

Efficient Approach to Determine the pK_a of the Proton Release Complex in the Photocycle of Retinal Proteins

Jia Wu,[†] Dewang Ma,[†] Yazhuo Wang,[†] Ming Ming,[†] Sergei P. Balashov,[‡] and Jiandong Ding^{*,†}

Key Laboratory of Molecular Engineering of Polymers of Ministry of Education, Department of Macromolecular Science, Advanced Materials Laboratory, Fudan University, Shanghai 200433, China and Department of Physiology and Biophysics, University of California, Irvine 92697, USA

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This work utilizes a photoelectrochemical approach to study the pH dependence of proton release and uptake in the photocycles of two retinal proteins, bacteriorhodopsin (BR) and archaerhodopsin 4 (AR4). By detecting photoinduced potentials that originate from the proton concentration changes (ΔH^+) generated by proteins near the indium tin oxide (ITO) electrode, we show that the kinetics of release and uptake can be followed in a broad pH range, and the pK_a of the proton release complex (PRC) can be easily determined under different conditions. Nonoriented protein films were deposited on the electrode, and photovoltage in an electrochemical cell was detected after illumination with a green flash. The kinetics of proton release and uptake could be measured as light-induced decreases and increases of the photopotential. A kinetic analysis was performed, and a formula describing proton fluxes of wild-type BR and AR4 and D96N mutant of BR was derived. Three components—fast proton release, slow proton release, and proton uptake—were found in the wild-type retinal proteins; two components, fast and slow proton releases, were found in the D96N mutant. The pH dependence of the fraction of fast release over the whole release was used to determine the pK_a for proton release in the photocycles of these retinal proteins. Measurements were also performed in conventional buffer solutions and crown ether. The presence of buffer in 10–50 mM concentration did not abolish the light-induced signals, indicating that the electrode response is much less sensitive to buffers than pH-sensitive dyes in a suspension due to a higher protein/buffer ratio near the electrode. This feature enables us to study effects of chemicals with high buffer capacity, and significant effects of buffers and crown ether on proton pumping behaviors of retinal proteins were revealed. In comparison with the classic pH-sensitive dye approach, the photoelectrochemical approach is convenient and efficient for measurements of transient proton concentration changes (ΔH^+) generated by a proton pump and thus might be utilized as a powerful tool for the investigation of light-driven proton pumping mechanisms in a wide pH range.

Introduction

An evolutionarily early and most simple biological design for capturing and storing light energy is based on transmembrane proton transport by retinal proteins, bacteriorhodopsin (BR)^{1,2} and archaerhodopsin (AR).³ The transport is driven by light-induced retinal chromophore isomerization, and both proteins exhibit several similar features, including the involvement of the so-called proton release complex (PRC or XH) in release of a proton from the reaction site buried inside the membrane to the extracellular surface. Along with the similarities, certain differences were found in the mechanism of proton transport, which is related to the function of the PRC⁴ at different pH values. This stimulated our efforts in developing an efficient approach for studying the kinetics of proton transport by membrane proteins in a wide pH range and at various conditions. Such an approach is expected to be useful for comparative studies of a variety of retinal-based proton pumps recently found in marine eubacteria (proteorhodopsins) and other organisms.^{5–7}

BR is a retinal protein found in a purple membrane, a specialized part of the cell membrane of *Halobacterium salinarum*.^{1,2} When excited by light, BR undergoes a photocycle

consisting of a series of intermediates, with different lifetimes and wavelengths of maximum absorption, K, L, M, N and O, of which, the M intermediate absorbing at 412 nm exhibits a lifetime of several milliseconds.⁸ The formation of M involves proton transport from the retinal Schiff base to the counterion Asp85. At neutral pH it is accompanied by a proton release to the extracellular surface from a group of residues (primarily Glu204, Glu194, and others) and interacting water molecules,^{9,10} which constitute the PRC, coupled to the counterion.^{10,11} The subsequent reactions of the photocycle involve reprotonation of the Schiff base from internal proton donor Asp96 (during the M-to-N transition) and proton uptake from the cytoplasmic side during the N-to-O transition.¹² Intramolecular proton transfer from Asp85 to PRC during the O-to-BR transition concludes the photocycle.

The natural variants of AR found in the so-called claret membrane³ of Halobacteria (presumably *Halorubrum*) were less extensively investigated, but it was noted that some features are different from those of BR.¹³ Thus, the order of proton release and uptake at neutral pH was reversed in AR1 compared to BR.¹⁴ A similar behavior was found for archaerhodopsin 4 (AR4)^{4,15,16} isolated from the *H. sp. xz515*, which was found in Tibet.^{17,18} Although the photocycle of AR4 is similar to that of BR with the same set of intermediates, the proton pump

* Corresponding author. Phone: +86 21 65643506. Fax: +86 21 65640293. E-mail: jdding1@fudan.edu.cn

[†] Fudan University.

[‡] University of California.

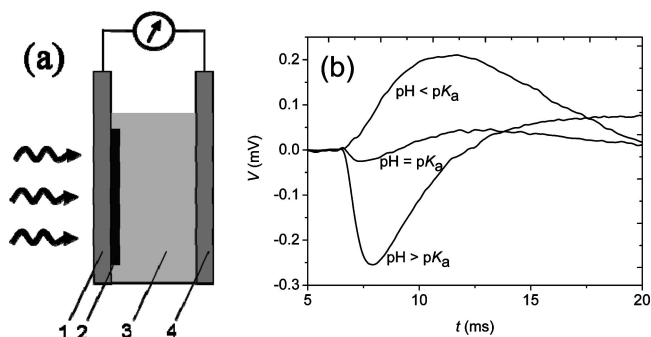


Figure 1. Scheme of the photoelectrochemical approach to determine the pK_a of the PRC in the photocycles of retinal proteins. (a) Parts of the photoelectrochemical cell: (1) an ITO transparent electrode; (2) a membrane protein layer randomly deposited onto the electrode; (3) an electrolyte solution; (4) a counter ITO electrode. Photovoltage is recorded upon a flash illumination. (b) The typical photoresponse (photovoltage *V* versus time *t*) from proteins deposited on ITO electrode under different pH values. The sample is set as the positive electrode in our voltage measurement, so that light-induced proton release from protein to the electrolyte solution leads to a negative voltage peak.

behaves differently—the temporal order of light-induced proton release and uptake in the photocycle is reversed at neutral pH.¹⁷

According to earlier studies,^{4,11,12,19} the difference in the pK_a values of PRC¹¹ in the M intermediate is directly related to different behaviors of the two proton pumps. The pK_a of the PRC of BR is about 5.7,^{12,19} and that of AR4 is about 8.4.⁴ When the bulk pH is higher than the pK_a of PRC in the M intermediate, a proton is released first; when the pH of medium is lower than the pK_a of the PRC, a proton is taken up first. The pK_a and a proper function of PRC are important for the kinetics of the overall proton transport by BR and AR4. Disabling the PRC of BR by mutations causes severe slowing of the turnover of its photocycle,^{10,20,21} because PRC is important for facilitation of deprotonation of the counterion at the end of the photocycle.^{4,10}

