Excitation Spectrum of the N Intermediate in the Photocycle of Bacteriorhodopsin

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We obtained the absorption spectrum of the N intermediate that plays an important role in the proton uptake during the photocycle of bacteriorhodopsin. The reported spectra of N that were reconstituted from the transient difference absorption spectrum depended on the assumed kinetic models such as sequential M → N → O reaction and M ⇄ N ⇄ O quasi-equilibrium. In this work we measured the excitation spectrum of N by monitoring the characteristic absorption of Q (a photoproduct of N) at 664 nm. The λ_{max} and ϵ_{max} of N were 559 ± 3 nm and (4.30 ± 0.75) × 10^4 M^{-1} cm^{-1}, respectively. The present spectrum is consistent with the results of the recent SVD analysis. This work provides independent evidence that Q is photochemically formed from N.

1. Introduction

Halobacterium salinarum (*H. salinarum*) is a halophilic archaebacterium.[1] Under anaerobic conditions it synthesizes a photoactive protein called bacteriorhodopsin (bR) that has a retinal chromophore covalently bound to the ϵ-amino group of Lys-216 via a protonated Schiff base (−C═NH^+—Lys). The all-trans isomer of bR (bR_{568}, λ_{max} = 568 nm) functions as a light-driven proton pump from the cytoplasmic side to the external side.[2] The proton pump is coupled with a cyclic photoreaction (photocycle) of bR_{568} via five states K, L, M, N, and O.[3] The photocycle is initiated by the retinal isomerization from all-trans to 13-cis conformation in the excited state of bR_{568}.[4] The photon energy is stored in the cisoid chromophore and is used for the changes in the conformation and the charge distribution in the protein.

The N state was reported in the earliest photocycle scheme as N_{520} (λ_{max} = 520 nm),[3] and Hwang et al.[5] and Shkrob and Rodionov[6] supported its formation. However, it has been a ghost species that appears only in high pH suspension because it has no visible absorption spectrum remarkably distinguished from bR_{568} though K, L, M, and O have. Kouyama et al.[7] clarified long-lived intermediates P_{560} and R_{350} that form between M and bR_{568}.[8,9] They attributed the absorption at 560 and 350 nm to the α- and β-bands of N, respectively, and pointed out that the N state plays an important role on the uptake of a proton from the cytoplasmic side.[7]

The chromophore structure of N has been clarified to be a 13-cis, 14-s-trans, C_{15}═N_{16} anti (trans) protonated Schiff base.[10] The electronic structure of N, however, has not necessarily been established. Some authors reconstituted its spectrum from the measured difference absorption spectra.[3,7,11,12] The estimation of the concentration of N at delay time t ($[N]_t$), which is essential to the reconstitution, is difficult even though both the excitation density and the reaction quantum yield were strictly known because of the complicated photocycle of bR_{568}. For example, Váró et al.[13] proposed the following two kinetic models.

Their model (1) requires us to know many rate constants for the estimation of $[N]_t$, and the model (2), furthermore, requires us to know the equilibrium constant between bR_I and bR_{II}. Thus, the reconstitution of the spectrum of N is not an easy task and the obtained spectrum is strongly model-dependent. Gergely and Váró[12] recently obtained the model-independent spectrum of N by using the singular value decomposition (SVD) method.

$$bR_{568} \xrightarrow{h\nu} K \rightarrow L \rightleftarrows M_I \rightarrow M_{II} \rightleftarrows N \rightleftarrows O \rightarrow bR_{568} \quad (1)$$

$$\begin{array}{l} bR_I \xrightarrow{h\nu} K_I \rightarrow L_I \rightarrow M_I \rightarrow N_I \rightleftarrows O_I \rightarrow bR_I \\ \updownarrow \\ bR_{II} \xrightarrow{h\nu} K_{II} \rightarrow L_{II} \rightarrow M_{II} \rightleftarrows N_{II} \rightleftarrows O_{II} \rightarrow bR_{II} \end{array} \quad (2)$$

We recently found that the detection of Q (a photoproduct of N[13,14]) is efficient for the quantitative analysis of N rather than the direct detection of N itself, because Q exhibits an intense red-shifted absorption band and a fluorescence quantum yield much higher than that of N.[15−17] In this work we measured the excitation spectrum by monitoring the absorbance change (ΔA^N) due to $N \overset{h\nu}{\rightsquigarrow} Q$. Thus the model-independent absorption spectrum of N was obtained.

