Existence of two O-like intermediates in the photocycle of *Acetabularia* rhodopsin II, a light-driven proton pump from a marine alga

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A spectrally silent change is often observed in the photocycle of microbial rhodopsins. Here, we suggest the presence of two O intermediates in the photocycle of *Acetabularia* rhodopsin II (ARII or also called Ace2), a light-driven algal proton pump from *Acetabularia acetabulum*. ARII exhibits a photocycle including a quasi-equilibrium state of M, N, and O (M⇄N⇄O→) at near neutral and above pH values. However, acidification of the medium below pH ~5.5 causes no accumulation of N, resulting in that the photocycle of ARII can be described as an irreversible scheme (M→O→). This may facilitate the investigation of the latter part of the photocycle, especially the rise and decay of O, during which molecular events have not been sufficiently understood. Thus we analyzed the photocycle under acidic conditions (pH ≤ 5.5). Analysis of the absorbance change at 610 nm, which mainly monitors the fractional concentration changes of K and O, was performed and revealed a photocycle scheme containing two sequential O-states with the different molar extinction coefficients. These photoproducts, termed O₁ and O₂, may be even produced at physiological pH, although they are not clearly observed under this condition due to the existence of a long M-N-O equilibrium.

Key words: retinal, isomerization, Schiff base switching, spectrally silent transition, microbial rhodopsin

Microbial rhodopsins undergo a cyclic photochemical reaction (photocycle) induced by photoexcitation of all-trans retinal (RET) as a chromophore. The photolyzed protein

The latter half of the photocycle of microbial rhodopsins remains incompletely understood. Although the transition from N to O intermediate is accompanied by thermal reisomerization of retinal chromophore and accessibility switch of the protonated Schiff base, it has been an unsolved problem which event occurs first, even in bacteriorhodopsin (BR), the most intensively-studied microbial rhodopsin. The present work revealed the formation of two O-states in the latter part of the photocycle of *Acetabularia* rhodopsin II, a BR-like light-driven proton pump from a marine alga. This finding would help us to understand the latter molecular event in the photocycle of microbial rhodopsins.
after all-trans to 13-cis isomerization of RET thermally returns to the initial unphotolyzed state. During their respective photocycles, several spectroscopically different photoproducts, referred to as K, L, M, N, and O, appear in a sequential manner [1]. The transitions between successive states are accompanied by several chemical and structural events, such as reisomerization of RET, proton (or other ion species such as Cl⁻ and Na⁺) movement, and conformational changes of the protein, playing a crucial role in exerting the respective functions of microbial rhodopsins [1]. Among four microbial rhodopsins in haloarchaea, the photocycles of bacteriorhodopsin (BR) as a light-driven proton pump and two sensory rhodopsins (SRI and SRII) commonly include a blue-shifted M with deprotonated retinal Schiff base (SB). However, the decay of M in BR is relatively fast, whereas those in SRI and SRII are slow due to a defect in efficient SB reprotonation. On the other hand, M is not found in the photocycle of halorhodopsin (HR), a light-driven inward chloride pump. Although the transitions between each photointermediate described above (K-O) occur with large spectral shift, transitions without apparent spectral changes are also often observed in their photocycles [2]. The most famous example of this is found in the first half of the photocycle of BR [3]. A spectrally silent transition between two M-like substates, M₁ and M₂, which works as the accessibility switching process for SB from the extracellular (EC) side to cytoplasmic (CP) side, leads to unidirectional outward proton transport by BR [3]. Another example is the L₁-L₂ transition that occurs in the photocycle of HR. In this transition, it has been deduced that the position of Cl⁻ changes inside the protein [1,4,5]. In addition, it is considered that the formation of multiple M species in SRI and SRII may participate in signal transduction to a transducer by F-helix tilting [6,7]. Therefore, spectrally unchanged processes during the photocycle may also happen on other intermediates and be worth further investigation if they exist.

In contrast with the first half of the photocycle, the second half of the photocycle has been incompletely understood. The presence of two N-states after M was reported in BR [3]. The N₁ (N) to N₂ (N') conversion changes the connectivity of a proton donor to SB (D96BR) from SB at the intracellular surface to take up a proton from the CP aqueous phase [3]. This process also serves for the regulation of the direction of proton translocation like the M₁-M₂ conversion. The N-O transition accompanies the isomerization. The isomerization-switch-transfer (IST) model by Haupts et al. assumes that the switch (orientation change of N-H of SBH+) between CP and EC) occurs after the isomerization [8]. On the other hand, Wang et al. carried out the molecular dynamic (MD) simulations during the N-O transition of BR, and the first switch is followed by the isomerization in the latter part of the photocycle, which is contrast to IST model [9]. Wang et al. thought the important role of the interaction of SBH+ with deprotonated D212BR in EC [9].

