequency factor, not a lower energy barrier for the reaction. The most plausible interpretation for this notable increase in the frequency factor is an enhancement in the accessibility of the water molecules to the Schiff base linkage inside the bR molecules; hydroxylamine is a small water-soluble molecule, and its penetration along with water molecules into the internal region of the bR is indispensable for the hydrolysis reaction. Interestingly, water molecules affect the stability of the bR molecules, although their contributions to the function of membrane proteins have been reported (see reviews on bR[29,30]). A similar phenomenon was reported with nuclear magnetic dispersion[31] and H−D exchanges in peptide groups with FT-IR spectroscopy[32] for *n*-octyl-*β*-glucoside-solubilized bR that undergoes photobleaching by continuous irradiation of visible light, even at room temperature.[13] In these experiments, the exchange of water molecules between the internal region of the molecule and the external water medium is considerably accelerated compared to native bR in the PM.

What structural changes are then responsible for the enhancement in hydroxylamine reactivity of bR molecules? In the temperature region corresponding to the high-temperature intermediate state revealed in this study, structural changes inside the bR molecules were revealed by spectroscopic studies. FT-IR studies by El-Sayed and Wang revealed protein conformational changes along with retinal isomerization from all-trans to 13-cis configuration.[15] They proposed that the secondary structural changes are α_{II}-to-α_I conformational transitions based on previous theoretical[33] and experimental[34−37] investigations. The coupling structural changes in the main chain and chromophore may be related to accelerated water penetration, because similar structural changes[32] and increased water exchange between the internal region and the external water media[31] are observed for bR upon solubilization with *n*-octyl-*β*-glucoside. Furthermore, a previous study on the hydroxylamine reactivity of bR with synthetic retinal analogues of fixed configuration[23] showed that the distorted structure with a cis configuration is preferred in the dark reaction, which supports this theory. In addition to structural changes in the backbone and chromophore of the bR molecules, Shnyrov and Mateo reported a small red shift in the intrinsic fluorescence, suggesting exposure of the hydrophobic core to a more hydrophilic environment, prior to a much larger shift above 70 °C.[17] Nonetheless, the structural changes already reported are relatively small and highly reversible in the dark. The H−D exchange experiments using FT-IR spectroscopy showed that deuteration of the amide hydrogens of the main chain is accelerated, but at a temperature >70 °C, although a significant increase in water accessibility inside the bR molecules at the 60 °C transition is suggested. The discrepancy in transition temperature between the enhancement in water accessibility suggested by hydroxylamine reactivity in the dark and the acceleration of H−D exchange in the backbone is attributable to relatively small structural changes inside the bR molecules during the thermal transition. It is plausible that H−D exchange in the main chain is suppressed by strong hydrogen bonds in the helical ordered structure despite the exposure of the internal region of the protein to water molecules, even after the α_{II}-to-α_I conformational transition. The high-temperature intermediate state may be a precursory one of "compact denatured state" with a large proportion of ordered structure in the fully denatured bR at further elevated temperatures proposed by Taneva et al.[38]

In addition to the structural changes mentioned above, recent CD measurements for PM in the visible region at high temperatures[9,10] revealed that the bilobe asymmetric band characteristic of trimeric structures in PM begins to change to a single positive band at ∼60 °C, indicating that the intermolecular interaction between the bR molecules inside the trimeric unit diminishes due to melting of the 2D crystals. It should be noted that the unique amide I features characteristic of the α_{II}-helix show up only when bR monomers interact with each other in the trimers.[37] Therefore, the changes in the oligomerization state of bR in the PM above 60 °C may be related to the α_{II}−α_I conformational transition mentioned above. It is probable that the disorder of the lattice increases the dynamic fluctuation of the bR molecules that retain relatively ordered secondary structures. A large dynamic fluctuation in bR molecules using NMR spectroscopy was reported when the lattice in the PM was disrupted by solubilization with a mild nonionic detergent such as Triton X-100 or *n*-octyl-*β*-glucoside.[31,39] Thus, we propose a schematic model for the high-temperature intermediate state of bR embedded in the PM as shown in Figure 6. In this model, the native structure is retained below ∼60 °C. Upon heating to ∼60−70 °C, bR molecules undergo reversible structural changes, not to an unfolded structure, but to a

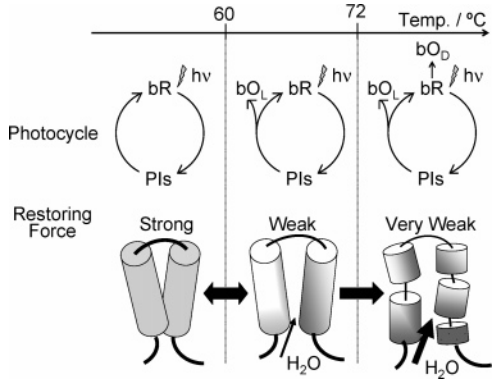

Figure 6. Model of the structural changes of bR in the purple membrane at high temperatures with possible pathways in the photocycle. PIs, photointermediates; bO_L, bacterioopsin generated by photobleaching under light illumination; bO_D, bacterioopsin generated by thermal bleaching in the dark.

relatively ordered structure with larger dynamic fluctuations due to melting of the 2D crystals, and H−D exchange in the peptide groups is suppressed by hydrogen bonds in the ordered secondary structure despite significant enhancement in the water accessibility inside the bR molecules. It is likely that the increase in dynamic fluctuation is closely related to weakening of the restoring force in the photocycle within the high-temperature intermediate state, which results in irreversible photobleaching of bR molecules in the temperature region corresponding to that reported by Yokoyama et al.[9,10] Dynamic fluctuation in the temperature range might enhance penetration of water molecules from the cytoplasmic side as well as the extracellular side, although Subramaniam et al. reported that reaction of hydroxylamine with bR in lipid/detergent micelles in the dark probably occurs from the extracellular channel preferentially.[21] At temperatures > ∼70 °C, several spectroscopic methods revealed larger structural changes.[6−10,15,17,38,40] This leads to further enhancement in hydroxylamine reactivity and thermal bleaching as well as photobleaching of bR. Acceleration of H−D exchange in the main chain also can be explained by larger structural changes in bR molecules, including those within the secondary structure. Nonetheless, the structural changes in bR molecules in the temperature range may be relatively small, compared to thermal denaturation of water-soluble proteins, because Taneva et al. reported that thermal denaturation of bR at more elevated temperature leads to a "compact denatured state" that retains a great proportion of ordered structure.[38]

In summary, the high-temperature intermediate state of bR exists prior to the premelting transition of the PM, as revealed by hydroxylamine reactivity measurements in the dark. It is suggested that recently reported irreversible photobleaching in the very similar temperature range[9,10] is attributable to a significant increase in water accessibility inside the bR molecules and an increase in dynamic fluctuations due to the disordering of the 2D crystals. The process returning to the original ground state through photointermediates after absorption of visible light is accomplished by intramolecular interactions within a bR molecule and intermolecular interactions within the 2D crystal structure, although these two interactions may be interdependent. To address the question of the restoring force of bR in the photocycle, extensive studies on the structural stability and changes of bR molecules embedded in PM at high temperature or solubilized with mild nonionic detergents are now underway, in addition to a primitive vibrational spectroscopic study of structural changes of bR upon solubilization with octyl-β-*n*-glucoside.[32]

Acknowledgment. This work was in part supported by grants-in-aid from the Ministry of Education, Culture, Science, Sports and Technology of Japan (Monbukagakusho) and by the Industrial Technology Research Grant Program of the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

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