2. Experimental Section

The purple membrane (PM) was prepared from *H. salinarum* (ET1-001) by standard procedures.[18] Absorption spectra of the samples were measured with a spectrophotometer (JASCO V-570 equipped with an integration sphere unit V-469) before and after photolysis experiments. An excitation light source (430−700 nm, 250−350 μJ, 5 ns) was an optical parametric oscillator pumped by a Nd:YAG laser (MOPO 700, Spectra-Physics). The excitation intensity (3.5−5.0 mJ/cm^2) was set so as not to induce the saturation of the transient absorption due to the formation of the bR_{568} ↔ K photostationary state in the pulse width. A continuous wave (CW) xenon lamp (150 W, Hamamatsu Photonics L2274) was used for a probe light source with appropriate glass filters. The CW light was also used as a background light source for the formation of the photostationary state purple membrane (PSPM) including N. The transmitted probe light was detected with a photomultiplier (Hamamatsu Photonics R3788-02) coupled with a triple monochromator (f = 257 mm, Acton Research Corp., SpectraPro-275). Signals

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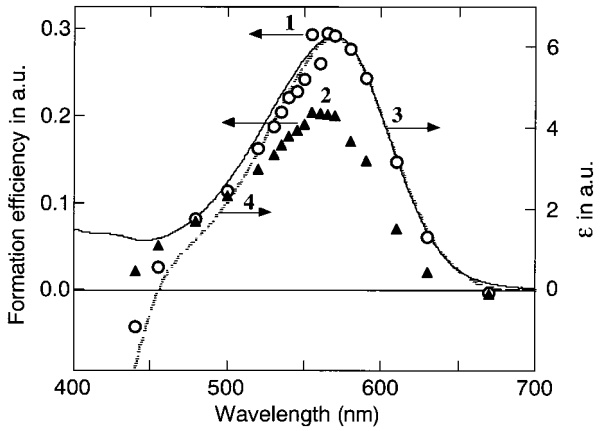

Figure 1. Excitation spectra of bR_{568} and N in an alkaline PSPM monitored by the transient absorption of M (open circles, $\lambda_{probe} = 408$ nm) and N (solid triangles, $\lambda_{probe} = 664$ nm), respectively. Conditions: pH 10, 17 μM protein, 22 °C. Key: solid line, absorption spectrum of bR_{568}; dashed line, the bR_{568} minus M difference spectrum reported by Fukuda and Kouyama.[20]

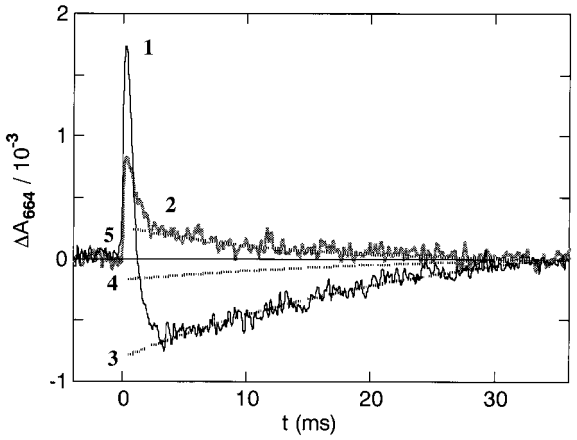

Figure 2. Kinetics in absorbance change at 664 nm following the pulsed excitation of an alkaline PSPM at pH 10: curve 1, $\lambda_{ex} = 555$ nm; curve 2, $\lambda_{ex} = 440$ nm; curves 3 and 4, the absorbance changes due to the recovery of bR_{568} ($\tau = 26$ ms) at $\lambda_{ex} = 555$ and 440 nm, respectively; curve 5, summation of the absorbance changes due to the decay of a photoproduct of M ($\tau = 17$ ms) and the recovery of bR_{568} at $\lambda_{ex} = 440$ nm.

(256−512 shots) were stored and averaged with a digital oscilloscope (Textronics TPS360).

The apparatus was calibrated with a light-adapted PM (LAPM) at pH 7 as standard. The characteristic absorption of M at 408 nm ($\Delta A^{LA}_{408}(\lambda_{ex},t)$) was measured, extrapolated at $t = 0$, and normalized by the photon number of the excitation pulsed light. The photon number at each λ_{ex} was measured by an energy meter (Teraherz Technologies Inc. P-444 pyroelectric probe, DigiRad R-752 Universal laser radiometer) just before and after the measurement. The calibration constant $\alpha(\lambda_{ex})$ was set so as to make $\Delta A^{LA}_{408}(\lambda_{ex},0)$ reproduce the molar extinction coefficient of bR_{568} (ϵ^{bR}) at λ_{ex}.

$$\Delta A^{LA}_{408}(\lambda_{ex},0) = \alpha(\lambda_{ex})\,\epsilon^{bR}(\lambda_{ex}) \quad (3)$$

3. Results and Discussion

The measured sample suspension was a PSPM at pH 10. The PSPM was composed of bR_{568} and the stationarily accumulated intermediates M and N with lifetimes elongated by alkalization. The sample suspension and the standard (pure bR_{568}, LAPM at pH 7) were alternatively measured at the same λ_{ex}. The absorbance change was normalized by the photon number of the excitation pulsed light for each measurement.