The predominant approach for pK_a determination of PRC in retinal proteins is based on the measurement of light-induced absorption changes of a pH-sensitive dye added to a membrane suspension or attached to membrane surface.^{4,12,19} Upon light absorption, the protein with a proton pumping function produces a transient pH change, from asynchronous proton release and uptake, which could be detected by a pH-sensitive dye. To determine the pK_a of the PRC, the pK_a of the dye should be close to that of the PRC to maintain sufficient sensitivity through the necessary pH range. Therefore, a dye suitable for one protein might not be applicable to another protein or its mutant. For example, pyranine used in BR is unsuitable for AR4. We found that thymol blue was more sensitive for pH higher than 8 and suitable for AR4.⁴ Another complication with dyes is that they do not work in some conventional conditions such as high buffer capacity. In recent years more and more mutants have been reported for fundamental or applied studies, such as D96N, D85N, R82Q, R134K, and E204Q of BR and others.^{22,23} Each mutant has its own pK_a of PRC. Moreover, even the same mutant or a wild-type protein could have different pK_a values under different environments. Some chemical additives might have significant effects on proteins. For example, Liang et al. found an interesting effect of a crown ether on the photochromic properties of wild-type BR and BR-D96N films.^{24,25} Its effect on proton pumping properties has not been examined due to the lack of an appropriate experimental approach. So developing a convenient and widely applicable approach for measurement

TABLE 1: Structures, Charges, and pK_a Values of the Additives

Additive	Structure	Charge under neutral pH	pK _a
Phosphate		−	2.12;7.21;12.32
Citric acid		−	3.10;4.65;6.40
Boric acid		−	9.24
MES		−	6.15
Hepes		+	7.55
Tris		+	8.30
TEMED		~0	6.0
diaz-15-crown-5		++	9.0

of light-induced proton release and uptake and determination of the pK_a of PRC is necessary and important.

The photoelectrochemical property of BR has attracted much attention in the recent decades.^{26–29} Three main photocurrent components (B1, B2, and B3) were detected from the purple membrane oriented in a polyacrylamide gel.^{27,30} Wang et al. reported that three components could be observed in films of oriented purple membrane deposited on a piece of conductive glass such as an indium–tin oxide (ITO) electrode.^{31,32} The tin-oxide and ITO electrodes coated with BR are pH sensitive and were used to study light-induced pH changes in the nonoriented films of purple membranes.^{32,33} The electrochemical property of a retinal protein reflects its light-driven proton pumping mechanism and is useful for studies of the photocycle.^{26,30,33–39}

This study employs a photoelectrochemical approach to determine the pK_a of PRC in the excited protein, as schematically presented in Figure 1. Differently from short-term continuous illumination used by Robertson and Lukashov,³³ we used flashes. That gives a similar form of signals to those detected in flash-induced kinetic and spectral measurements. Moreover, it provides an opportunity to study kinetics of light-induced pH changes with high signal-to-noise ratio, which we will discuss later. Furthermore, measurements can be conducted at high buffer concentrations and a wide pH range.

A nonoriented protein film was prepared on a transparent glass electrode coated with an ITO conductive layer. The flash-induced voltage changes were measured at a series of pH values. The intensity and polarity of the signals correspond to the amount and time sequence of proton release/uptake during the photocycle. At pH > pK_a, a negative response is caused by

the fast proton release; at $\text{pH} < \text{pK}_a$, a positive signal appears because the proton uptake now precedes the release. At $\text{pH} = \text{pK}_a$, the fast and slow phases of proton release equally contribute to the overall proton changes according to the previous studies via the dye approach.¹⁹ In agreement with this, the photoelectric signals obtained in our measurements in a wide pH range contain three components: fast proton release, slow proton release, and proton uptake. We developed a quantitative approach to describe such a complex proton flux for BR, AR4, and BR-D96N. The results were fitted with an equation to obtain time constants and fractions of three components at different pH values. The change of the fraction of fast release was used to determine the pK_a of PRC. This extends our earlier study of the pH dependence of proton release and uptake for AR4^{4,16} to a wider pH range with a much higher accuracy. BR and its D96N mutant were used for a comparison and as a well studied “standard”.

The pK_a determination was also carried out upon addition of diaza-15-crown-5 and for a series of conventional buffer solutions. The pH-sensitive dyes cannot be used under these situations, but the photoelectrochemical approach is feasible. We found that both the diaza-15-crown-5 and buffer systems caused a decrease of the pK_a of PRC. The advantage of the present photoelectrochemical approach over the pH-sensitive dye is that the signals reflect the proton activity of light-driven proton pump directly and in a wide pH range.

Experimental Section

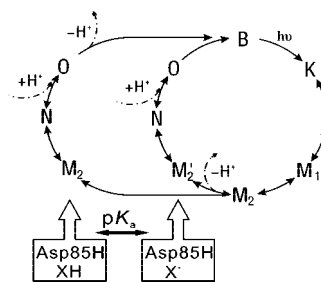
The purple membrane containing BR was isolated from R1M1, a *Halobacterium* strain. Bacteria culture and protein isolation followed the standard procedures for BR.² A suspension of AR4 in the “claret membrane” was obtained from the strain *H. sp. xz515* by the same procedure as used for BR. A centrifugation under a sucrose density step gradient, with concentrations (wt %) of sucrose of 30, 35, 40, and 45%, was performed to further purify the claret membrane. After spinning the sample (35 000 rpm, in the SW41 rotor of a Beckman centrifuge for 12 h), the claret membrane was collected from the 40% sucrose layer. It was washed and suspended in deionized water with a concentration of about 0.1 mg/mL.

A film of the purple or claret membrane was formed in the following way. A piece of ITO glass of 8×35 mm was washed with distilled water. A 100 μL aliquot of protein suspension (0.1 mg/mL) was deposited on a piece of glass and dried at room temperature. Three milliliters of an electrolyte solution was prepared in a cuvette, and its pH was adjusted by adding 1 M NaOH or HCl.

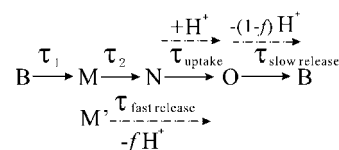
Both the electrode with a sample (film of a membrane protein) and the reference electrode (blank ITO glass) were immersed in the electrochemical cell filled with an electrolyte solution. The distance between the two electrodes was 6–8 mm. The cell was illuminated with a camera flash (YINYAN BY-26AZ) in combination with a filter, which provided green light with an intensity-maximum at around 570 nm. Kinetics of photoinduced electrical potentials between the electrodes were monitored on an oscilloscope (XINJIAN XJ4316A) and stored in a wavesaver with about 1000 amplification. The signals were collected and processed on a personal computer.

The electrolyte solution used in measurements was composed of 100 mM NaCl and 20 mM KCl unless otherwise indicated. Phosphate, citric acid, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), 2-(N-Morpholino) ethanesulfonic acid monohydrate (Mes), boric acid, and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were used as buffers. Tetramethyl ethylene diamine (TEMED) was also used as

SCHEME 1: A schematic presentation of proton release and uptake in a photocycle. “B” denotes the light-adapted background state of the retinal protein; “K”, “L”, “N”, “M₁”, “M₂”, “M₂’” and “O” refer to different intermediate states. Solid lines and arrows denote the route of the photocycle; the combination of dot-dashed lines and arrows indicate the route of proton uptake (proton-in); that of fast proton release (proton-out), which happens ahead of proton uptake; and that of slow proton release (proton-out), which happens after proton uptake. The number of the total protons released in the fast and slow processes is equal to the number of protons taken up. The proportion of fast or slow release is decided by a pK_a of PRC in excited retinal protein at a given pH.



