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Photoreaction of N_{560} intermediate in the photocycle of bacteriorhodopsin

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Sophisticated measurements were made on the nanosecond time-resolved absorbance change of the purple membrane of *Halobacterium halobium* under cw background light irradiation (440–800 nm, 11–441 mW/cm²). A red-shifted transient species R_{660} (K_N, Q) was found in alkaline conditions (pH > 9.3). Background light intensity effect shows that (i) R_{660} is photochemically formed from N_{560} intermediate which is accumulated under background light irradiation because of the elongated lifetime in alkaline suspension, and that (ii) the slow decaying M_{412} is not photochemically formed from N_{560} but from bR_{568}.

Bacteriorhodopsin; Purple membrane in alkaline suspension; Secondary photocycle; N_{560}; R_{660}

1. INTRODUCTION

The purple membrane of *Halobacterium halobium* contains a chromoprotein bacteriorhodopsin (bR_{568}). On absorbing a photon, a bR_{568} molecule undergoes a photochemical cycle via several intermediates: $bR_{568} \rightsquigarrow K \rightarrow L \rightarrow M \rightarrow N \rightarrow O \rightarrow bR_{568}$ [1,2]. The bR_{568} molecule exhibits proton pumping activity in a broad pH range (3-12 [2]). The efficiency of proton pumping complicatedly depends on salt concentration [3] and pH value [4]. These behaviors have not been derived from the simple Lozier's scheme [2].

Photochemical reactions of bR_{568} have recently been interpreted with multiple cycles [5–7] and/or back reactions [8–11]. Furthermore, photo-branching reactions have been proporsed: photoreaction of an intermediate with a long lifetime such as N_{560} accumulated under cw visible light irradiation. Kouyama et al. [12] suggested that the slow-decaying M_{412} (M^s) is a photoproduct of N_{560}. Balashov et al. [13] and Váró and Lanyi [9] reported the formation of a red-shifted photoproduct (K_N) of N_{560}. Recently Ohtani et al. [14] have found a fluorescent intermediate Q and attributed it to a photoproduct of N_{560}.

In this work, a secondary photocycle initiated from N_{560} in alkaline purple membrane was studied with the aid of the time-resolved absorption spectroscopy. We found that (i) N_{560} is photochemically converted to an O-like red-shifted intermediate R_{660} which is similar to K_N. (ii) The effect of pH value on the behavior of R_{660} was same as that of Q measured by fluorometry [14]. Thus we show evidence for the photoreaction of an intermediate N_{560} at room temperature. We furthermore clarified that M^s should not be located in the photocycle of N_{560} but in that of bR_{568}.

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2. MATERIALS AND METHODS

The culture of *H. halobium* (ET1-001) and the isolation of the purple membrane were performed as described by Oesterhelt and Stoeckenius [15]. The isolated membrane was suspended in distilled water and the pH value was adjusted with NaOH solution. Chromoprotein concentrations were 9–15 μM. The sample suspensions were exposed to a cw yellow light (440–800 nm, 150-W Xe lamp) just before each experiment. All experiments were carried out at room temperature (20.7–25.8°C). Absorption spectra of samples were measured with a spectrophotometer (Shimadzu, UV-3000) before and after the photolysis experiment.

A nanosecond absorption spectroscopy was performed with a conventional system (Applied Photophysics). Photolysis of the sample was accomplished with a 532-nm pulsed light (5-ns FWHM, 0.33 mJ, 5 Hz) obtained from a Nd:YAG laser (Spectra Physics, DCR3F). A probe light was detected with a photomultiplier (Hamamatsu Photonics, R3825) coupled with a grating monochromator (1200 grooves/mm). A cw Xe lamp was used as a background light source (11–441 mW/cm²) which controlled the stationary fraction of photointermediates in the sample suspension. Here the intensity was adjusted with neutral density glass filters (HOYA). For the detection of the slight intensity change in the probe light, 512–2048 output signals from a photomultiplier were stored and averaged with a digital oscilloscope (Textronics, 11401). The data were transferred to a personal computer for the further analysis.

3. RESULTS AND DISCUSSION

3.1. *Formation of a new red-shifted transient species R_{660}*

N_{560} intermediate with a long lifetime [12] was accumulated when the alkaline purple membrane suspension (pH 11.1) was irradiated with a cw background light (390–800 nm, 350 mW/cm²). As shown in Fig. 1, the time-resolved difference absorption spectrum was ob-

