Fluorescence spectra of bacteriorhodopsin and the intermediates O and Q at room temperature

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Abstract An unequivocal answer is given to the question of why the reported fluorescence spectra of bacteriorhodopsin (bR_{568}) have been different from one another. The inconsistency is shown to arise from the accumulation of the fluorescent intermediates O and Q (K_N) by cw excitation light. Their fractions in the photo-stationary states depend on the excitation power and the suspension pH. We report the intermediate-free fluorescence spectrum of bR_{568} obtained with a weak excitation source (632.8 nm, $5.3 \times 10^{15} - 1.9 \times 10^{16}$ photons $cm^{-2} \cdot s^{-1}$) and a near-IR sensitive intensified photodiode array system. The fluorescence maxima of the spectra, $F(\lambda)$ and $f(\tilde{\nu})$, are located at 755 ± 10 nm and 12700 ± 200 cm^{-1}, respectively. The spectrum of O is identical to that of the deionized purple membrane bR_{605} (F_{max} = 750 ± 5 nm, f_{max} = 13,000 ± 100 cm^{-1}). Q (K_N) exhibits a blue-shifted spectrum more than that of bR_{568} (F_{max} < 720 nm, f_{max} > 13,400 cm^{-1}).

Key words: Bacteriorhodopsin; Purple membrane; Infrared fluorescence; O_{640} intermediate; Q intermediate

1. Introduction

The light-adapted purple membrane of *Halobacterium halobium* contains bacteriorhodopsin (bR_{568}), which is a rhodopsin-like protein with an all-*trans* retinal chromophore [1]. It is not a visual pigment but a light-driven proton pump [2]. The function is initiated by the all-*trans*→13-*cis* photoisomerization of retinal [3]. The primary step is an efficient, selective, and ultrafast isomerization. It grounds on the structure of the retinoid protein in the excited state. Since Lewis et al.'s first reports [4–8], many authors have challenged the measurement of the fluorescence spectrum of bR_{568} at room temperature. However, their results have been inconsistent with one another.

We previously found that the fluorescence spectrum of the purple membrane suspension shifted to the shorter wavelength region with irradiation by intense 570-nm light at 22°C, and that two fluorescent intermediates with fluorescence lifetimes of 9 ± 5 ps and 50 ± 13 ps were formed [9]. They appeared in the photo-stationary state under irradiation of a pulse train (82 MHz) and returned back to the original pigment bR^{568} in the dark. Therefore, we suspected that the inconsistencies in the reported spectra arise from the accumulation of such intermediates, which fluoresce more than bR^{568}. We made a search for these intermediates using a highly sensitive time-resolved fluorometer and found that the intermediates O and Q are fluorescent [10]. The former efficiently fluoresced both in neutral and in acidic suspensions, and the latter, which is photochemically formed from N [10–13], fluoresced in alkaline and/or high salt concentration suspension.

$$bR_{568} \overset{h\nu}{\rightsquigarrow} K \rightarrow L \rightarrow M \rightarrow [N \underset{-H^+}{\overset{H^+}{\rightleftharpoons}} O(F1.)] \rightarrow bR_{568}$$
$$\underset{h\nu}{\downarrow}$$
$$Q(F1.)$$

In the present work we have measured the fluorescence spectra of bR_{568}, O, and Q, and clarified that the discrepancy in the reported spectra arises from contamination with the fluorescence of Q.

2. Materials and methods

The culturing of *Halobacterium halobium* and the preparation of purple membranes were performed according to the established method [14]. The sample suspension contained in a quartz cuvette was light-adapted with a visible light (440–800 nm) just before the measurement of each spectrum and was stirred continuously. The excitation source was a cw laser (632.8 nm, NEC, GLG 5360 or 514.5 nm, Spectra-Physics 2020; irradiated area 0.05–0.06 cm^2). A 90° collecting geometry was used. The scattered excitation light was rejected by a J-aggregate thin film filter [15] and sharp cut-off glass filters. Fluorescence was detected with an IR-sensitive intensified photodiode array system (Princeton Instruments; SMA RE/IRY-700 detector head, ST-1000 controller) coupled with a spectrograph (Jobin-Yvon; HR-320, f 32 cm, 150-grooves/mm grating, 100-μm slit width). The photocathode and the diode array were cooled to decrease thermal electron emission and dark current. Framing rate was set at 1/60 Hz to decrease the read-out noise. The spectral response of the whole system was calibrated with a standard light source.