SCHEME 2: The simplified scheme of proton release and uptake during the photocycles of BR and AR, in analogy to a sequential series of chemical reactions with the reverse reaction ignored. M': part of M undergoing fast proton release; τ_n : time constants of the n th steps; f : fraction of fast proton release over the total number of protons released or taken up in a photocycle of retinal proteins, which is dependent upon the pK_a of PRC in M'.



additive. 1,4,10-Trioxa-7,13-dioxa-cyclopentadecane (diaza-15-crown-5) was purchased from Aldrich. Table 1 shows some features of the additives. A point mutant of BR, D96N was a gift from Professor Norbert Hampp. All electrochemical measurements were performed at room temperature.

Model and Formulas Derivation. We analyzed the pH dependence of kinetics of proton release and uptake in the photocycle of BR¹⁰ and AR4. On the basis of the similarity of two proteins, and on the simplified models of their photocycles, we deduced a formula to describe the kinetics of proton release and uptake in BR and AR4 at different pH values. The routes of proton release and uptake along with the photocycle are shown in Scheme 1.

The events are shown in the form similar to a sequential series of chemical reactions in Scheme 2. Here, the reversed reactions are ignored.

According to Scheme 2, the change of proton concentration due to the net proton flux $\Delta[\text{H}^+]$ is composed of three parts, proton uptake $[\text{H}^+]_{\text{uptake}}$, fast proton release $[\text{H}^+]_{\text{fast_rel}}$, and slow proton release $[\text{H}^+]_{\text{slow_rel}}$, expressed as

$$\Delta[\text{H}^+] = [\text{H}^+]_{\text{uptake}} + [\text{H}^+]_{\text{fast_rel}} + [\text{H}^+]_{\text{slow_rel}} \quad (1)$$

The kinetics of the three components are related to the concentration of the N intermediate, [N]; that of the effective

proportion of M intermediate, [M']; that of the O intermediate, [O]; and the fraction of fast proton release over the proton totally released f ($f \in [0,1]$):

$$\frac{d[H^+]_{\text{uptake}}}{dt} = -[N]/\tau_{\text{uptake}} \quad (2a)$$

$$\frac{d[H^+]_{\text{fast_rel}}}{dt} = (f/\tau_{\text{fast_rel}})[M'] \quad (2b)$$

$$\frac{d[H^+]_{\text{slow_rel}}}{dt} = [(1-f)/\tau_{\text{slow_rel}}][O] \quad (2c)$$

Here τ_{uptake} , $\tau_{\text{fast_rel}}$, and $\tau_{\text{slow_rel}}$ denote the time constants for proton uptake, fast proton release, and slow proton release, respectively. Considering the expressions for concentrations of N, M', and O as indicated in the Supporting Information, equations 2a–2c could be resolved as

$$[H^+]_{\text{uptake}} = [B]_0(e^{-t/\tau_{\text{uptake}}} - 1) \quad (3a)$$

$$[H^+]_{\text{fast_rel}} = f[B]_0(1 - e^{-t/\tau_{\text{fast_rel}}}) \quad (3b)$$

$$[H^+]_{\text{slow_rel}} = (1-f)[B]_0 \left(\frac{e^{-t/\tau_{\text{slow_rel}}/\tau_{\text{uptake}}} - e^{-t/\tau_{\text{uptake}}/\tau_{\text{slow_rel}}}}{1/\tau_{\text{slow_rel}} - 1/\tau_{\text{uptake}}} + 1 \right) \quad (3c)$$

Here, $[B]_0$ is the initial concentration of the retinal protein. The combination of eqs 1 and 3a–3b leads to eq 4, the expression for the transient proton concentration change triggered by a flash.

$$\Delta[H^+] = [B]_0 \left[\frac{f\tau_{\text{uptake}} - \tau_{\text{slow_rel}}}{\tau_{\text{uptake}} - \tau_{\text{slow_rel}}} e^{-t/\tau_{\text{uptake}}} - f e^{-t/\tau_{\text{fast_rel}}} + \frac{(1-f)\tau_{\text{slow_rel}}}{\tau_{\text{uptake}} - \tau_{\text{slow_rel}}} e^{-t/\tau_{\text{slow_rel}}} \right] \quad (4)$$

The equation could be simplified under the following asymptotic conditions:

(a) For a wild-type retinal protein, if the medium pH is much higher than the pK_a of PRC, for instance, pH > pK_a + 1, then slow proton release disappears ($f = 1$) and only proton uptake and fast proton release occur:

$$\Delta[H^+] = [B]_0(e^{-t/\tau_{\text{uptake}}} - e^{-t/\tau_{\text{fast_rel}}}) \quad (5)$$

(b) When pH < pK_a - 1 for a wild-type retinal protein, fast proton release is suppressed, and just proton uptake and slow proton release appear ($f = 0$).

$$\Delta[H^+] = \frac{[B]_0}{1 - \tau_{\text{uptake}}/\tau_{\text{slow_rel}}} (e^{-t/\tau_{\text{uptake}}} - e^{-t/\tau_{\text{slow_rel}}}) \quad (6)$$

(c) For BR-D96N, $\tau_{\text{uptake}} \gg \tau_{\text{slow_rel}}$ since the active proton uptake is inhibited. Then, eq 4 is simplified as

$$\Delta[H^+] = f[B]_0(e^{-t/\tau_{\text{uptake}}} - e^{-t/\tau_{\text{fast_rel}}}) \quad (7)$$

Under an extremely low pH, the fast proton release from PRC cannot be observed at all, only a slow proton uptake and a very slow proton release occurs. In this case, $f = 0$, and eq 4 is simplified as eq 6.

The signal detected in the photoelectrical approach, $V - V_0$, is proportional to the ΔpH according to the Nernst equation. The value of ΔpH depends on buffer capacity β of solution near the electrode, as well as upon the change of proton concentration due to the transient net proton flux out of proteins $\Delta[H^+]$. As a result,

$$V - V_0 = C'\Delta\text{pH} = \frac{C'}{\beta}\Delta[H^+] \quad (8)$$

Here, V_0 denotes the baseline value, C' is a constant related to the signal amplitude, β is the parameter of buffer capacity defined as $\beta = d[H^+]/d\text{pH}$.