Open circles in Figure 1 show the corrected excitation spectrum of the PSPM monitored at 408 nm ($\Delta A^{PS}_{408}(\lambda_{ex},0)/\alpha(\lambda_{ex})$). It is in good agreement with the absorption spectrum of bR_{568} (solid line) in the ≥455 nm region. The $\Delta A^{PS}_{408}(\lambda_{ex},0)/\alpha(\lambda_{ex})$ in the <450 nm region was negative because not only bR_{568} but also the accumulated M was excited (see eq 4). The absorbance change at 408 nm due to the excitation of N was neglected.

$$\Delta A^{PS}_{408}(\lambda_{ex},0) = \Delta A^{bR/M}_{408}(\lambda_{ex},0) + \Delta A^{M/Y}_{408}(\lambda_{ex},0)$$
$$= (\epsilon^{M}_{408} - \epsilon^{bR}_{408})[M]_{t=0} + (\epsilon^{Y}_{408} - \epsilon^{M}_{408})[Y]_{t=0} \quad (4)$$

Here, Y denotes the photoproduct of M with a bacteriorhodopsin-like absorption spectrum ($\lambda_{max} = 570$ nm) which corresponds to P_{585} found at low temperatures.[14,19] Thus, the term $\Delta A^{M/Y}_{408}(\lambda_{ex},0)$ in eq 4 gives the negative value because ϵ^{M}_{408} is much larger than ϵ^{Y}_{408}. The excitation spectrum of N in the PSPM was measured by monitoring the characteristic absorption of Q at 664 nm.[16] The measured $\Delta A^{PS}_{664}(\lambda_{ex},t)$ was composed of $\Delta A^{bR/M,N}_{664}(\lambda_{ex},t)$, $\Delta A^{N/Q}_{664}(\lambda_{ex},t)$, and $\Delta A^{M/Y}_{664}(\lambda_{ex},t)$ due to the photocycles of bR_{568}, N, and M, respectively, given by the following equations.

$$\Delta A^{PS}_{664}(\lambda_{ex},t) = \Delta A^{bR/M,N}_{664}(\lambda_{ex},t) + \Delta A^{N/Q}_{664}(\lambda_{ex},t) + \Delta A^{M/Y}_{664}(\lambda_{ex},t) \quad (5)$$
$$= (\epsilon^{M}_{664}[M]_t + \epsilon^{N}_{664}[N]_t)l - \epsilon^{bR}_{664}([M]_t + [N]_t)l + (\epsilon^{Q}_{664} - \epsilon^{N}_{664})[Q]_t l + (\epsilon^{Y}_{664} - \epsilon^{M}_{664})[Y]_t l$$
$$\approx -\epsilon^{bR}_{664}([M]_t + [N]_t)l + \epsilon^{Q}_{664}[Q]_t l + \epsilon^{Y}_{664}[Y]_t l \quad (6)$$

Here, ϵ^{M}_{664} and ϵ^{N}_{664} are negligibly small.

When λ_{ex} was set in the ≥470 nm region (see curve 1 in Figure 2), the measured kinetics $\Delta A^{PS}_{664}(\lambda_{ex},t)$ was composed of a slow bleaching recovery (curve 3) and a fast absorption decay. The former and the latter were attributed to the recovery of bR_{568} and the decay of Q to N,[15,16] respectively. The absorbance change $\Delta A^{M/Y}_{664}(\lambda_{ex},t)$ was negligibly small in the ≥470 nm λ_{ex} region. The net absorbance change due to the formation of Q at 0.5 ms ($\Delta A^{N/Q}_{664}(\lambda_{ex},0.5\text{ms})$) was obtained by the subtraction of the $\Delta A^{bR/M,N}_{664}(\lambda_{ex},t)$ from the measured absorbance change.

When λ_{ex} was set in the <470 nm region, the third component appeared in the measured $\Delta A^{PS}_{664}(\lambda_{ex},t)$ (curve 2). The slow absorption decay was attributed to the decay of Y, i.e., $\Delta A^{M/Y}_{664}(\lambda_{ex},t)$. The accumulated M in the PSPM was pumped by the <470 nm pulsed light. Both the contribution of the third component and the recovery of bR_{568} were subtracted from the measured $\Delta A^{PS}_{664}(\lambda_{ex},t)$.