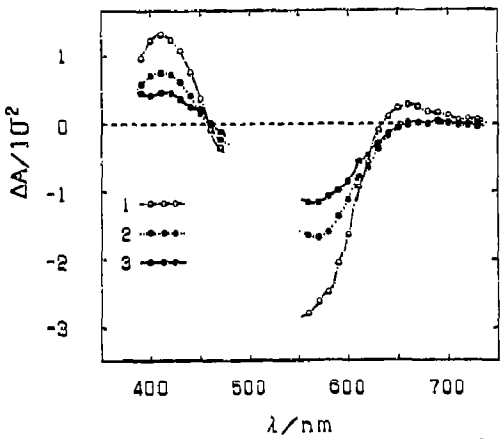

Fig. 1. Time-resolved difference absorption spectrum following the 532-nm pulsed excitation of the purple membrane suspension (11 μM, 25.1°C) in the photo-stationary state at pH 11.1 under irradiation of cw background light (390–800 nm, 350 mW/cm²). Here, transmitted background light was used for the probe light. Delay times are 0.5 ms (curve 1), 8.8 ms (curve 2), and 40.3 ms (curve 3).

tained after the 532-nm pulsed excitation of this sample suspension in the photo-stationary state. Curves 1, 2, and 3 stand for the spectra at delay times of 0.5 ms, 8.8 ms, and 40.3 ms, respectively. Negative absorbance change around 570 nm was due to the disappearances of both bR_{568} and N_{560}. Positive absorbance change in the blue region (< 460 nm) was attributed to the formation of M_{412}. Another positive absorbance change in the red region (> 620 nm) was observed at 0.5-ms delay time (curve 1). The maximum of this band was located at 660 nm, so we hereafter denoted the *red-shifted* species as R_{660}. Fig. 2A shows that R_{660} is formed just after the pulsed excitation (< 0.5 ms) and that it decays with a time constant of 1.5 ± 0.5 ms.

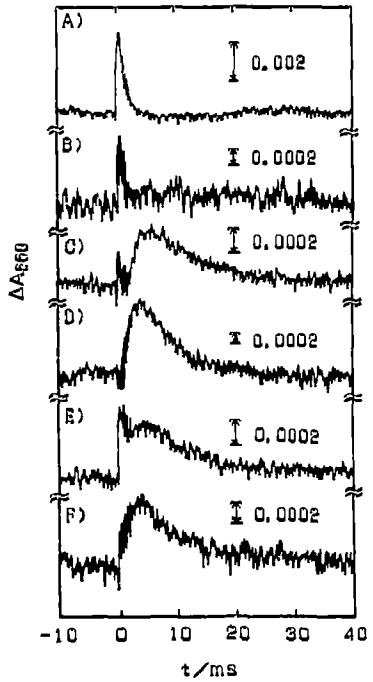

Fig. 2. Effect of pH on the transient absorbance change monitored at 660 nm. (A) Experimental condition was described in the caption of Fig. 1. (B) pH 10.1, (C) pH 9.2, (D) pH 7.9, (E) pH 7.9, 0.83 M KCl, (F) pH 7.5, 0.83 M KCl. Two cw xenon lamps with appropriate glass filters were used for the background light (390–800 nm, 430 mW/cm²) and probe light sources (11 mW/cm²) for Fig. 2B–F. [protein] = 12 μM.

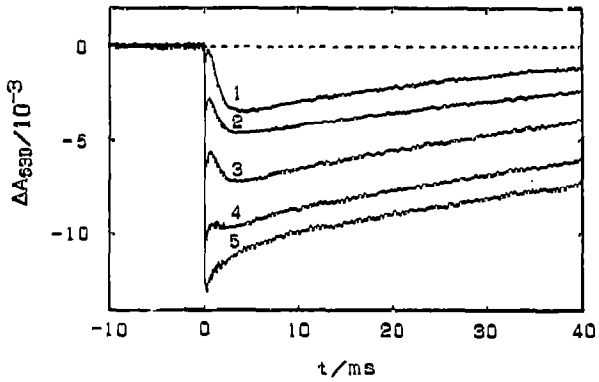

Fig. 3. Effect of cw light intensity on the yield of R_{660} at pH 10.9. The powers of the background light (440–800 nm) were 430 mW/cm² (curve 1), 211 mW/cm² (curve 2), 105 mW/cm² (curve 3), 31.5 mW/cm² (curve 4), and 0 (curve 5). The power of the probe light was 11 mW/cm². The average power of the 532-nm pulsed light (0.33 mJ/pulse, 5 Hz) was 2.76 mW/cm². [protein] = 14.5 μM. T = 22.7°C.

Fig. 2B–D shows the pH effect on the kinetics of the transient absorbance change. The lifetime of R_{660} at pH 10.1 (Fig. 2B) was identical with that measured at pH 11.1 (Fig. 2A). Its lifetime was insensitive to pH value and only the formation efficiency of R_{660} decreased with pH value. R_{660} was not observed in neutral or weak alkaline suspension (< pH 9.3). Another species appeared with a time-lag of a few milliseconds (see Fig. 2C). This species should be attributed to O_{640} intermediate. Fig. 2C clearly shows that R_{660} is a different species from O_{640}.

The yield of R_{660} was not only enhanced by alkalization but also by adding salts. It was clearly observed at pH 7.9 and even at pH 7.5 when KCl concentration was set at 0.83 M (see Fig. 2E,F). These behaviors of R_{660} were in good agreement with those of Q measured by time-resolved fluorometry [14].

