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Enhancement of Hydroxylamine Reactivity of Bacteriorhodopsin at High Temperature

Masashi Sonoyama* and Shigeki Mitaku
Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan

Recent denaturation experiments of bacteriorhodopsin (bR) in the dark and under illumination at high temperatures revealed that irreversible thermal bleaching occurs above ~70 °C and the preceding reversible structural changes in the dark above 60 °C are closely related to irreversible photobleaching observed in the same temperature range (Yokoyama et al. (2002). *J Biochem.* 131, 785). In this study, structural properties of bacteriorhodopsin (bR) at high temperatures were extensively probed by hydroxylamine reactivity with the Schiff base in the dark and hydrogen-deuterium (H-D) exchange in the peptide groups. In the Arrhenius plot from kinetics measurements of the hydroxylamine reaction, a good linear relationship between the reaction time constant and the inverse of the absolute temperature was observed below 60 °C, while significant increase started above 60 °C, suggesting that remarkable increase in water accessibility of the Schiff base in the temperature region. FT-IR spectroscopic studies on the H-D exchange suggested increase in the deuterium exchanges rate of the peptide hydrogen in the same temperature region.

Key words: bacteriorhodopsin, high-temperature state, photobleaching, hydroxylamine reaction, water accessibility

INTRODUCTION

Bacteriorhodopsin (bR), a light driven proton pump, forms the two-dimensional crystalline lattice called purple membrane (PM) with lipid molecules in the cytoplasmic membrane of *Halobacterium salinarum* [1]. Upon absorption of visible light, bR molecules under physiological condition undergo cyclic structural changes through many identified photointermediate states (J, K, L, M, N, O) [2]. It is generally thought that PM mainly has two thermal transitions: irreversible melting one above 90 °C and reversible premelting one at approximately 80 °C [3]. Nonetheless, recent extensive denaturation kinetics and reversibility experiments on PM showed that in the dark, irreversible bleaching occurred above 70 °C after reversible structural changes above 60 °C, while irreversible photobleaching of bR is induced by continuous light irradiation when heated up to 60 °C [4]. These experimental results indicate that bR molecules in the temperature region of approximately 60-70 °C is in the very interesting state, in which irreversible photobleaching is induced by continuous light illumination for bR molecules showing no irreversible bleaching in the dark. Furthermore, it was shown that the photobleaching is closely related to the reversible structural changes in the dark above 60 °C.

In the present study, structural perturbations in bR molecules at high temperatures were examined by reactivity with hydroxylamine, a water-soluble reagent for hydrolyzing the Schiff base linkage, in the dark and spectroscopic methods for elucidating molecular structures of bR molecules, which undergo irreversible photobleaching by continuous irradiation of visible light. It was strongly suggested that significant increase in the water accessibility of the Schiff base is related to photobleaching of bR.

*To whom correspondence should be addressed.
E-mail: sonoyama@cc.tuat.ac.jp

MATERIALS AND METHODS

Purple membranes of *Halobacterium salinarum*, strain R1M1, were isolated and purified according to the standard

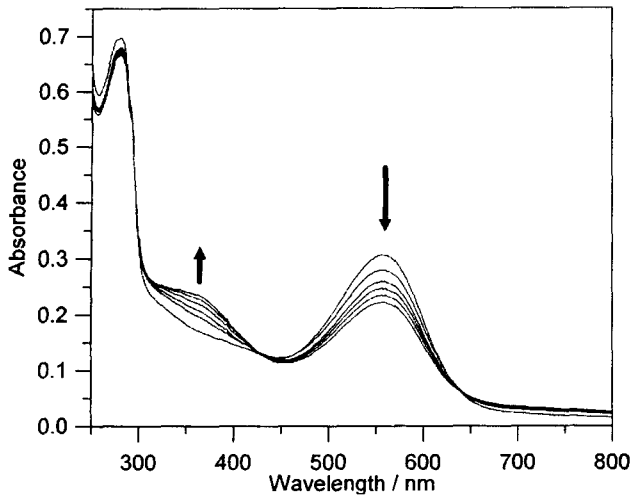

Figure 1. Absorption spectra of bR after mixing with hydroxylamine solution at 50 °C in the dark.

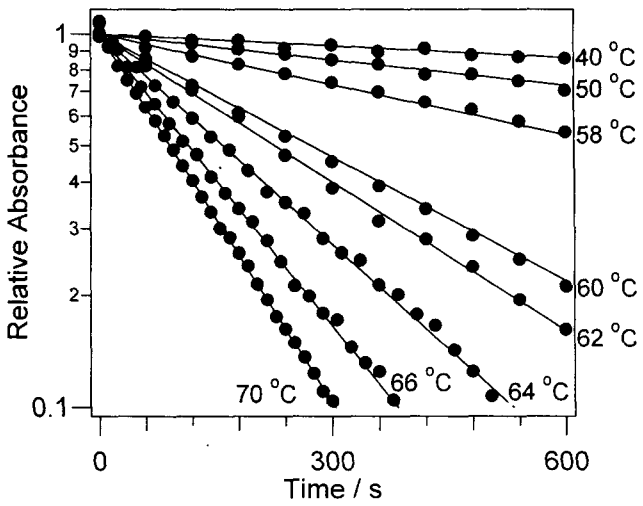

Figure 2. Decay curves of relative absorbance at 560 nm during hydroxylamine reaction of bR in the dark in the temperature range of 40-70 °C.