In this article, we report the presence of two O states in the photocycle of Acetabularia rhodopsin II (ARII or also called Ace2), which is one of two eukaryotic light-driven proton pump homologues from the marine alga Acetabularia acetabulum [10,11]. As reported previously, the latter half of the photocycle of ARII at neutral pH is described as M→N→O→ARII→ARII [11]. The existence of reversible reactions between M, N, and O makes analysis of the photocycle complicated. However, the equilibrium between M and N in the above scheme shifts toward M due to a rapid back reaction at acidic pH (<~5.5). In addition, the N→O reaction is fast under this condition because the medium pH is below the pK₅ (5.9 or 6.3) of D92 ARII (corresponding to D96ARII) during H⁺-uptake at N-decay [11]. Therefore, N is not accumulated at pH <~5.5, allowing the scheme to be simplified as M→O→ARII→ARII. Consequently, the kinetic analysis becomes easy, and we found two O intermediates in the photocycle of ARII.

Materials and Methods

Sample preparation

The procedure for the synthesis of ARII protein by cell-free expression and its purification method were the same as previously described [10,11]. ARII solubilized with 0.05% n-dodecyl-β-D-maltoside (DDM) was used.

Flash photolysis

Measurements were performed by using the same apparatus and procedure described previously [12]. Absorbance changes generated by the excitation of proteins with a laser pulse (Nd:YAG 532 nm, 7 ns, 5 mJ/pulse) were collected at three characteristic wavelengths (400, 520, and 610 nm) at 20°C. The experimental medium was a solution containing 400 mM NaCl and 10 mM NaOH at 2°C. The absorbance signals were measured at three characteristic wavelengths (400, 520, and 610 nm) at 20°C. The absorbance changes at 610 nm over the early time range prior to ~0.02 ms signals mainly reflect concentration changes of M and the original pigment (ARII), respectively. On the other hand, two red shifted photointermediates, K- and O-states, have an absorbance maximum wavelength (λmax) at a similar red-shifted spectral range [11]. It is worth noting that the absorbance change at 610 nm over the early time range prior to ~0.02 ms represents the time-dependent fractional concentration change of K, whereas the rise and decay of O are detected at the latter time range.

In agreement with the simplified scheme described above,
Figure 1  Simulation of the observed $\Delta A_{610}$ signal in the photocycle of ARII under acidic condition (pH≤5.5) by three analytical models. The data at pH 4.1 are analyzed by (A) a model with simple O (see Eq. (4)), (B) a model with two O-states, $O_1$ and $O_2$ ($\varepsilon_{O_1} = \varepsilon_{O_2}$) (see Eq. (6)), and (C) a model with $O_1$ and $O_2$ ($\varepsilon_{O_1} \neq \varepsilon_{O_2}$) (see Eq. (7)). The upper panels show the fitting results. In these figures, the observed and fitting curves are shown as black noisy and red smooth lines, respectively. The original state (ARII), M, and K plus O were monitored at 520, 400, and 610 nm, respectively. The red dotted, broken, and chain lines in these panels stand for the calculated fractional concentration changes of K, $O_1$ (or simple O), and $O_2$, respectively (see Eqs. (1), (3), and (5)). A small negative absorbance change in the $\Delta A_{400}$ signal at the time range from ~0.1 to ~10 ms may originate from the contribution of the small absorbance of the original pigment at this wavelength (also see Fig. 3B), which may lead to a fitting error with Eq. (2). The lower thin panels represent the difference at 610 nm between the observed and regression curves. The columns (D)–(F) and (G)–(I) show the corresponding fitting results for the data at pH 4.8 and 5.5, respectively. The assumptions are: a single O-intermediate (Eq. (4)) is adopted in the left column, two O-intermediates with the same $\varepsilon$ (Eq. (6)) are in the middle column, and two O-intermediates with different $\varepsilon$s (Eq. (7)) are in the right column. The ratio of $\alpha_{O_1}$ and $\alpha_{O_2}$ were estimated to be 0.63 and 0.64 at pH 4.8 and 5.5, respectively. Measurements were performed using the ARII protein solubilized by 0.05% DDM in medium containing 400 mM NaCl buffered with 2 mM 6-mixed buffer at 20°C.
Below pH equilibrium with increasing pH.

