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Time-resolved fluorometry of purple membrane of *Halobacterium halobium*

O_{640} and an O-like red-shifted intermediate Q

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The photocycle of the light-adapted purple membrane was studied with a time-resolved fluorometry apparatus: fluorescence of the sample suspension (>660 nm) was pumped with a 633-nm cw laser and the temporal change induced by a 532-nm pulsed laser was measured with a photon-counting-type transient recorder. The formation and the decay of the O_{640} intermediate were clearly observed in the pH region between 4.0 and 11.4. A photochemical cycle of N_{560} was apparently driven in alkaline suspension (pH > 9.3). An O-like fluorescent intermediate Q appears and decays with time constants of <0.1 ms and 1.7 ± 0.2 ms, respectively.

Bacteriorhodopsin; Purple membrane; Photocycle of N_{560}; Fluorescence of O_{640}; Q intermediate; *Halobacterium halobium*

1. INTRODUCTION

Purple membrane of *Halobacterium halobium* contains a single protein with a retinal chromophore. The rhodopsin-like protein is called bacteriorhodopsin (bR). Its light-adapted form, bR_{568}, functions as a light-driven proton pump [1]. An outline of the molecular mechanisms of this proton pump has been established: the reaction of bR_{568} is initiated with the photo-isomerization of retinal and is followed by successive thermal reactions via K_{590}, L_{550}, M_{412}, N_{560}, and O_{640} intermediates with different absorption spectra [2,3]. The release and uptake of proton(s) occur in the $L_{550} \rightarrow M_{412}$ process and the $N_{560} \rightarrow bR_{568}$ process, respectively [4,5]. However, the reported quantum yields of the photocycle [6] and the efficiencies of the proton pump [3,4,7] have not been consistent with each other. It is expected that these complexities are caused by the existence of more than one photocycle driven in parallel [6,8] and/or branched [9] in neutral and alkaline suspensions, as well as in acidic suspensions [10].

Multiple cycles have not been fully discriminated by conventional absorption technics. Resonance Raman spectra are sensitive to structure changes in detail, but the two-photon reaction products are sometimes formed because of the high density excitation of samples. We recently found that some intermediates (O_{640} and/or N_{560}) are more fluorescent than bR_{568} [11] and confirmed that fluorescence spectroscopy with low-power light (several mW/cm^2) for fluorescence pumping could be applied to the kinetic studies of photocycles. Thus, we developed a highly sensitive time-resolved fluorimetry with 50-ns resolution time and clarified the kinetics of O_{640} in the pH region between 4.0 and 11.4 and also found an O-like fluorescent intermediate which was photochemically formed from N_{560} intermediate.

2. MATERIALS AND METHODS

The purple membrane of *Halobacterium halobium* (ET1-001) was prepared by the conventional method [12]. The membrane suspension was light-adapted with a yellow light (>440 nm, 150-W xenon or 100-W tungsten lamp) just before each measurement.

The photocycle of bR was driven with a second harmonic (532 nm, 5-ns FWHM) of a Q-switched Nd:YAG laser (Spectra Physics, DCR-3F). A cw He-Ne laser (633 nm, NEC, GLG5320) was used for the continuous fluorescence excitation of the membrane suspension. The polarizations of the two lasers were adjusted parallel to each other. A sample cell (1 × 1 × 4 cm^3) was set in a temperature controlled holder. The sample was always stirred with a magnetic stirrer. The temporal change in the fluorescence intensity (>660 nm) was detected with a photon-counting system with 50-ns resolution time (Hamamatsu Photonics, C2550 Universal Photon Counter equipped with a R649 photomultiplier). The scattering lights of lasers were eliminated with an O56 and two R66 sharp cut-off filters (Hoya).