procedure. The purified purple membrane was suspended in 20 mM Tris-HCl buffer at pH 7.2. Reaction kinetics measurements were performed using a Beckman Coulter DU7500 photo-diode array spectrophotometer at the temperature region of 35-70 °C. Hydroxylamine reaction was initiated by mixing bR suspension and hydroxylamine solution incubated at each temperature. The final concentration of bR suspension and hydroxylamine solution was 5 μM and 0.2 M, respectively. Samples for IR measurements were prepared by incubating bR suspension at the temperature of 30-70 °C in the dark for 20 or 120 minutes, followed by drying it on a BaF_2 disk. IR spectra of bR were measured with a Bio-Rad FTS-6000 FT-IR spectrometer.

RESULTS AND DISCUSSION

UV absorption spectra were successively measured after mixing bR suspension and hydroxylamine solution at the temperature range of 35-70 °C. Spectra measured at 50 °C are shown in Figure 1. The band at 560 nm diminishes in concomitant with the increase in the absorbance at 360 nm. This indicates that bR is notably hydrolyzed to retinaloxime and bacterioopsin hydroxylamine even in the dark at 50 °C. Spectral changes at other temperatures were very similar to those at 50 °C, although time dependences of the changes were different from one another. Below room temperature, no significant decrease in absorbance at 560 nm was observed for 1 hour. This is in striking contrast with bR molecules under illumination. It is thought that higher hydroxylamine reactivity of bR under illumination is due to much enhancement of water accessibility in photointermediate(s) of bR [5].

In Figure 2, decay curves of relative absorbance at 560 nm at various temperatures are shown as a function of time. Relative absorbance is obtained by dividing the absorbance at a time by the one of the starting point. All the decay curves were well expressed as a single exponential function.

Decay time constants were calculated by fitting experimental data in Figure 2 into a single exponential function. In Figure 3, the results are shown as a function of the inverse of the absolute temperature. Below 60 °C, a good linear relationship was clearly observed in the Arrhenius plot. The kinetic parameters obtained from the plot are 15.5 kcal/mol for the activation energy and 1.77×10^7 s^{-1} for the frequency factor. These values are very similar to the previous results by Rousso et al. [5]. Upon heating above 60 °C, decay time constants show remarkable increase, leading to deviation from the linear line below 60 °C in the Arrhenius plot. This indicates that water accessibility of the Shiff base is enhanced above 60 °C. At further elevated temperatures above ~ 64 °C, on the other hand, a linear relationship is observed again in the Arrhenius plot. In the temperature range, the activation energy and the frequency factor are 16.4 kcal/mol and 2.38×10^8 s^{-1}, respectively. The activation energy is very similar to that below 60 °C, while the frequency factor is approximately ten times higher.

These experimental results on hydroxylamine reaction

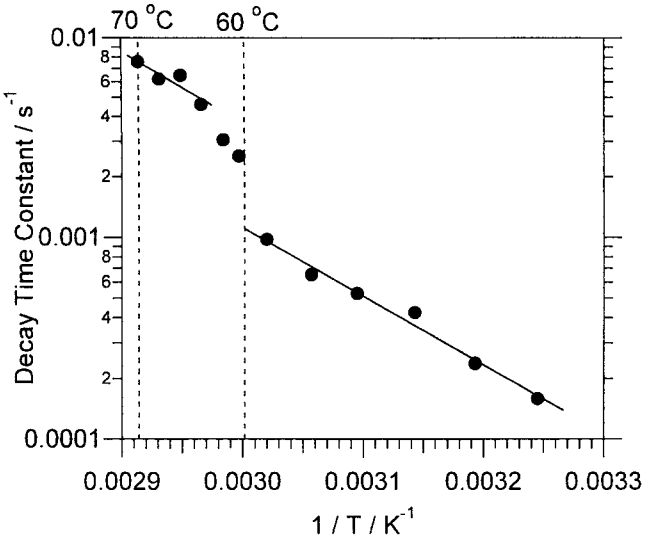

Figure 3. Arrhenius plot of hydroxylamine reaction kinetics of bR in the dark. Decay time constants were obtained from the data in Figure 2.

kinetics revealed that bR molecules undergo a transition at approximately 60 °C to a high-temperature state from the viewpoints of water accessibility of the Schiff base. It was suggested from extensive studies on hydroxylamine reactions of various bR mutants that the reaction occurs preferentially from the extracellular side of the protein [6]. The increase in hydroxylamine reactivity above 60 °C might indicate that the extracellular domain of bR molecules is more open to the water media in the temperature range. Hydrogen-deuterium exchange experiments by FT-IR spectroscopy indicated that deuterium exchanges in the peptide groups are slightly accelerated above 60 °C (data not shown). This also suggests increase in water accessibility of bR molecules in the temperature region. Recent denaturation experiments at high temperatures revealed that irreversible photobleaching occurs above ~60 °C and is closely related to reversible structural changes in the dark in the corresponding temperature range [4]. It is likely that the enhancement in water accessibility is related to irreversible photobleaching of bR molecules.

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