$$\Delta K = e^{-k_1 t}$$

$$\Delta M = \frac{k_0}{k_1 - k_0} (e^{k_1 t} - e^{k_0 t})$$

$$\Delta O = -\frac{k_0 k_1}{(k_0 - k_1) (k_1 - k_2) (k_2 - k_0)} [(k_1 - k_2) e^{k_2 t} + (k_2 - k_0) e^{k_0 t}]$$

where $k_0$, $k_1$, and $k_2$ signify the rate constants of K-, M-, and O-decay, respectively. When scaling constants for the amplitude of the absorbance of K and O at 610 nm, which contain molar extinction coefficients ($\varepsilon$) for their respective intermediates, defined as $\alpha_k$ and $\alpha_o$, respectively, $\Delta A_{610}$ signals can be expressed as the following equation.

$$\Delta A_{610} = \alpha_k \Delta K + \alpha_o \Delta O$$

Eq. (4) was applied to the analysis for the $\Delta A_{610}$ signal. This analysis, however, did not give a good fitting result (see Fig. 1A, D, and G). As seen in the lower panels of these figures, the fitting deviation is especially prominent in the absorbance change originating from the rise and decay of O (the time region from ~0.02 to ~10 ms). At first glance, the rise of O is not monophasic, irrespective of the single decay of M (see the strain line in the inset of Fig. 2A). This discrepancy can be solved by assuming a scheme that contains another O-like state after the first O, and these two sequential O-states are referred to as $O_1$ and $O_2$, respectively.

The decay of M ($\Delta A_{400}$) is single exponential (see the data below pH 4.8 in the inset of Fig. 2A), and its decaying phase seems to match the rising phase of O ($\Delta A_{610}$) (also see Table 1). When assuming that the concentration of K at the initial moment ($t=0$) is 1, the time-dependent fractional concentration changes of K, M, and O in the simplified scheme ($K\rightarrow M\rightarrow O\rightarrow \cdots$) can be derived as the following equations, (1), (2), and (3), respectively.

$$k_0 - k_1 = k_2$$

$$k_0 - k_1 = k_2$$

$$k_0 - k_1 = k_2$$

$$k_0 - k_1 = k_2$$

Where $k_0$, $k_1$, and $k_2$ are the rate constants for the $\Delta A_{400}$ signals with Eq. (2) and distinguished from the corresponding values ($k_0$, $k_1$, and $k_2$) estimated from the fitting for the $\Delta A_{610}$ signals. Two $k_0$ and $k_1$ values are similar each other, although there are small differences between them presumably due to fitting error for the $\Delta A_{400}$ signals (also see the legend of Fig. 1).

**Table 1 Decay rate constants of various photointermediates under acidic conditions**

| pH     | $k_0$ (K-decay) | $k_0'$ (K-decay) | $k_0''$ (M-decay) | $k_0'''$ (M-decay) | $k_2$ (O-decay) | $k_2$ (O-decay) |
|--------|-----------------|-----------------|-------------------|-------------------|----------------|----------------|
| 4.1    | 99.3            | 79.8            | 40.0              | 30.2              | 2.25           | 0.285          |
| 4.8    | 86.4            | 66.8            | 17.7              | 13.1              | 1.53           | 0.324          |
| 5.5    | 74.8            | 67.7            | 6.08              | 5.46              | 1.36           | 0.340          |

*The unit of all values is ms⁻¹.

$^b k_0$ and $k_1$ were estimated by the fitting for the $\Delta A_{610}$ signals with Eq. (2) and distinguished from the corresponding values ($k_0$, $k_1$, and $k_2$) estimated from the fitting for the $\Delta A_{400}$ signals. Two $k_0$ and $k_1$ values are similar each other, although there are small differences between them presumably due to fitting error for the $\Delta A_{400}$ signals (also see the legend of Fig. 1).
We first performed fitting analysis using the following equation under the assumption that the $e$s for the two O-states are equal.

$$\Delta A_{610} \approx a_K \Delta K + a_{O1} \Delta O_1 + a_{O2} \Delta O_2$$

This fitting result is shown in Figure 1B, E, and H. The fit is largely improved, but a small difference is yet observed (see the lower thin panels of Fig. 1B, E, and H).