3. Results and discussion

3.1. Fluorescence of O

bR_{568} is thermally converted to its 13-*cis* isomer, bR_{548}, the fluorescence yield of which is not negligible ($\Phi_F^{cis} = 0.4\,\Phi_F^{trans}$ [7]). Sample suspensions were light-adapted just before each measurement and the 632.8-nm line was used for excitation so as not to pump bR_{548}. Curves 1 and 2 in Fig. 1 show the fluorescence spectra of a native purple membrane suspension at pH 6.3 measured at 5.3×10^{15} photons·$cm^{-2} \cdot s^{-1}$ and 1.9×10^{17} photons $cm^{-2} \cdot s^{-1}$ excitation powers, respectively. The integrated fluorescence intensity F ($= \int_{670\,nm}^{850\,nm} F(\lambda)\,d\lambda$) was proportional to the excitation power $P_{632.8}$ in the $\leqq 1.9 \times 10^{16}$ photons·$cm^{-2} \cdot s^{-1}$ region ($\log F / \log P_{632.8} = 1.0 \pm 0.1$, see filled circles in Fig. 2). Then the broad spectrum shown by curve 1 is attributed to the fluorescence of bR_{568}. The fluorescence efficiency of the suspension increased with $P^{632.8}$ in the $> 1.9 \times 10^{16}$ photons·$cm^{-2} \cdot s^{-1}$ power region ($\log F / \log P_{632.8} = 1.2$). Spectrum 2 in Fig. 1 ($P_{632.8} = 1.9 \times 10^{17}$ photons·$cm^{-2} \cdot s^{-1}$) exhibits the intensity

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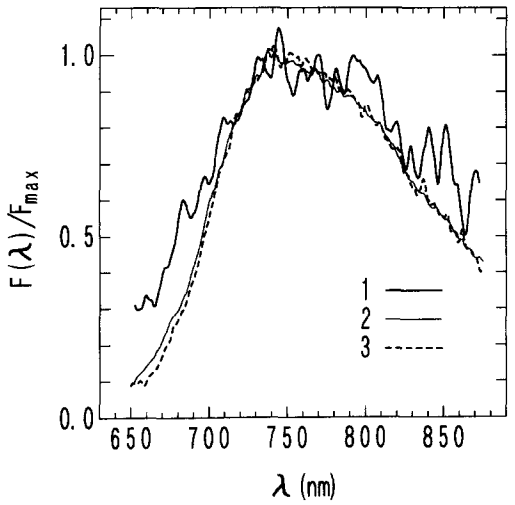

Fig. 1. Fluorescence spectrum of the purple membrane suspension pumped by 632.8 nm light at 22°C. The spectral response of the system was calibrated. Curve 1, pH 6.3, P_{ex} 5.3×10^{15} photons·cm^{-2}·s^{-1}, 17 μM bR; curve 2, pH 6.3, P_{ex} 1.9×10^{17} photons·cm^{-2}·s^{-1}, 17 μM bR; curve 3, pH 5.0, P_{ex} 1.9×10^{16} photons·cm^{-2}·s^{-1}, 13 μM bR.