The following formula is used to fit the experimental photovoltage data in each trajectory of a flash-induced photovoltage change as

$$V = V_0 + \frac{C}{\beta} \left[-e^{-(t-t_0)/\tau_{\text{uptake}}} + \frac{f(\tau_{\text{slow_rel}} - \tau_{\text{uptake}})}{\tau_{\text{slow_rel}} - f\tau_{\text{uptake}}} e^{-(t-t_0)/\tau_{\text{fast_rel}}} + \frac{(1-f)\tau_{\text{slow_rel}}}{\tau_{\text{slow_rel}} - f\tau_{\text{uptake}}} e^{-(t-t_0)/\tau_{\text{slow_rel}}} \right] \quad (9)$$

Here, C is a constant, t_0 indicates the starting moment of flash. In dealing with our experimental data, we usually fit the asymptotic regions of pH at least one unit from the pK_a of PRC to obtain three characteristic relaxation times, then obtain f , the fraction of fast proton release around pK_a. The pH change caused by proteins upon each flash illumination is very small, and β can be treated as a constant in every fitting. However, β is a variable in a broad range of pH. So it is necessary to plot the fraction f instead of magnitude to get an accurate pK_a value.

Results

pK_a Determination under Normal Saline Solutions. Figure 2A shows the photoresponse profiles from a BR film deposited on an ITO electrode in the electrolyte solution of 100 mM NaCl and 20 mM KCl at different pH's. The amplitude and polarity of the observed signals changed with pH. The signals were negative at pH above 6, but became positive at pH below 5. The transition thus took place between 5 and 6. All signals were fitted by eq 9 derived from the models we suggested in Schemes 1 and 2. The pH dependence of time constants of proton release and uptake are shown in Figure 2b. From the fraction of fast release at a series of pH, we determined the pK_a value of PRC of BR as shown in Figure 2c. The obtained value (5.6 ± 0.1) is consistent with the value determined with the pH-sensitive dye (~ 5.7).^{12,19}

Figure 3 shows the response profiles of AR4 under a similar condition. It has been reported that AR4 shows a reversed time sequence of proton release and uptake at neutral pH compared to that in BR.^{4,17} The time-order difference of proton pump activity in BR and AR4 is clearly seen from comparison of the

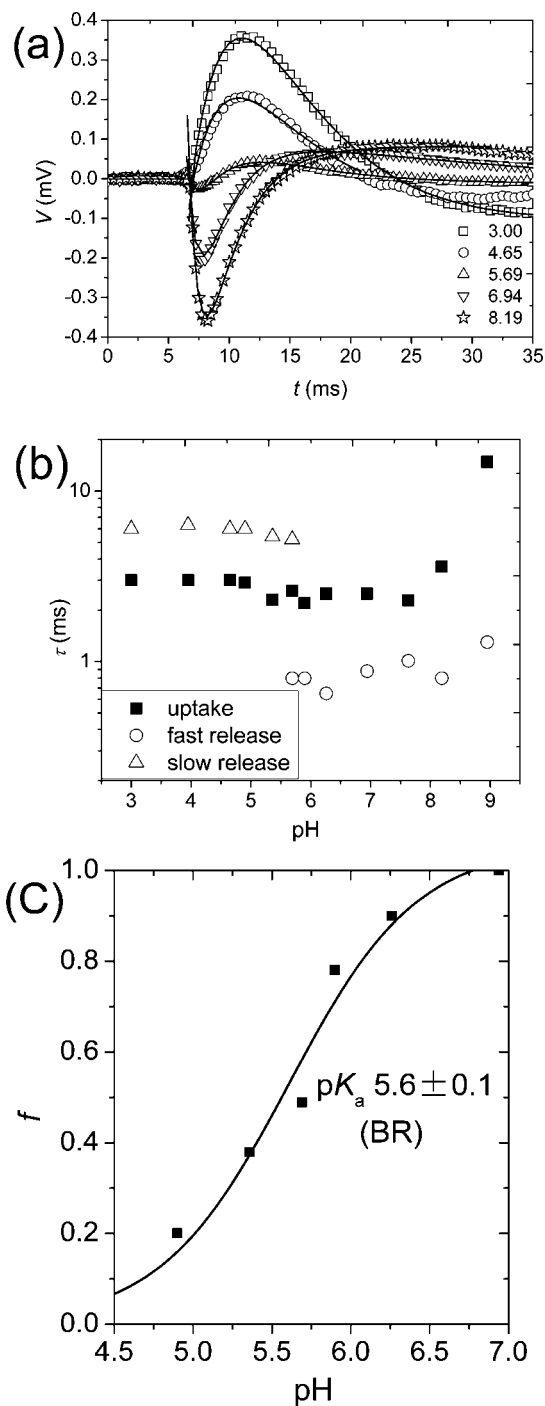


Figure 2. Kinetics of light-induced transient potential changes from a BR film deposited on an ITO electrode, measured in 100 mM NaCl and 20 mM KCl at different pH values, and estimation of the pK_a of the PRC. (a) Flash-induced photovoltages under the indicated pH values. Although all of the data points were used in fitting according to eq 9, just one data point for every 20 data collected was, for clarity, shown in (a) for each pH; only 5 out of 12 sets of data are plotted, although 12 pH values were examined. (b) The time constants of proton release and uptake at different pH values obtained from the fit. When pH was below 5, only proton uptake and slow release were observed. (c) The fraction of fast proton release over all of protons released, f , as a function of pH to determine the pK_a of PRC during the photocycle (in the M state). The data were fitted with the Henderson–Hasselbalch equation $y = A_1 + (A_2 - A_1)/[1 + 10^{n(x-pK_a)}]$ with a pK_a of 5.6 ± 0.1 ($n = 1.5$).

