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Kinetic Measurements of Irreversible Photobleaching of Bacteriorhodopsin in A High Temperature State

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Irreversible photobleaching of bacteriorhodopsin (bR), namely denaturation induced by illumination of visible light, was investigated by absorption kinetic measurements. The denaturation kinetics revealed that light illumination significantly enhanced the structural decay of bR. The kinetic analyses showed that the molecular structure of bR denatures according to a single-exponential decay, whereas irreversible photobleaching has two decay components. The decay constant of the slow component of photobleaching is almost same as that in the dark. An Arrhenius plot of the denaturation kinetic constants for the fast and slow components showed similar activation energies of approximately 19 kcal/mol.

Key words: bacteriorhodopsin, photobleaching, thermal denaturation, structural stability, denaturation kinetics

INTRODUCTION

Bacteriorhodopsin (bR), which is a light-driven proton pump of *Halobacterium salinarum*, is one of the membrane proteins whose molecular structure and functional mechanism have been well studied [1]. The crystallographic studies demonstrated that this protein consists of seven transmembrane helices, small loop segments and the retinal chromophore covalently bound to the protein via the Schiff base [2]. The function of bR is achieved along the photocycle comprised of several photo-intermediate states (J, K, L, M, N and O) [1]. The molecular structure of the photo-intermediate state has recently been reported, revealing structural switching during the photocycle [3]. The Schiff base is closed on the cytoplasmic side in the ground state, while the molecular structure switches to the open state during the M- and N-intermediate states, resulting in vectorial proton translocation [3]. It still remains to be revealed, however, why the photo-intermediates can recover to the ground state over these structural changes. The information of the structural stability of the photo-intermediates as well as the ground state would be helpful to resolve these questions.

The structural stability of the ground state of bR have been extensively investigated by denaturation experiments [4]. The denaturation experiment using organic solvents revealed that the molecular structure of the ground state of bR is stabilized mainly by the polar interaction, including the electrostatic interaction and hydrogen bonds [4].

The denaturation experiments of bR under light illumination have also been carried out in order to study the stability of the photo-intermediate states, demonstrating that irreversible photobleaching occurred at high temperature [5]. The experimental result strongly suggested that the structural stability of the photo-intermediate states is lower than that of the ground state. The difference in the structural stabilities between illuminated and dark conditions, however, is not evaluated yet.

In this work, we investigated the denaturation of bR by absorption kinetic measurement under various temperature and light illumination conditions. The analysis of denaturation kinetics indicated that the structural decay of bR in the dark was responsible for a single structural component. However, it was shown that light illumination significantly accelerated the structural decay of bR and generated an additional fast decay component. The denaturation events of bR at high temperature were discussed in combination with the results of the kinetic analyses.

MATERIALS AND METHODS

Purple membrane of *Halobacterium salinarum*, strain R1M1, were isolated and purified according to the standard procedure [6]. The purified purple membranes were suspended in Tris-HCl buffers (pH 7.0).

Absorption spectra at high temperature were recorded us-

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ing a photo-diode array spectrophotometer with a thermostated cell holder (DU7500, Beckman Coulter, USA) in the temperature range 25 to 90 °C. For illumination of visible light, a light illumination system, which was previously reported [5], was used.

In absorption kinetics measurements, absorption spectra were measured successively at interval of 150 seconds both in the dark and under illumination.

RESULTS AND DISCUSSION

We measured the decay kinetics of absorbance at the spectral peak around 560 nm, comparing the photobleaching and denaturation in the dark. The time courses of the relative absorbance in the dark and under illumination were shown in Fig. 1(a). It was possible to reproduce the decay of the relative absorbance in the dark using a single-exponential curve (Fig. 1(a) upper). The rate constant of the absorption change in the dark was found to be on the order of 10^{-5} s^{-1}. On the other hands, under illumination, the absorption decay had two componets (fast and slow) that could be well reproduced by a double-exponential curve. The kinetic analysis revealed that the slower decay time of photobleaching corresponded to that of the denaturation in the dark. The faster decay component was observed to be about 10 times faster than the slower decay component.