Careful inspection reveals a small second increasing phase in the $\Delta A_{610}$ signal originating from O, which may imply that the $e$ of the second O ($O_2$) is different from that of the first one ($O_1$). Moreover, the results of the multieponential global fitting analysis under acidic conditions (pH 5.0) also support this idea (see Fig. 3). This analysis reveals a photocycle scheme containing five photointermediates: $P_0$ (ARII)→$P_1$ (K)→$P_2$ (M)→$P_3$ (O$_1$)→$P_4$ (O$_2$)→$P_5$ (ARII’), which is almost consistent with the above described scheme, although a small contamination of K is observed in the $P_2$-state. The maximum absorbance of $P_5$ (O$_2$) is apparently larger than that of $P_3$ (O$_1$). Therefore, this together with the discrepancy of Eq. (6) with observed data led us to consider that $e_{O_1}$ (i.e. $\alpha_{O_1}$) are different each other. The following combined function of Eqs. (1), (3), and (5) was used for the fitting.

$$\Delta A_{610} \approx a_K \Delta K + a_{O1} \Delta O_1 + a_{O2} \Delta O_2$$

Eq. (7) simulates the observed $\Delta A_{610}$ signal well (see Fig. 1C, F, and I). Therefore, we concluded that there were two O-intermediates with different $\alpha$ values. The estimated values of the $k_i$s ($i=0$–3) are shown in Table 1. The values of $a_{O_1}$ and $a_{O_2}$ at pH 4.1 were 30.3 and 45.3, respectively, and the ratio of these values ($a_{O_1}/a_{O_2}$) was 0.67. This approximately 0.7 value is reflected by the ratio of $e_{O_1}$ to $e_{O_2}$, which is almost the same as the ratio of the peak values (Fig. 3B and C).

As the medium pH increases, the rise of O becomes monophasic (see the lower panel B in Fig. 2). This may be attributed to the lack of accumulation of O$_2$ due to the prolonged decay of its precursor (N). Indeed, it appears that lack of contribution of O$_1$-formation to the $\Delta A_{610}$ signal occurs simultaneously with the appearance of the biphasic M-decay in the $\Delta A_{610}$ signal originating from the formation of M-N equilibrium due to the delay of N-decay, which was detectable at a pH above 5.5 (Fig. 2). Thus, $\Delta A_{610}$ signals are observed as a sum of K and simple O ($O_2$) at a physiological neutral or weak alkaline pH. However, we infer that O$_1$ exists even under these pH conditions, although it cannot be detected because of the appearance of the M-N-O quasi-equilibrium. It is worth noting that Chizhov et al. reported the existence of two Os in HR from _Natronomonas pharaonis_ (NpHR) [13].

What molecular event does occur during the transition from O$_1$ to O$_2$? Wang et al. proposed the following two possible sequences at the N→O transition of BR [9]:

![Figure 3](image-url)
1) Sequence 1: N (13-cis RET, 15-anti SBH\textsuperscript{+})
   \rightarrow (all-trans RET, 15-syn SBH\textsuperscript{+})
   \rightarrow (all-trans RET, 15-anti SBH\textsuperscript{+}).

2) Sequence 2: N (13-cis RET, 15-anti SBH\textsuperscript{+})
   \rightarrow (13-cis RET, 15-syn SBH\textsuperscript{+})
   \rightarrow (all-trans RET, 15-anti SBH\textsuperscript{+}).