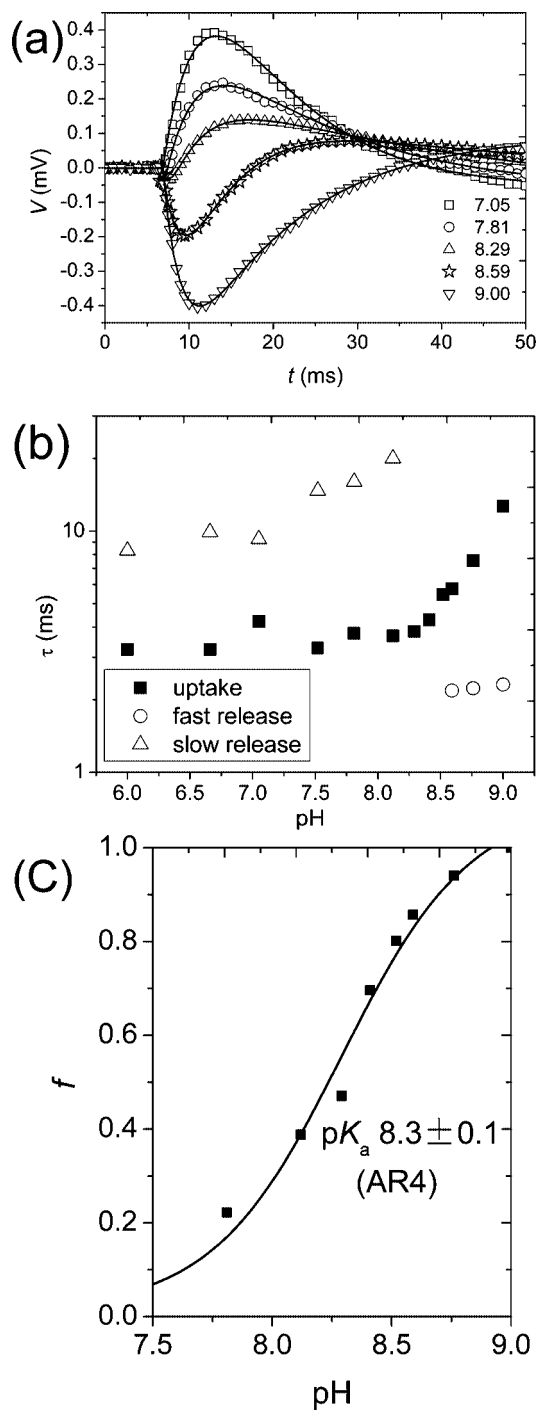


Figure 3. Kinetics of light-induced transient potential changes from an AR4 film deposited on an ITO electrode, measured in 100 mM NaCl and 20 mM KCl at different pH values, and estimation of the pK_a of the PRC. (a) Flash-induced photovoltages under the indicated pH values. Although all of the data points were used in fitting according to eq 9, just one data point of every 20 data collected was, for clarity, shown in (a) for each pH and only 5 out of 12 sets of data are plotted. (b) The time constants of proton release and uptake at different pH values obtained from the fit. When pH was low (pH < 6), only uptake and slow release were observed, and fast release disappeared. (c) The fraction of fast proton release as a function of pH to determine the pK_a of PRC in excited retinal proteins. The data were fitted with the Henderson–Hasselbalch equation with pK_a of 8.3 ± 0.1 ($n = 1.1$).

data close to the neutral pH in Figures 2a and 3a. The data for AR4 could also be fitted well with eq 9. Figure 3 shows that

the pK_a value of the PRC of AR4 is about 8.3 ± 0.1 , which supports our previous results for AR4 (~ 8.4) determined with the pH-sensitive dye thymol blue.⁴ The data in Figure 3b indicate

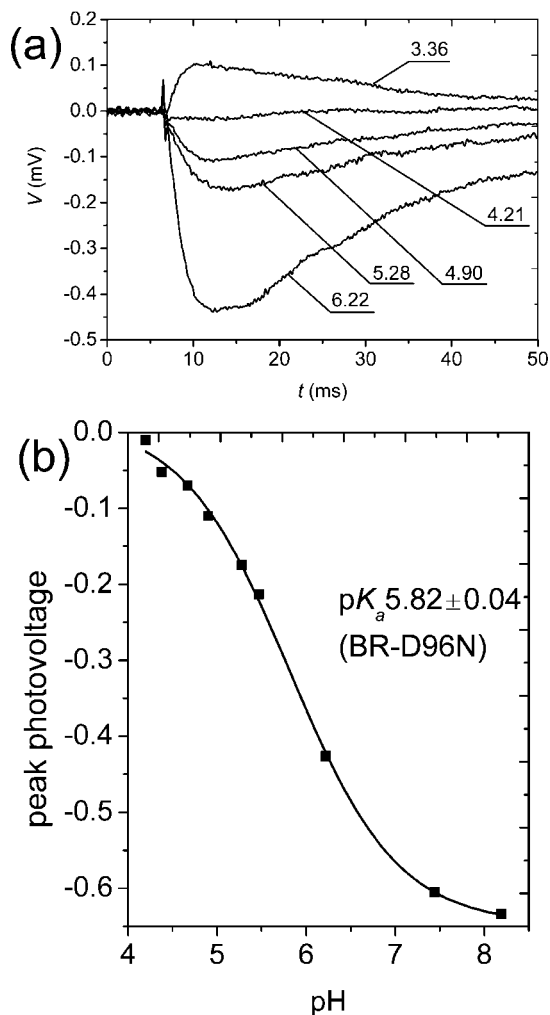


Figure 4. Photoresponse from BR-D96N deposited on an ITO electrode and measured in 100 mM NaCl and 20 mM KCl. (a) Flash-induced photovoltages at the indicated medium pH values. (b) The peak photovoltage associated with fast proton release as a function of pH, obtained by fitting the kinetic traces with eq 9 as a function of pH. The midpoint corresponds to the pK_a of PRC in the M state. The data were fitted by the Henderson–Hasselbalch equation with pK_a of 5.82 ± 0.04 ($n = 0.72$).

that the rate of proton uptake decreases at high pH values but is almost constant below pH 8.

To check how inhibition of light-induced proton uptake affects the kinetics and crossover point of the photoresponse, we examined the D96N mutant of BR, in which the Asp96 was replaced by an asparagine. Asp96 is the key residue for the reprotonation of the Schiff base and proton uptake.^{12,40,41} In the D96N mutant of BR, the proton uptake is depressed at neutral and high pH.^{23,33,42} We used it to verify our photoelectrochemical approach. Figure 4a shows the photoresponse profiles of BR-D96N in a saline solution under a series of pH. For clarity, the data at only a few pH values are shown in Figure 4a. In a wide pH range, just negative signals from fast proton release were seen. The signal of proton uptake did not appear until at a very low pH (~4.1). It is consistent with the previous measurement by Robertson and Lukashev.³³ From the amplitude of the negative transient signal originating from the fast proton release, the pK_a of PRC in D96N was determined. (The reason we can use magnitude change to get the pK_a in this case is given in the Discussion.) Figure 4b shows the fitting curve and the determined pK_a (~5.8) of PRC for D96N. The value is, reasonably, almost the same as that of wild-type BR.

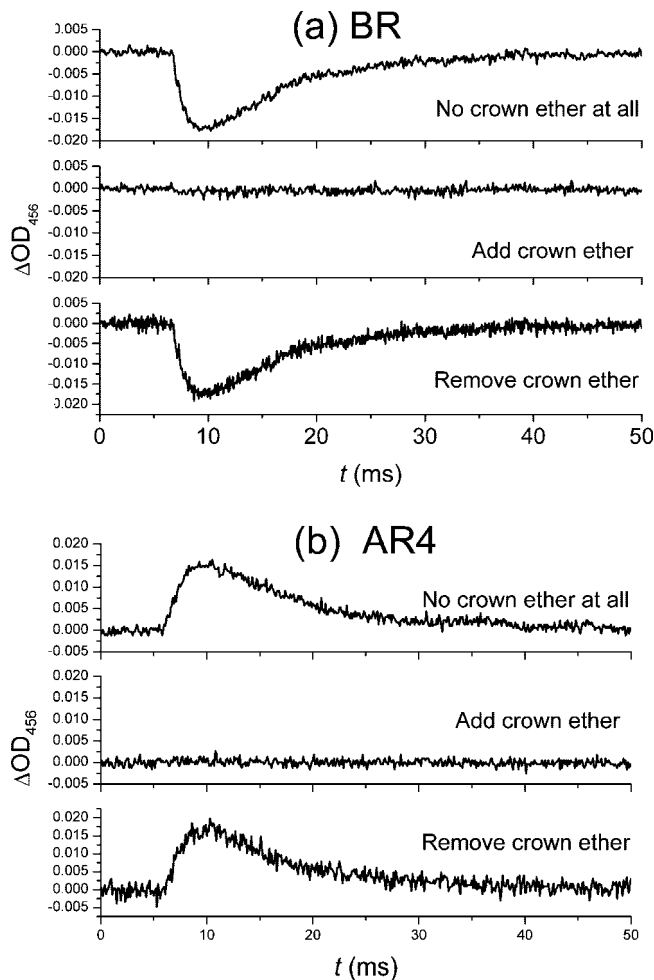


Figure 5. Absorption changes of a pH-sensitive dye pyranine at 456 nm caused by light-induced transient pH changes in suspension of (a) BR and (b) AR4 in 100 mM NaCl and 20 mM KCl with or without the presence of 10 mM diaza-15-crown-5 at pH 7.0.