Figure 1(b) shows the Arrhenius plot of the denaturation kinetics in the dark and under illumination. The decay constants of denaturation in the dark are almost same as those of the slower decay components of photobleaching. The activation energies were approximately 19.0 kcal/mol for the fast component and 19.1 kcal/mol for the slow components which were calculated from the two broken lines in Fig. 1(b). The difference of the activation energies between the two conditions was negligibly small, and only the frequency factor of the decay kinetics was changed by the illumination.

Figure 2 shows the reaction scheme of bR inferred from the present kinetic measurements. Bacteriorhodopsin molecules undergo the structural changes to the high-temperature intermediate state (bR^{HT}) above 60 °C in the dark, as discussed in elsewhere [7]. The denaturation of bR in the dark, however, is not observed below 70 °C [7]. The present study revealed that the denaturation of bR in the dark observed above 70 °C had a single decay constant, suggesting that the denaturation of bR in the dark was due to a single molecular reaction. It is likely that bR spontaneously denatures above 70 °C through the bR^{HT} state which is stable at the temeratures between 60 and 70 °C if it is not activated by the light absorption.

Under the condition which bR molecules are illuminated by visible light above 60 °C, a photocycle which is different from that at the physiological temperature might exist, corresponding to bR^{HT} in the dark. Wang and El-Sayed [8] reported that the M-intermediate state is observable at high temperature range [8]. It was also reported that irreversible photobleaching of bR started to observe above 60 °C at which the structural changes to bR^{HT} also began in the dark [7]. These facts strongly suggest that the photocycle at high temperature proceeds

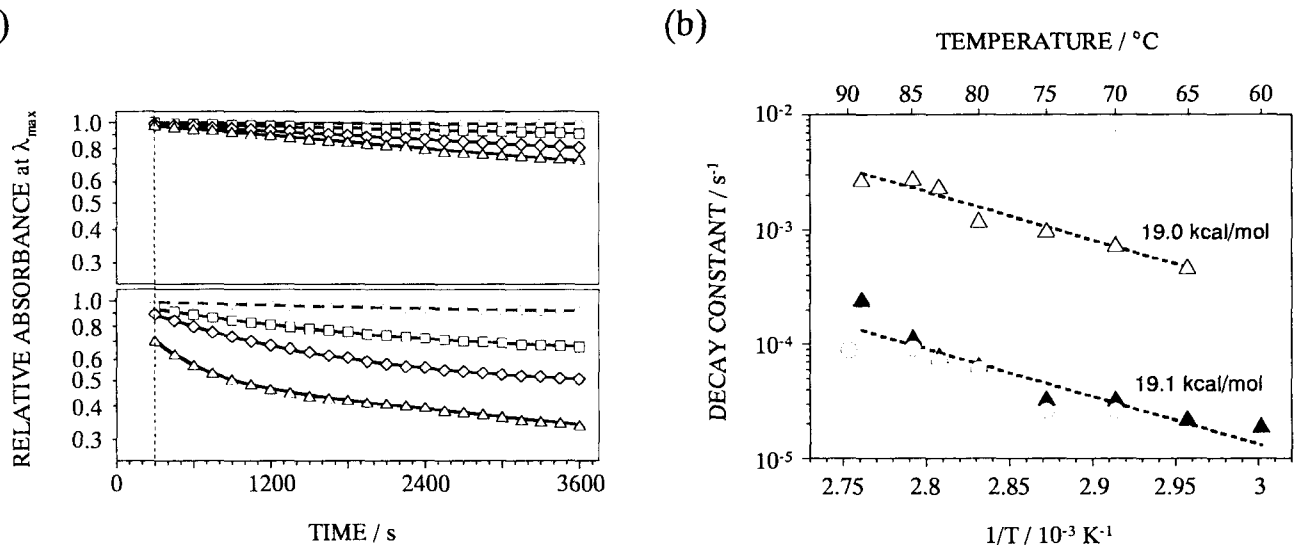

Figure 1. (a) Decay curves of the relative absorbance at maximum wavelength around 560 nm in the dark (upper) and under illumination (lower) at various temperatures: 60 °C (circles), 70 °C (squares), 80 °C (diamonds) and 85 °C (triangles). Only data points after the dead time of 300 seconds are shown. (b) Arrhenius plot of the denaturation of bacteriorhodopsin in the dark (circles), and the faster (open triangles), the slower components (closed triangles) under illumination.