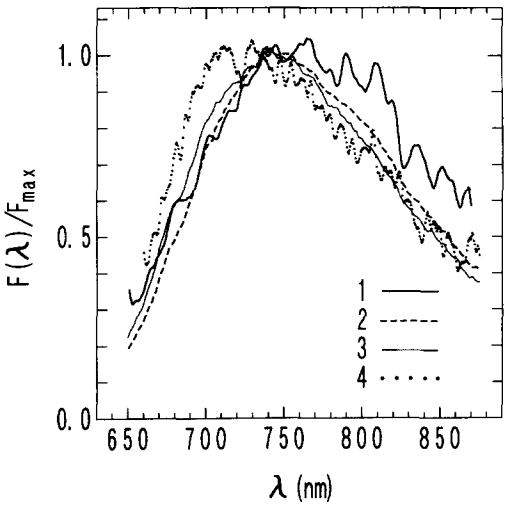

Fig. 3. Fluorescence spectrum of the purple membrane suspension pumped by 632.8 nm light at 22°C. The spectral response of the system was calibrated. Curve 1, pH 10.3, P_{ex} 1.9×10^{16} photons·cm^{-2}·s^{-1}, 15 μM bR; curve 2, pH 10.3, P_{ex} 2.6×10^{17} photons·cm^{-2}·s^{-1}, 15 μM bR; curve 3, pH 11.5, P_{ex} 2.6×10^{17} photons·cm^{-2}·s^{-1}, 14 μM bR; curve 4, pH 11.5, 1 M KCl, P_{ex} 7.3×10^{16} photons·cm^{-2}·s^{-1}, 21 μM bR.

ratio $F_{680\ nm}/F_{750\ nm}$ (= $\lambda_{680/750}$ = 0.30 ± 0.01) lower than that of spectrum 1 (0.52 ± 0.04). The spectral change is due to the accumulation of a fluorescent intermediate with a spectrum narrower than that of bR_{568}. We expected that the intermediate is O, according to the results of the previous time-resolved fluorometry [10].

We examined the accumulation of O in weakly acidified purple membrane suspensions (pH 5.0), in which O is more stable than N, and found that the measured threshold power for the accumulation of the fluorescent intermediate (5.3×10^{15} photons·cm^{-2}·s^{-1}) was lower than that at pH 6.3 (1.9×10^{16} photons·cm^{-2}·s^{-1}). The slope log F/log$P_{632.8}$ was 1.2 and no spectral change was found in the 5.3×10^{15}–2.6×10^{17} photons·cm^{-2}·s^{-1} power region. The spectrum shown by curve 3 in Fig. 1 was measured at 1.9×10^{16} photons·cm^{-2}·s^{-1} excitation power. The origin of the narrow spectrum was attributed to the intermediate O. Spectrum 2, measured at pH 6.3, is in good agreement with the fluorescence spectrum of O. Thus we concluded that the accumulation of O yields the narrowing of the measured spectrum of the purple membrane suspension.

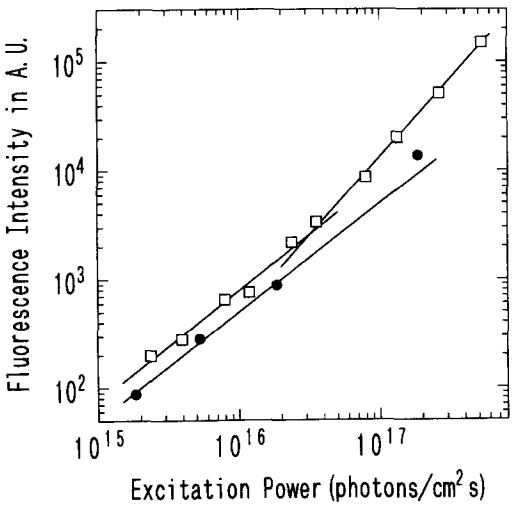

Fig. 2. Effect of excitation power on the fluorescence intensity F (= $\int_{670\ nm}^{850\ nm} F(\lambda)\ d\lambda$) at 22°C. (●) λ_{ex} 632.8 nm, pH 6.3, 38 μM bR; (□) λ_{ex} 514.5 nm, pH 11.5, 1 M KCl, 27 μM bR.

The fluorescence spectrum of O was identical to that of the deionized (or acidified) purple membrane bR_{605} (data not shown). It is reported that O and the all-*trans* component of bR_{605} (t-bR_{605}) have common photochemical properties: the lack of the M-form in their photocycles [16,17] and the formation of 9-*cis*-retinal-containing photoproducts [18,19]. We found that the structure of O in the excited state is same as that of t-bR_{605}.