The pH titrations of both BR and AR4 show that the photoelectrochemical approach is suitable to estimate the pK_a of PRC. The following subsections will demonstrate that this approach is also applicable to determine pK_a values of PRC in retinal proteins under conditions where the spectroscopic observations based on a pH-sensitive dye are not available.

Examinations of Crown Ether Effects. A significant effect of diaza-15-crown-5 on photochromic properties of BR was reported by Liang et al. when they examined BR embedded in poly(vinyl alcohol) films with addition of crown ether. A strongly increased lifetime of the M intermediate was found,^{24,25} and interaction between diaza-15-crown-5 and BR was implied. In this study, we investigated the crown ether effect on photoelectric properties of BR and on the pK_a value of PRC. Also, the effect of crown ether on AR4 was examined for the first time. The crown ether was added to the electrolyte solution in a final concentration of 10 mM.

The dye approach was also applied to detect the photoinduced pH changes. Figure 5 shows the results measured with the dye at neutral pH with or without diaza-15-crown-5. The buffer capability of the additive might be responsible for the signal disappearance when diaza-15-crown-5 was added. To check that effect, pH titration of diaza-15-crown-5 was carried out in 100 mM NaCl and 20 mM KCl (without proteins), and strong buffer capacity at pH above 7 was observed (data not shown), which was responsible for the elimination of the dye signal. Figure 5

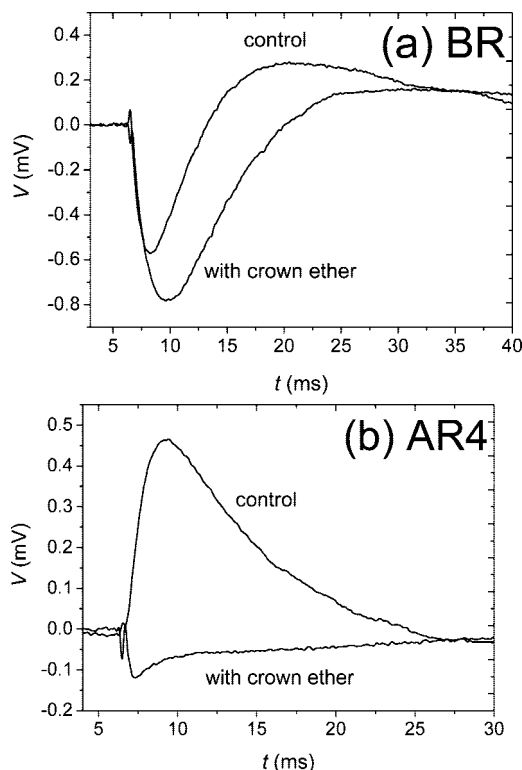


Figure 6. Flash-induced photovoltage of (a) BR and (b) AR4 in 100 mM NaCl and 20 mM KCl solution with or without addition of 10 mM crown ether at pH 7.0.

also indicates that the interaction between crown ether and protein is reversible, since the photoelectrical signals were recovered after removal of crown ether via centrifugation.

The buffer capacity of the crown is a limitation for the dye approach but not for the photoelectrochemical approach. Figure 6 shows the kinetic curves of BR and AR4 with 10 mM diaza-15-crown-5 in the electrolyte solution. Clear signals were obtained successfully, from which the pK_a was determined unambiguously. For BR, the resulting pK_a is about 4.5; for AR4, the pK_a is near 6.9. Because the lowered pK_a for AR4 is less than 7.0, the addition of crown ether even led to a reversed time order of proton uptake and release of AR4 at neutral pH, as illustrated in Figure 6.

pK_a Determination in Buffer Solutions. A buffer can affect the amplitude and decay time of photoelectric signals.^{23,32,43–45} It was shown with pH-sensitive dyes that buffers accelerate release of a proton from a membrane surface to the bulk.²⁶ The present studies examine buffer effects via the photoelectrochemical approach. Figure 7 shows the magnitude change of signals for BR and AR4 as a function of buffer concentration in different buffers and at different pH values. Significantly different buffer effects were found at low and high concentrations.

A. Effects of Low-concentrated Buffers (~0–10 mM). Significant buffer-induced photovoltage changes were observed, and the trends were dependent upon medium pH: a photovoltage increase occurred at relatively high pH values such as pH 7.00 and pH 8.10 for BR films in PBS (Figure 7a) and pH 9.00 for AR4 films in either PBS or boric acid solution (Figure 7b), and a decrease occurred at relatively low pH values such as pH 4.80 for BR films in citric acid solution (Figure 7a) or pH 7.00 for AR4 films in PBS.

We interpret these increase/decrease phenomena as united by a decrease of pK_a of PRC of the retinal proteins in the presence of a buffer. Considering also that photovoltage is

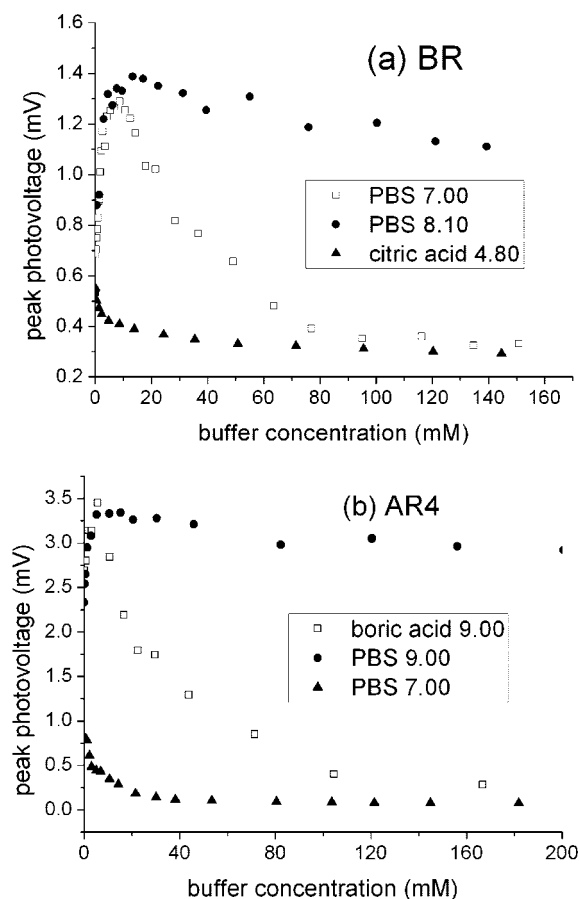


Figure 7. Photovoltage amplitude of (a) BR and (b) AR4 as a function of buffer concentration at the indicated pH values close to or far away from the pK_a values of the corresponding buffers. The pK_a of phosphate buffered saline (PBS) solution is about 7.2, and that of citric acid saline solution is about 4.7. The ionic environments in measurements were kept constant (100 mM NaCl, 20 mM KCl).

minimum when medium pH is close to the pK_a of PRC of a retinal protein and is larger when pH is away from the pK_a (as also shown in Figures 2 and 3 without buffers), the decrease of pK_a of PRC in both BR and AR4 films in buffers leads to the a larger difference between medium pH and PRC pK_a at high pH values and smaller at low pH values, and thus to the opposite changes of photovoltage magnitude. At high pH values, early proton release occurs, and the proton exchange from film surface to bulk solution might also influence the photoelectrical signal. So, as the photovoltage increase in buffers at relatively high pH values is concerned, part of the increase might be caused by an accelerating effect of buffers on proton exchange from surface to bulk.