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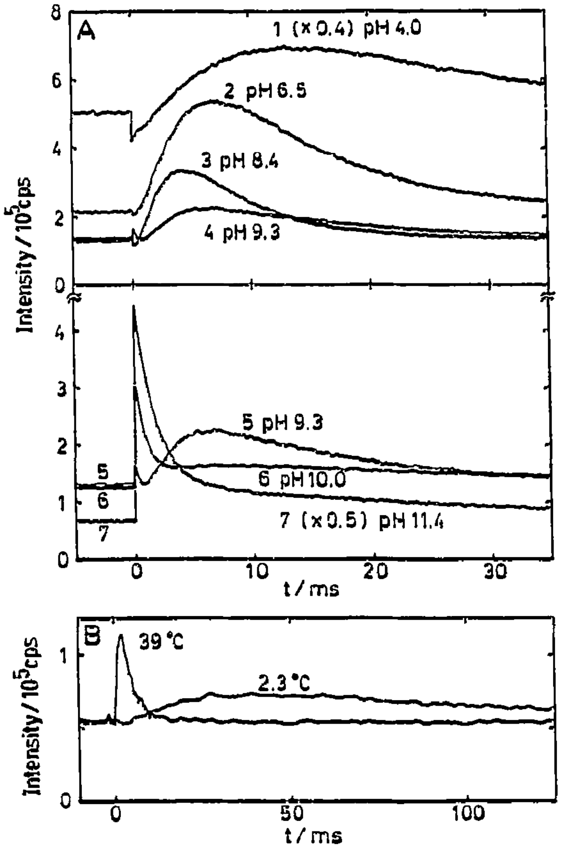

Fig. 1. Effects of pH (A) and temperature (B) on the kinetics of the transient fluorescence intensity (>660 nm) of the light-adapted purple membrane. The photocycle was driven with a 532-nm pulsed light (5-ns FWHM). Fluorescence excitation light source was a 633-nm cw He-Ne laser. (A) 22°C; $[bR_{568}] = 3.9 \times 10^{-6}$ M; photocycle driver, 3.1 mJ/cm^2 · pulse, 1.25 Hz; fluorescence probe, 7.5 mV/cm^2. (B) pH 6.5; $[bR_{568}] = 1.4 \times 10^{-6}$ M; photocycle driver, 1.5 mJ/cm^2 · pulse, 0.625 Hz; fluorescence probe, 1.3 mW/cm^2.

3. RESULTS AND DISCUSSION

3.1. *Dynamics of the O_{640} intermediate*

Curve 2 in Fig. 1A shows the typical time dependence of the fluorescence intensity (>660 nm) at pH 6.5. A weak bleaching was observed just after drive of the photocycle and it was followed by a build-up and a decay of the fluorescence intensity change. The fluorescent transient was attributed to the O_{640} intermediate because of the following reasons: (1) O_{640} has an intense absorption band at the wavelength of the fluorescence excitation laser (633 nm), but neither N_{560} nor M_{412} has any band [3]. (2) The measured time constants for the build-up and the decay of the transient fluorescence (τ_2 = 4.0 ± 0.6 ms and τ_1 = 7.9 ± 0.6 ms, respectively) are consistent with those of O_{640} (2–4 ms and 8 ms, respectively [13– 15]) measured by absorption spectroscopy. (3) Dependencies of both the pH value and temperature on the formation yield of the fluorescent transient are also consistent with those of O_{640} [3]. Fig. 1A and B show that the transient signal increases with the decrease in the pH value from 4.0 to 11.4 and also increases with the increase in temperature from 2.3°C to 39°C, respectively.

Thus, we could clearly observe the behavior of O_{640} with the newly developed time-resolved fluorometry even in alkaline suspension. O_{640} is stable (τ_1 > 15 ms) at pH 4.0. The lifetime decreased with an increase in pH value. O_{640} has the shortest lifetime in the pH region between 8.4–9.3. The lifetime increases again in the pH region higher than 10.0. Measured time constants are given in Table I.