3.2. Fluorescent Q and non-fluorescent N

The spectrum 1 in Fig. 3 was measured with a low excitation power ($P_{632.8}$ = 1.9×10^{16} photons·cm^2·s^{-1}) at pH 10.3. The spectrum ($\gamma_{680/750}$ 0.56 ± 0.04) is in agreement with that of bR^{568} shown by curve 1 in Fig. 1. Spectrum 2 in Fig. 3 was measured with the higher excitation power ($P_{632.8}$ = 2.6×10^{17} photons·cm^{-2}·s^{-1}). The decrease in $\gamma_{680/750}$ from 0.56 ± 0.04 to 0.49 ± 0.01 may be due to the formation of O. However, the ratio $F_{700\ nm}/F_{800\ nm}$ (= $\gamma_{700/800}$) increased from 0.81 ± 0.05 to 0.90 ± 0.02. Such an increase in $\gamma_{700/800}$ was not seen at pH 6.3. The ratio rather decreased with the accumulation of O (for example, 0.76 and 0.67 at $P_{632.8}$ = 5.3×10^{15} and 1.9×10^{17} photons·cm^{-2}·s^{-1}, respectively). The increase in $\gamma_{700/800}$ shows the accumulation of a photoproduct with a blue-shifted fluorescence spectrum. The ratio $\gamma_{700/800}$ increases with the increase in pH (1.1 ± 0.0 at pH 11.5; curve 3 in Fig. 3). The fluorescence in the shorter wavelength region was markedly enhanced by adding 1 M KCl ($\gamma_{700/800}$ = 1.3 ± 0.1; curve 4 in Fig. 3).

The lifetime of N and its fraction in the N⇌O quasi-equilibrium increase with the increase in the suspension pH and/or salt concentration [20]. Q (K_N) is photochemically formed from the accumulated N [10–13]. The alkaline purple membrane suspension in the photo-stationary state is composed of bR_{568}, M, N, and Q (and a small amount of O). We attribute the fluorescence

Table 1
Fluorescence properties of bR_{568}, O, and Q

Pigment	$\tilde{\nu}_{max}$ (cm^{-1})	FWHM (cm^{-1})	λ_{max} (nm)	$F_{680\ nm}/F_{750\ nm}$	$F_{700\ nm}/F_{800\ nm}$
bR_{568}	12,700 ± 200	>3500	>755 ± 10	0.56 ± 0.04	0.81 ± 0.05
O	13,000 ± 100	2800	750 ± 5	0.28 ± 0.01	0.63 ± 0.01
Q	>13,400	<3000	<720	>0.75	>1.3

Fluorescence maxima $\tilde{\nu}_{max}$ and λ_{max} were obtained from the corrected spectra $f(\tilde{\nu})$ and $F(\lambda)$, respectively.

band in the <720 nm region to Q, according to the results of the power dependence of the fluorescence intensity.

The open squares in Fig. 2 show the integrated intensity F ($= \int_{670\ nm}^{850\ nm} F(\lambda)\,d\lambda$) of a purple membrane suspension (pH 11.5, 1 M KCl) measured with a 514.5-nm excitation line. The fluorescence of bR_{568} was measured up to the 1.2×10^{16} photons·cm^{-2}·s^{-1} excitation power (log F/log $P_{514.5} = 1.0 \pm 0.1$). This upper limit was higher than that for the 632.8-nm excitation of the same sample ($< 0.77 \times 10^{16}$ photons·cm^{-2}·s^{-1}, data not shown), although the accumulated N absorbs the green light more efficiently than the red light. This is due to the non-fluorescent property of N. The fluorescence of Q becomes dominant in the $\geqq 3.6 \times 10^{16}$ photons·cm^{-2}·s^{-1} region (log F/ log $P_{514.5} = 1.4 \pm 0.0$). The intense fluorescence of Q is due to its high quantum yield, which is 100 times higher than that of bR_{568} (Kamiya, N., Ishikawa, M., Kasahara, K., Kaneko, M., Yamamoto, N. and Ohtani, H., unpublished results).