B. Effects of High-concentrated Buffers. A gradual magnitude decrease was observed in high buffer capacity as in the previous work.⁴³ The decrease depends on buffer capacity and indicates that the electric signals are caused by pH change, which can be almost completely eliminated by adding a large amount of buffer, for instance, >50 mM for a thin AR4 film as shown in Figure 7b.

We quantitatively compare the pK_a values of PRC of BR and AR4 in a buffer. An intermediate buffer concentration was chosen, because we think that this case might lead to a significant pK_a change compared to low buffer concentrations while keeping sufficient signals compared to high buffer concentrations. Figure 8 shows the pH titration of BR and AR4

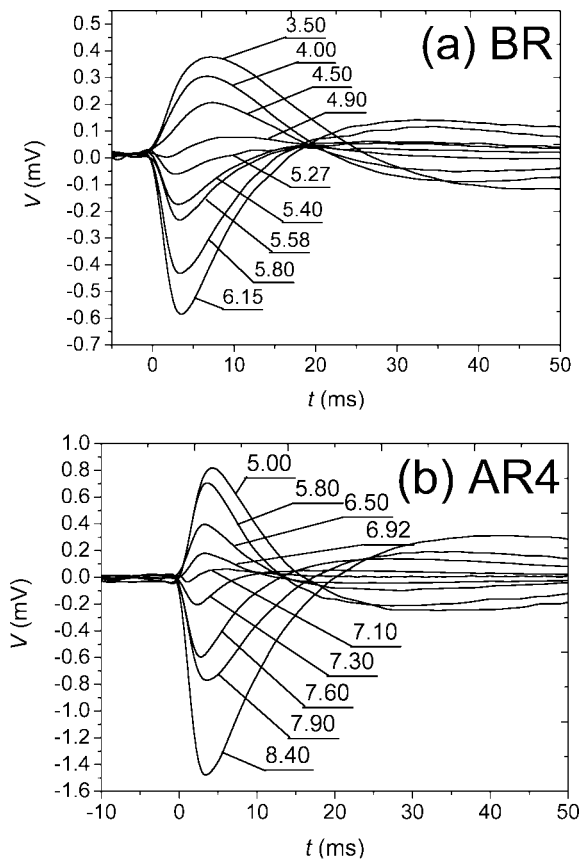


Figure 8. Flash-induced photovoltage of (a) BR and (b) AR4 in 100 mM NaCl and 20 mM KCl solution with 25 mM PBS at indicated pH values.

in 25 mM PBS. The parameter f was obtained after fitting, and the pH value at which $f = 0.5$ was determined to be the pK_a of PRC (5.1 for BR and 7.2 for AR4). The pK_a values of PRC in the M state of BR and AR4 under various conditions are summarized in Table 2. The PBS-induced pK_a change for AR4 (from 8.4 to 7.2) was found to be larger than that of BR (from 5.7 to 5.1).

We further examined a series of buffer solutions, taking AR4 as the model protein, since its pK_a change in the presence of buffer is more significant than for BR. The results are shown in Figure 9, together with the pK_a values for saline solutions and crown ether. The pK_a of the PRC of AR4 decreased upon increase in salt concentrations. It reached the minimum of 7.7 in the saturated salt solution. The pK_a values of PRC determined in five buffer solutions were all in the range of 7.2~7.4. Figure 9 also indicates that buffer effects are more significant than those of the salt solutions, and the crown ether has the strongest effect.

Discussion

Origin of the Photovoltage in the Nonoriented Films on the ITO. Previous studies reported such photovoltage signals as the result of the transient local pH change near the electrode caused by the asynchronized proton release and uptake, which could be described by Nernst relationship $\Delta E = A\Delta\text{pH}$, where ΔE denotes potential change, and A is the constant of the Nernst slope (-0.059 V/pH or -0.037 V/pH).^{33,38} Our results support this explanation. Moreover, the present studies shed light on some other features of the signals.

1. Resolve the puzzle of the ΔpH interpretation, which comes from the apparent inconsistency of medium pH change, ΔpH , and the change of proton concentration due to the transient net

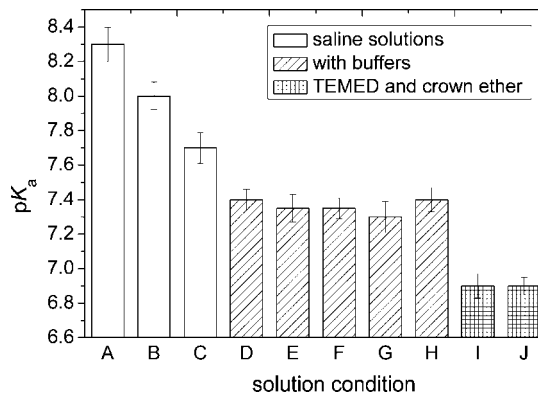


Figure 9. The pK_a values of PRC in AR4 determined under different conditions. (A) Saline solutions of 100 mM NaCl and 20 mM KCl; (B) saline solutions of 400 mM NaCl and 80 mM KCl; (C) saturated solutions of KCl (about 4 M); (D) buffer solutions of 10 mM phosphate in 100 mM NaCl and 20 mM KCl; (E) buffer solutions of 10 mM boric acid in 100 mM NaCl and 20 mM KCl; (F) buffer solutions of 10 mM Hepes in 100 mM NaCl and 20 mM KCl; (G) buffer solutions of 10 mM Tris-HCl in 100 mM NaCl and 20 mM KCl; (H) buffer solutions of 10 mM MES in 100 mM NaCl and 20 mM KCl; (I) 10 mM diaza-15-crown-5 in 100 mM NaCl and 20 mM KCl; (J) 10 mM TEMED in 100 mM NaCl and 20 mM KCl.

TABLE 2: pK_a Values of PRC of BR and AR4 in the M State

	pK _a of BR	pK _a of AR4
reference	5.7 ^a	8.4 ^a
electrolyte solution	~5.6 ^b	~8.3 ^b
PBS	~5.1 ^b	~7.2 ^b
diaza-15-crown-5	~4.5 ^b	~6.9 ^b

^a Determined by the dye approach.^{4,12,19} ^b Determined by the photoelectrochemical approach in the present paper.

proton flux out of proteins $\Delta[\text{H}^+]$. For instance, the ΔpH -induced voltage by the same amount of protons at pH 7 should be much larger than at pH 5 considering the background protons in water at a low pH. But the pH dependence of the photovoltage amplitude in either our measurements (see, for instance, Figure 2) or in the literature^{32,33} has little pH dependence.