3.2. *A new intermediate 'Q' under alkaline conditions*

A small signal with a short decay constant (τ_3) appears just after the excitation of the sample at pH 9.3 (see curve 5 or 6). It precedes the formation of O_{640}. The signal grows with an increase in pH value. The origin of the newly found fluorescence is tentatively denoted as species 'Q'. The lifetime of Q, 1.7 ± 0.2 ms, was insensitive to the pH value. Q was formed within 0.1 ms. The build-up process of Q was hidden by an intense emission with a very fast decay time (τ_4 = 10–30 μs) in the early stages (data not shown). The origin of the fastest component has not been clarified.

No change in the absorption spectrum of the sample before and after the photolysis was seen. Q is not an irreversible photoproduct but an intermediate which is finally converted to bR_{568}. This species has an absorption band at 633 nm as well as O_{640} but exhibits different kinetics and pH dependence from those of O_{640}. It is attributed neither to L_{550} nor to N_{560}. Any intermediate with such a lifetime has been reported in the primary photocycle of bR_{568} at room temperature.

Fig. 2A shows that the kinetics of the transient fluorescence of a dark-adapted sample at pH 11.4 (all measured samples except this sample were measured after full light adaptation). The signals of both Q and O_{640} are seen at the early stage of the measurement (see curve 1).

Table I

Effects of the pH value on the time constants for the decay (τ_1) and the formation (τ_2) of O_{640} intermediate at 22°C

pH	τ_1/ms	τ_2/ms
4.0	>15	8.0 ± 2.0
6.5	7.9 ± 0.6	4.0 ± 0.6
7.6	6.4 ± 0.4	2.8 ± 0.1
8.4	5.3 ± 0.5	2.4 ± 0.1
9.3	5.6 ± 0.5	2.4 ± 0.1
10.0	15 ± 5 (150 ± 50)[b]	–[a]
11.4[c]	9.6 ± 0.8 (>100)[b]	–[a]

[a] Not determined.

[b] Lifetime τ_1' longer than τ_1 was measured. Its origin has not been clarified.

[c] A dark-adapted sample was measured.

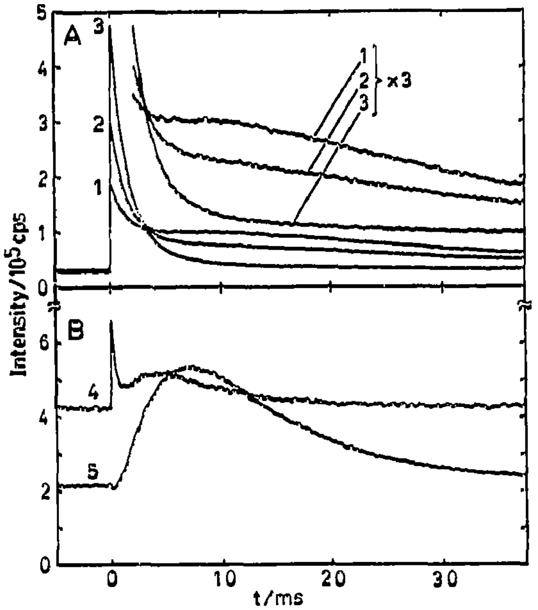

Fig. 2. Irradiation power effect on the formation and the decay of Q intermediate at 22°C. (A) Dark-adapted purple membrane suspension at pH 11.4 was irradiated with a probe (633 nm, 7.5 mW/cm^2) and a drive (532 nm, 3.1 mJ/cm^2 · pulse) lasers. Curve 1, driver 2.5 Hz; stirrer ON; curve 2, driver 10 Hz; stirrer ON; curve 3, driver 10 Hz; stirrer OFF. These data were sequentially taken without any pauses. (B) Light-adapted purple membrane suspension at pH 6.5 was irradiated with a cw probe and a 1.25-Hz drive laser. Curve 4, lasers were tightly focussed (75 mW/cm^2 and 24 mJ/cm^2 · pulse) onto a sample (3.6×10^{-5} M); curve 5, the same data as curve 2 in Fig. 1A.