Thus, we obtained the excitation spectrum of N ($\Delta A^{N/Q}_{664}(\lambda_{ex},0.5\text{ ms})/\alpha(\lambda_{ex})$) shown by solid triangles in Figure 1. N exhibits a λ_{max} at 559 ± 4 nm. Hereafter we denote N as N_{559}. We could reproduce the N_{559} minus bR_{568} difference spectrum reported by Fukuda and Kouyama[20] when we set the ϵ_{max} of N_{559} at 43 050 M^{-1} cm^{-1}. Thus we obtained the ϵ_{max} of N_{559} ($(4.30 \pm 0.75) \times 10^4$ M^{-1} cm^{-1}). The reported spectra of N distributed around our spectrum (see Figure 3). Our ϵ_{max} is smaller than that reported by Kouyama et al.[7] ($(4.5-5.3) \times 10^4$

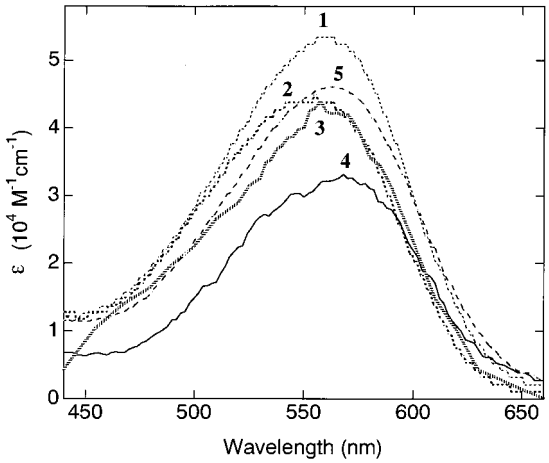

Figure 3. Absorption spectrum of N: curves 1 and 2, calculated for $\epsilon_{580}^{N}/\epsilon_{580}^{bR} = 0.78$ and 0.60, respectively, according to the unidirection and no-back-reaction scheme (Kouyama et al.[7]); curve 3, this work; curve 4, calculated according to $M_{II} \rightleftarrows N \rightleftarrows O$ scheme by Váró et al.;[11] curve 5, obtained by using the SVD method (Gergely and Váró[12]).

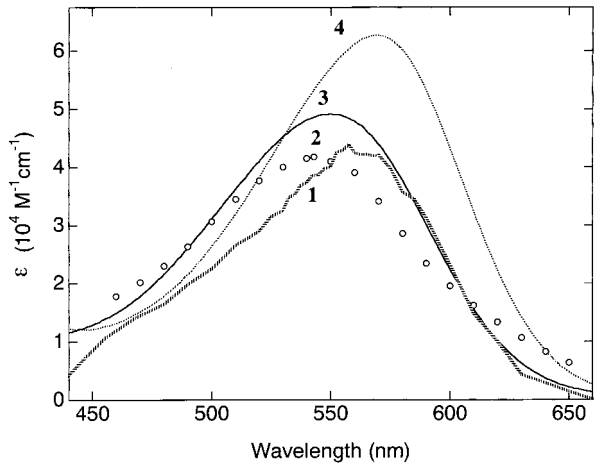

Figure 4. Absorption spectra of 13-cis pigments and bR_{568}: curve 1, N_{559} (this work); curve 2, L_{543} (Shichida et al.[21]); curve 3, bR_{550}; curve 4, bR_{568}.

M^{-1} cm^{-1}, $\lambda_{max} = 555-560$ nm) and is much higher than that reported by Váró et al.[11] (3.3×10^4 M^{-1} cm^{-1} at 568 nm). Our spectrum resembles the recent one reported by Gergely and Váró[12] (4.6×10^4 M^{-1} cm^{-1} at 562 nm). Their spectrum was obtained by using the SVD analysis.

Absorption spectra of 13-cis pigments are shown in Figure 4. The ϵ_{max} of N_{559} is nearly equal to that of L_{543} (4.3×10^4 M^{-1} cm^{-1} at 543 nm[21]) and smaller than that of bR_{550} (the 13-cis isomer of bR_{568}, 4.8×10^4 M^{-1} cm^{-1} at 550 nm). Here, the spectrum of bR_{550} was calculated from the spectrum of DAPM according to the results of photocurrent measurement (trans: 13-cis = 55:45).[22]

The λ_{max} of N_{559} is longer than those of L_{543} and bR_{550}. Pigments with an all-trans chromophore show the remarkable red shift of λ_{max} by the protonation of the carboxyl group of Asp-85. For example, the λ_{max} of O with COOH (624 ± 4 nm, H. Ohtani and S. Kanematsu, unpublished) is longer than that of bR_{568} with COO^-. The red-shifted spectrum of N_{559} is consistent with the previous observations that Asp-85 is protonated in the N_{559} state.[23,24] The difference in the λ_{max} between L_{543} and bR_{550} may be due to the difference in the retinal conformation, i.e., the distance between the protonated Schiff base and its counteranion ($-COO^-$ of Asp-85), because L_{543} and bR_{550} have C=N anti and C=N syn conformations, respectively.[25,26]

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