We have compared the results of the titration of a BR suspension and pure water (data not shown) and have found that the suspension of the retinal protein has substantial buffer capacity in the pH range of measurement. The buffer capacity of protein decreases pH change and makes it less pH dependent. The effect might be stronger in a concentrated protein suspension or a protein film. The buffer capacity of proteins near the electrode is expected to be approximately constant in a wide pH range. As a result, $\Delta\text{pH} \propto \Delta[\text{H}^+]$. This is also the reason that we could use magnitude change to determine the pK_a of PRC of BR-D96N as shown in Figure 4 when a direct determination of f via data fitting based upon eq 9 is not available for this mutant.

2. Our studies led to an unexpected finding that the presence of buffers with even a high buffer concentration such as 50 mM did not abolish the photovoltage signals in our photoelectrical measurements, whereas light-induced pH changes in bulk solutions as measured via the pH-sensitive dyes were completely abolished in this condition. This phenomenon of strong photoelectrical responses could be interpreted from the much higher ratio of buffer to protein in the film layer facing the electrode (neighbor layer) compared to that in suspension. We have checked films of different thickness (data not shown) and found the more significant decrease of photovoltage upon addition of

a buffer in cases of thinner protein films, which might be due to relatively lower protein–buffer ratio in the neighbor layer.

3. Overshooting behavior in the transient photoelectrical responses. In measurements of both BR and AR4 in various conditions (see, for instance, Figures 2a, 3a, 8a, and 8b), we observed the phenomenon of “overshoots” with time, namely, the transient photovoltage was, after reaching the peak, decreased and even crossed the zero line with the opposite polarity to the initial peak. The amplitude of the overshoot was approximately proportional to the amplitude of the signal both for thin and thick films (data not shown). The overshoot behavior was also found in some other studies that showed that the orientated films with different directions gave a similar signal.^{31,38} So the overshoot was not caused by orientation or measurement problems but might reflect some intrinsic charge or proton movements. In our opinion, the diffusion of protons in solution driven by proton gradient formed by proton release or uptake might be responsible for the overshoot. Assuming proton uptake happens, the loss of protons in the electrolyte solution near the electrode must cause diffusion of protons toward the electrode from the bulk electrolyte solution and thus might induce an additional current in the electrode with the opposite direction to the initial one. Due to the slow rate of diffusion, this effect could only be observed in the late stage, forming a late “overshoot”.

The Photoelectrochemical Approach Is Efficient in Determination of the pK_a of PRC in the Photocycle of Retinal Proteins. The order of proton release and uptake could be reflected by the peak direction of the photovoltage upon a flash illumination with a high signal-to-noise ratio, as shown in the previous figures. Further analysis based upon our model and derived equations could be used to obtain convincing values of pK_a of PRC of an excited retinal protein. In our opinion, the photoelectrochemical approach shows significant advantages over the classic pH-sensitive dye approach in three aspects. (1) Simple procedure of sample preparation and condition changes in a wide pH range. (2) Less requirement of equipment and more straightforward reflection of light-driven proton pumping characteristics. (3) Versatility: as mentioned above, the dye approach is neither available under a high buffer concentration nor at pH values far from its pK_a . The dye approach is thus strongly dependent upon a suitable dye free of any interaction with proteins and with its pK_a close to the pK_a of PRC of the retinal protein. However, these limitations are relaxed for the photoelectrochemical approach. Under conventional saline solutions, both approaches are available, and the resulting pK_a values agree with each other satisfactorily. In a solution with a substantial buffer capacity such as PBS solution or a chemical additive with significant buffer capacity such as crown ether, the electrochemical approach is still feasible, whereas the dye approach does not work.

Factors Influencing the pK_a Values. Table 2 lists some pK_a values determined via the electrochemical approach reported in this paper as well as the available data obtained via the dye approach from the literature. Via the photoelectrochemical approach, an interesting phenomenon was discovered. When we added buffers and other chemicals to the electrolyte solution, the pK_a values of the PRCs of BR and AR4 were significantly altered. The decrease of pK_a of PRC of an excited retinal protein in a saline or buffer solution is discussed below.

The pK_a was decreased upon increasing salt concentration (Figure 9 and Table 2), which might originate from the screening effect of ions on protein surface charges. Retinal proteins such as BR and AR4 have an asymmetric charge distribution on their

N and C- terminuses. The high concentration of cations in solution affects the surface potential of protein and decreases the local proton concentration (“the surface pH”), which leads to a lower apparent pK_a .^{30,46}

The effects of buffer and crown ether are more significant compared with those of salts as indicated in Figure 9. When buffers or crown ether were present in a highly concentrated saline solution, an even lower pK_a was observed (data not shown). Our observation implies some interaction between crown ether or buffers with the retinal proteins. As is known, BR and AR4 have the same set of key amino acids in the proton transfer passage, and it is likely that AR4 has basically a similar mechanism of proton release as BR. During the photocycle, the protonation of Asp85 causes Arg82 reorientation toward the extracellular side to form a network of hydrogen bonds with water molecules and the dyad Glu194/Glu204.⁴⁷ The region containing a protonated water cluster and the dyad Glu194/Glu204 constitutes the PRC.^{10,47–50} The hydrogen bonds and charge distribution are the main interactions in the domain. According to the chemical structures of the buffers we used in measurements, we found that all the additives (Table 1) had strong binding capacities toward proton or cations and potential to form hydrogen bonds because of the N or O atoms in their structures. Previous researches have shown that some additives have a direct access to PRC and affect the domain.^{51,52} Recent structure of AR by Yoshimura and Kouyama indicates that the kinetics of light-induced proton release is probably dependent on how the PRC is configured in the unphotolysed state.⁵³ We suppose that the additive molecules in solution could interact with the domain of PRC so that the original structure of PRC was altered to a certain extent, causing a pK_a decrease. The detailed mechanism of buffers effect on the pK_a , especially that of crown ether, is not clear, but we can conclude that such an interaction of the ether with the protein is reversible according to Figure 5.

Conclusions

This work develops a photoelectrochemical approach to determine the pK_a of PRC in BR and AR4 and to examine the kinetics of proton release and uptake in films of the membrane proteins in a wide pH range. The proteins were randomly deposited on an ITO electrode that was placed in an electrolyte solution, and the photovoltage was recorded after a flash illumination. The signal reflects the order of proton release and uptake during a photocycle. The high sensitivity of the approach comes from the signal being directly proportional to the change of pH generated (protons released or taken up). The underlying model was suggested, and the equations for quantitative data analysis were derived. Such an approach was efficient and especially useful in the cases of solutions with buffers that otherwise can not be examined via the conventional dye approach. It also provides an opportunity to study the kinetics of proton pumping activities with a high signal-to-noise ratio in a broad pH range. The significant photoelectric effects of the crown ether and buffer solutions on BR and AR4 were found. The effects of crown ether are so large that the time order of proton uptake and release of AR4 could be altered even at neutral pH. Different proton-pumping behaviors of AR4 and BR were observed and attributed to a significant difference in the pK_a of PRC during the photocycle.

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Supporting Information Available: A detailed formulas derivation of eq 4 including all approximate conditions we used. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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