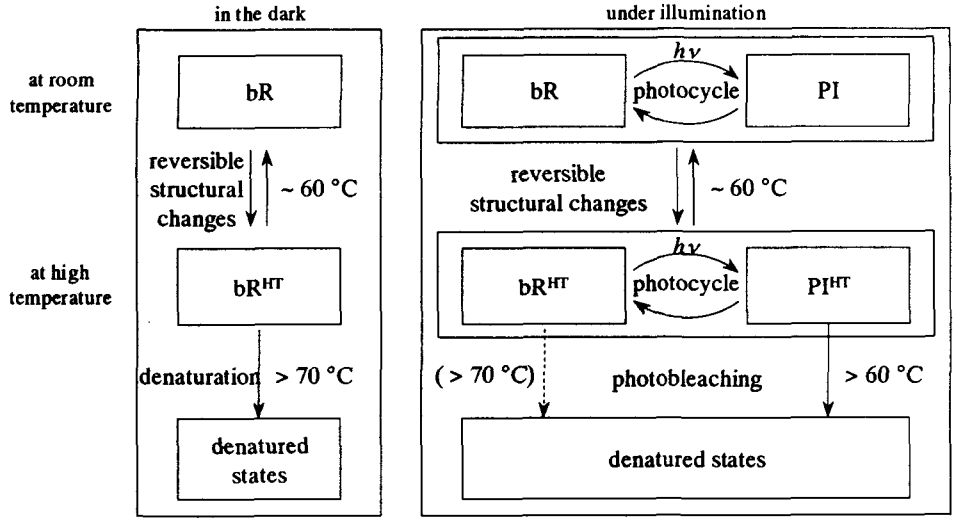

Figure 2. Schematic diagram of structural changes in bacteriorhodopsin induced by light illumination and heat. The ground state of bacteriorhodopsin (bR) undergoes reversible structural changes to the high-temperature intermediate state (bR^{HT}) above 60 °C in the dark. Above 70 °C, bR^{HT} begins to decay to denatured states in the dark. Under illumination, the photo-intermediate state from bR^{HT} (PI^{HT}) might appear above 60 °C. The fast decay component generated by light illumination would be responsible for the decay of PI^{HT}. The solid and broken arrows represent reasonable reactions and plausible reactions for which sufficient evidence is unavailable, respectively.

under illumination and that the structural changes to PI^{HT} results in the irreversible photobleaching. The result of the present work demonstrated that the structural decay of bR under illumination could be reproduced by a double-exponential function, and that the decay constants of the slower component of photobleaching was very similar to those of the denaturation in the dark. These results allow the supposition that PI^{HT} decays according to the fast decay time.

The kinetic analysis in this study revealed that the fast decay component was generated by visible light illumination above 60 °C at which the structural decay was not observed in the dark. This fact strongly suggests that the "restoring force", which assists the recovery to the ground state from the photo-intermediate states, is weakened at high temperature. The condition under which bR is stable in the dark but unstable under illumination would be very useful for elucudating the restoring force. Since irreversible photobleaching phenomenon may be related to the molecular movement during the photocycle such as the opening of the proton channel in the cytoplasmic side, the interaction between the cytoplasmic loop segments might aid the recovery to the ground state. It is also important to comprehend the photoreaction of bR at high temperature that the detailed molecular structure of bR^{HT} is resolved and PI^{HT} are identified.

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