3.2. *Effect of cw background light intensity*

The yield of R_{660} depended on cw light intensity as shown in Fig. 3. The temporal absorbance change at 630 nm was composed of the fast decay of R_{660} (within 4 ms) and the slow recovery of bR_{568} from N_{560} (> 5 ms). The amplitude of the fast component increased with the intensity of the cw light (see curves 1–4). The slow recovery process was dominant when the light intensity was weak (curve 5). Here, a fast recovery in the 0–8 ms region was attributed to the formation of N_{560} from M_{412}. Their quantitative relation is given in Fig. 4. N_{560} has a long enough lifetime to be accumulated under background light irradiation in alkaline and/or high ionic suspension [12]. The amount of N_{560} ($-\Delta A^{cw}_{570}$) in the photo-stationary state is given by solid squares. The

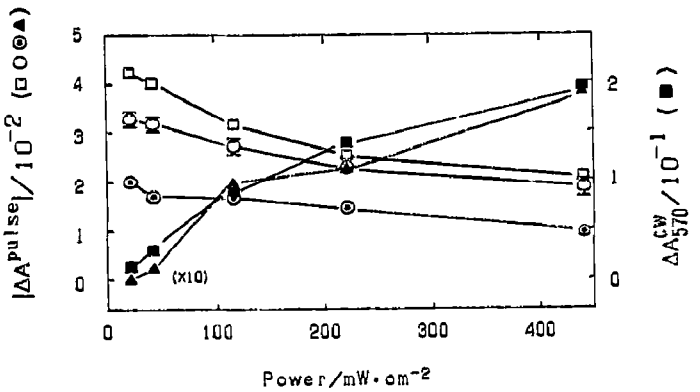

Fig. 4. Effect of cw light on the yields of N_{560} in the photo-stationary state [ΔA^{cw}_{570}] and transients, N_{560}, R_{660} and M_{412}, formed with a pulsed-light excitation of the stationary state ΔA^{pulse} at pH 10.9. Solid and open squares denote the yield of N_{560} monitored at 570 nm formed by cw and pulsed lights, respectively. Solid triangles denote the yield of R_{660} at 0.3-ms delay time (magnified by 10). Open and open-dot circles denote the yields of the initially formed M_{412} and its slow component (M^s), respectively, monitored at 410 nm. The summation of the probe light power (11 mW/cm²) and additional background light power is given in the figure. The average power of 532-nm pulsed light (0.33 mJ/pulse, 5 Hz) was 2.76 mW/cm². [protein] = 14.5 μM. T = 22.7°C.

yield of R_{660} (solid triangles) formed with a pulsed light was well correlated with the concentration of N_{560} in the photo-stationary state. N_{560} efficiently absorbs 532-nm light. So we concluded that R_{660} was photochemically formed from N_{560} intermediate in the photocycle of bR_{568}.

The effects of cw background light, pH value, and salt concentration on the behavior of R_{660} were quite similar to those of an O_{640}-like fluorescent intermediate Q reported previously [14]. Its spectroscopic property was similar to that of K_N found at low temperatures [13]. Therefore we concluded that R_{660}, Q and K_N are the same species.

3.3. *Two Photocycles in alkaline suspension*

Fig. 4 shows that the yield of R_{660} was in proportion to the amount of the accumulated N_{560} in the photo-stationary state. On the other hand the yield of initially formed M_{412} decreased with the increase in the cw light intensity. The fraction of bR_{568} under background light irradiation evidently decreased. These results show that there are at least two cycles in alkaline suspension: one is an ordinary cycle of bR_{568} and the other is that of N_{560} via R_{660}.

Two kinds of M_{412} have been known [16,17]: M^f (a fast decaying M) and M^s (a slow decaying M). Kouyama et al. [12] reported that M^s was a photoproduct of N_{560} in alkaline and high ionic strength suspensions, but their results are inconsistent with our present results. If M^s is a photoproduct of N_{560}, the concentration of M^s formed by a pulsed laser should increase with that of N_{560} in the photo-stationary state. Fig. 4, however, shows the opposite results. M^s is not a photoproduct of N_{560}. Here it should be noted that the amount of M^s is in proportion to those of both the initially formed M_{412} and N_{560} formed pulsed light. The results suggest that M^s and M^f do not exist in the different photocycles but rather in the cycle of bR_{568}. The following two photocycles are driven in alkaline suspension.

$$bR_{568} \xrightarrow{h\nu} M_{412} \rightleftharpoons N_{560} \longrightarrow bR_{568} \quad (1)$$

$$N_{560} \xrightarrow{h\nu} R_{660} \longrightarrow br_{568} \quad (2)$$

R_{660} finally goes back to bR_{568}. We, however, have not clarified whether R_{660} directly relaxes to bR_{568} or not. The secondary cycle should be considered in the quantitative measurements of quantum yields and reaction rates. The absorption of the second photon by N_{560} causes an internal filter effect and the enhancement of the recovery rate of bR_{568} from N_{560}.

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