The fraction of the former is enhanced with light-irradiation time and/or the repetition rate of the driver. The fluorescence intensity of Q is maximized when the escape rate of the irradiated molecules from the observed area is reduced (turn off the stirrer). Therefore Q is not attributed to a red-shifted intermediate ^{610}C [16] which is formed from the 13-*cis* component (bR_{548}) of the dark-adapted purple membrane.

The present results show evidence for a photochemical branching from an intermediate with a long lifetime, which is accumulated under the irradiation of bR_{568} with a 633-nm cw light and is converted to Q with a 532-nm pulsed light. We concluded that Q is derived from N_{560} because of the following results: (1) the formation yield of Q increases with both the pH value (pH > 9.3: see Fig. 1A) and the ionic strength (pH 8.4, [KCl] and/or [$KHCO_3$] > 10 mM: data not shown) of the suspension. There is a common ground to the conditions for both the formation of Q and the accumulation of N_{560} [5]. (2) Q was found even in the almost neutral suspension (pH 6.5, without salt) when the sample was irradiated with an intense pulsed laser (see Fig. 2B) to increase the transient concentration of N_{560}.

We concluded that both the photocycle of bR_{568} and a photo-branching reaction from N_{560} are driven under cw visible-light irradiation and that the fraction of the secondary cycle affects the quantum yields for the photocycle and the proton pump reported previously. These results, photoreaction of N_{560} and formation of a red-shifted intermediate, are supported by a recent nanosecond-absorption spectroscopy study (Yamamoto, N., Ohtani, H. and Naramoto, S., unpublished results).

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REFERENCES

[1] Stoeckenius, W. (1980) Accounts Chem. Res. 13, 337–344.
[2] Kung, M.C., Devault, D., Hess, B. and Oesterhelt, D. (1975) Biophys. J. 15, 907–911.
[3] Lozier, R.H., Bogomolni, R.A. and Stoeckenius, W. (1975) Biophys. J. 15, 955–962.
[4] Lozier, R.H., Niederberger, W., Bogomolni, R.A., Hwang, S. and Stoeckenius, W. (1976) Biochim. Biophys. Acta 440, 545–556.
[5] Kouyama, T., Nasuda-Kouyama, A., Ikegami, A., Mathew, M.K. and Stoeckenius, W. (1988) Biochemistry 27, 5855–5863.
[6] Birge, R.R., Cooper, T.M., Lawrence, A.F., Masthay, M.B., Vasilakis, C., Zhang, C.-F. and Zidovetzki, R. (1989) J. Am. Chem. Soc. 111, 4063–4074.
[7] Grzesiek, S. and Dencher, N.A. (1986) FEBS Lett. 208, 337–342.
[8] Bitting Jr., H.C., Jang, D.-J. and El-Sayed, M.A. (1990) Photochem. Photobiol. 51, 593–598.
[9] Kalisky, O., Ottolenghi, M., Honig, B. and Korenstein, R. (1981) Biochemistry 20, 649–655.
[10] Ohtani, H., Kobayashi, T., Iwai, J. and Ikegami, A. (1986) Biochemistry 25, 3356–3363.
[11] Ohtani, H., Ishikawa, M., Itoh, H., Takiguchi, Y., Urakami, T. and Tsuchiya, Y. (1990) Chem. Phys. Lett. 168, 493–498.
[12] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–679.
[13] Korenstein, R., Scherman, W.V. and Caplan, S.R. (1976) Biophys. Struct. Mech. 2, 267–276.
[14] Sherman, W.V. and Caplan, S.R. (1975) Nature 258, 766–768.
[15] Groma, G.I. and Dancshazy, Zs. (1986) Biophys. J. 50, 357–366.
[16] Kalisky, O., Goldschmidt, C.R. and Ottolenghi, M. (1977) Biophys. J. 19, 185–189.