3.3. Fluorescence of bR_{568}

It has been shown that the fluorescence spectra of the purple membrane suspensions are composed of those of bR_{568}, O, and Q. Their spectroscopic properties are summarized in Fig. 4 and Table 1. The background fluorescence of O is not negligible in neutral and in acidic suspensions when $\gamma_{680/750}$ is less than 0.5. The fluorescence spectrum of bR_{568} (curve 1 in Fig. 1) was measured by a weak 632.8 nm line ($P_{ex} = 5.3 \times 10^{15}$ photons·cm^{-2}·s^{-1}) at pH 6.3, although the signal-to-noise ratio was not high. The N⇌O equilibrium is shifted toward the non-fluorescent N with the increase in pH and/or in salt concentration. Although Q is the most fluorescent intermediate, its fluorescence (3-photon process, $bR_{568} \overset{h\nu}{\rightsquigarrow} N \overset{h\nu}{\rightsquigarrow} Q \overset{h\nu}{\rightsquigarrow} Q^*$) can be eliminated more easily than that of O (2-photon process, $bR_{568} \overset{h\nu}{\rightsquigarrow} O \overset{h\nu}{\rightsquigarrow} O^*$) by lowering the excitation power. Thus, the fluorescence spectrum of bR_{568} (curve 1 in Fig. 3 and curve 1 in Fig. 4) can be measured by taking advantage of the power dependence in an alkaline suspension. The fluorescence maxima λ_{max} and $\tilde{\nu}_{max}$ are located at 755 ± 10 nm and 12700 ± 200·cm^{-1}, respectively.

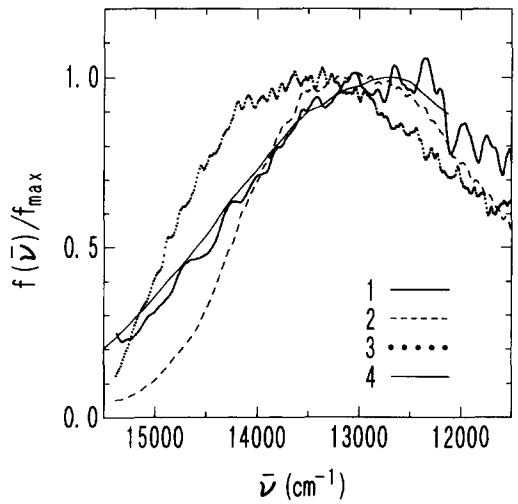

Fig. 4. Fluorescence spectra of bR_{568}, O, and Q measured at 22°C. The spectral response of the system was calibrated. Curve 1, bR_{568}, conditions as curve 1 in Fig. 3; curve 2, O_{640}, pH 5.0, $P_{632.8}$ 2.6×10^{16} photons cm^{-2} s^{-1}, 13 μM bR; curve 3, mixture of Q and bR_{568}, pH 11.5, 1 M KCl, $P_{514.5}$ 5.3×10^{17} photons cm^{-2} s^{-1}, 19 μM bR; curve 4, spectrum of bR_{568} measured by Lewis et al. [4] with a 514.5-nm excitation light.

Our spectrum (curve 1 in Fig. 4) is in best agreement with that measured by Lewis et al. [4] (curve 4 in Fig. 4). The spectra reported by Kouyama et al. [7] and by Atkinson et al. [8] are similar to that in the present work but the spectra are slightly blue-shifted. Atkinson et al. eliminated the fluorescent intermediates O and Q by using the sample-jet-flow system but a small amount of K with a λ_{max} at 731 nm might be accumulated within the duration of excitation light (8-ps full width at half maximum). The spectrum measured by Kouyama et al. is more intense than our spectrum in the <700-nm wavelength region. The difference may be due to the formation of Q. The spectra reported by Gillbro et al. [5] and by Govindjee et al. [6] exhibit peaks at 730 nm and 700 nm, respectively. We attribute them to the fluorescence of Q. The inconsistency in the reported spectra arise from the different fractions of Q accumulated in the sample suspensions, because the previous authors used intense excitation light to measure the ultraweak fluorescence of bR_{568} ($\Phi_F = 2.5 \times 10^{-5}$–$2.7 \times 10^{-4}$ [4,6,7]) with a photomultiplier, the sensitivity of which is low in the near-IR region. The contribution of O is not clear in all the reported spectra because samples were excited with green or yellow light instead of red in the previous experiments. However, the fluorescence of O may not be neglected in the measurement of the fluorescence quantum yield of bR_{568}.

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