The MD simulation by Wang et al. showed the possibility of Sequence 2 and the interaction of N-H with deprotonated D212\textsuperscript{HR} was pointed out [9]. Furthermore, all-trans RET, 15-syn SBH\textsuperscript{+} in Sequence 1 is considered to be metastable [14]. Considering these facts, Sequence 2 may be more plausible. As a broadly received conception, the O-state in BR has a twisted all-trans RET [15] and the large spectral red-shift during the N-O transition is attributed to the isomerization of RET from 13-cis to all-trans. In contrast, Subramaniam and coworkers reported the long life-time of O with 13-cis RET in L93A\textsuperscript{HR} mutant which induces the slow reisomerization of RET due to abolishment of van der Waals interaction between the 13-methyl of RET and the terminal methyl groups of L93\textsuperscript{HR} [16]. In addition, Zhang et al. solved its X-ray crystal structure and concluded that this long-lived O took 13-cis RET, 15-syn SBH\textsuperscript{+} configuration [17]. These findings demonstrate at least the existence of a 13-cis O in L93A\textsuperscript{HR}. Tóth-Boconádi et al. presumed two substates of O in L93A\textsuperscript{HR}, and described that their distinction is the difference of the configuration of RET, 13-cis or all-trans [18,19]. Furthermore, Milder postulated the presence of two consecutive O-intermediates having 13-cis and all-trans RET in the wild-type BR [20]. Hence, the O\textsubscript{1}-to-O\textsubscript{2} transition in this study may be also accompanied by the cis-to-trans isomerization of RET. In this respect, we can see that in Figure 3B, \( \lambda_{\text{max}} \) of P\textsubscript{1} (O\textsubscript{1}) is a little bit smaller than P\textsubscript{2} (O\textsubscript{2}) (also see Fig. 3C), and that \( \varepsilon \) of P\textsubscript{1} (O\textsubscript{1}) is clearly smaller than the other. The 13-cis isomer in the dark-adapted BR has smaller \( \lambda_{\text{max}} \) and \( \varepsilon \) values compared with those of the all-trans isomer [21].

Other microbial rhodopsins such as HR from Halobacterium salinarum and Anabaena sensory rhodopsin showed similar characteristic [22,23]. This spectral property is consistent with the assumption that O\textsubscript{1} and O\textsubscript{2} take the RET configuration of 13-cis and all-trans, respectively. In the anion-pumping photocycle of NpHR proposed by Kouyama et al., two O-intermediates (O’ and O) with 13-cis and -trans form of RET were assumed [5], which is also consistent with our present assumption. Thus, together with the model by Wang et al. [9], we propose a conformational scheme of RET and SBH\textsuperscript{+} upon the transition from N to O, which is shown in Figure 4.

In the acidic condition adopted here, N-decay is very fast and the rate of the isomerization is relatively \( \varepsilon \)-independent, which may result in the observation of O\textsubscript{1}. Under neutral or alkaline conditions, N-decay is slow and the O\textsubscript{1}-to-O\textsubscript{2} conversion rate is presumably faster, which results in the lack of detection of O\textsubscript{1}. Allthough O-intermediate in the crystal structure of L93A\textsuperscript{HR} takes 13-cis RET, 15-syn SBH\textsuperscript{+} in agreement with the expected configuration of O\textsubscript{1} in the present paper [17], the precise RET configuration of O\textsubscript{1} of the wild-type BR should be determined in further study. In addition, we should explore its existence for other microbial rhodopsins. However, there is a possibility that in many microbial rhodopsins, observation of O\textsubscript{1} (13-cis RET, 15-syn SBH\textsuperscript{+}) is difficult (although its existence is in the case) maybe due to M-N-O equilibrium and/or its short life time. In this case, a problem arises why this intermediate can be observable in ARII. This is an intriguing subject in future.

Figure 4 Schematic of configuration change of RET and SBH\textsuperscript{+} in the latter half of the photocycle. A picture is depicted in the case of BR.

**Conclusion**

In this study, we analyzed the photocycle of ARII under acidic conditions (pH < 5.5) at which accumulation of N is almost negligible. The kinetic analysis with three models revealed that the scheme containing two sequential O-intermediates (O\textsubscript{1} and O\textsubscript{2}) with different \( \varepsilon \) values is best. Considering the smaller \( \lambda_{\text{max}} \) and \( \varepsilon \) of O\textsubscript{1} than those of O\textsubscript{2}, we expected that the O\textsubscript{1}-to-O\textsubscript{2} transition is accompanied by the RET isomerization from 13-cis to all-trans, which agrees with the proposed sequence of two configurational changes in RET and SBH\textsuperscript{+} based on the MD simulations by Wang et al. [9].
et al. [9]: thermal reisomerization of RET is preceded by switch of the accessibility of SBH. Whether there are two O-intermediates in the photocycle of other microbial rhodopsins including BR should be investigated in future. However, the presence of the M-N-O equilibrium may make the detection of the first O (O1 in the present study) difficult.

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Conflicts of Interest

All authors declare that they have no conflict of interest.

Author Contributions

J.T., T.K., K.S., and N.K. directed the research. J.T. and N.K. co-wrote the manuscript. K.S. prepared ARII samples. T.K. performed flash photolysis measurements. J.T. and T.K. analyzed flash photolysis data. T.N., M.D., T.K.-S., M.S., S.Y., and S.M. helped to draft the manuscript. All authors critically reviewed and approved the final manuscript.

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