A new system for the measurement of electrogenicity produced by ion pumps using a thin polymer film: examination of wild type bacteriorhodopsin and the D96N mutant over a wide pH range

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Abstract We developed a new assay system for the measurement of capacitive electric currents generated by ion pumps using the thin polymer film 'Lumirror' (Toray Co., Japan). This system enables us to examine the electrogenicity of ion pumps over a wide range of experimental conditions with high reproducibility due to the mechanical and chemical stability, the high electric resistance and the high electric capacitance of the thin polymer film. Using this method, we examined the photoelectric response of wild type bacteriorhodopsin and its D96N mutant over a wide pH range (2.8–10.0). The results were explained in terms of the affinities of the proton binding sites for translocated protons. A possibility that the direction of the proton transfer from the Schiff base was influenced by the protonation/deprotonation state of the surrounding proton binding sites was suggested. We also found that this film can be used as a substrate for atomic force microscopy (AFM) samples and hence the active purple membrane was observed with AFM.

Key words: Bacteriorhodopsin; D96N; Electrical measurement; pH dependence; Atomic force microscopy

1. Introduction

Ion transport processes in biological membranes play many essential roles such as energy transduction, information processing, and co- or counter-transport of nutrients in living organisms. When these transport processes are electrogenic they can be measured electrically. One of the widely used systems for electrical measurement features membrane fragments (or vesicles) adsorbed onto various support materials. This system was originally developed by Drachev et al. [1,2]. They adsorbed vesicles containing cytochrome oxidase, H^+-ATPase or bacteriorhodopsin (bR) onto black lipid films and directly demonstrated their electrogenic activity. More stable systems were obtained using collodion films [3] or porous filters impregnated with a lipid solution [4–6] instead of a black lipid membrane as support material. Using these systems, the voltage generation was measured with high time resolution and the molecular mechanisms of electrogenic processes of bacteriorhodopsin [7–9] and other membrane proteins [10–12] were investigated. However, problems such as the inherent instability of the black lipid films, the effect of the solvent remaining in the membrane, and the rough heterogeneous surface of the support materials were still unsolved. To overcome these difficulties, many efforts have been undertaken: SnO_2 electrodes were used as a solid support onto which the membrane fragments were electrophoretically sedimented [13], deposited by the Langmuir-Blodgett film method [14–16], or immobilized by antibodies [17]. It was reported that the electrical signal detected using SnO_2 electrodes reflected the local pH changes in addition to the charge displacement [18]. Other metal substrates were also used as solid supports [13,19] but light induced artifacts were reported [19].

Therefore, a mechanically stable and inert material which does not respond to changes of pH, ionic concentration or light remains to be developed as solid support for the membrane adsorbed system. Further, low specific conductance and high specific capacitance of the support material are required for low noise and high sensitivity in the measurements. In the present study, we applied a thin polymer film (Lumirror, Toray Co., Japan) originally designed for film condensers as a solid support onto which membrane fragments containing bacteriorhodopsin were adsorbed. The photoelectric response by bacteriorhodopsin was detected in this system. Experimental conditions such as the pH or the composition of the buffer solution could be readily changed, although an ionic concentration gradient or a membrane potential could not be imposed on the adsorbed ion pumps in this system. Here, we report the measurement of the charge displacement upon illumination by both wild type bacteriorhodopsin and its D96N mutant over a wide pH range (2.8–10.0). The effect of different lengths of dark intervals (5 s to 5 min) was also investigated. Apparently, the effects of different affinities of the proton binding sites for the translocated proton in the bacteriorhodopsin molecule are responsible for the observed pH dependence.

2. Materials and methods

Purple membrane sheets were isolated according to Oesterhelt and Stoeckenius [20]. A recombinant *Halobacterium salinarium* strain which over-produces the D96N mutant bacteriorhodopsin was a kind gift from Dr. Janos Lanyi.

The chamber for the electrical measurements was the same as described by Montal et al. [21]. Between the two compartments of the chamber, a polyester film of 900 nm thickness (Lumirror, Toray Co., Japan) was inserted instead of the Teflon film described in [21]. Lumirror was a kind gift from Toray Co.

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Abbreviations: bR, bacteriorhodopsin; D96N, a mutant bR where Asp^{96} is replaced by Asn; AFM, atomic force microscopy

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The purple membrane sheets were adsorbed onto the film after it was set up within the chamber compartments. The chamber was first placed on its side so that the film was deposited horizontally. Typically, 80 μl of the purple membrane suspension (OD_{570}=4, in distilled water) was directly applied on the film sandwiched between the chamber compartments and left for 40 min at room temperature. The purple membrane solution was removed and 1.5 ml of buffer containing 100 mM NaCl, 20 mM Tris-maleate, pH 7.0 was filled in both compartments. In case of the purple membrane side, the buffer was exchanged two or three times to wash the residual unbound purple membrane. This could simply be carried out by pipetting due to the mechanical stability of Lumirror. To remove loosely attached purple membrane sheets, 80 μl of 10% octylglucoside solution was added (final concentration 0.5% w/v) and incubated for 1 min. The solution was exchanged against the original buffer several times to remove residual octylglucoside.

We measured the peak height of the photoelectric current rather than the peak area to evaluate the transport activity because the former reflects the initial (maximum) rate of charge movement whereas the latter is affected by non-linear voltage dependence of the rate of charge movement [22].

Solutions of various pH values ranging from 2.8 to 10.0 in 0.6 steps were prepared by mixing 20 mM Tris base and 20 mM maleic acid solutions both of which contained 100 mM NaCl. The pHs of the solutions were stable during the experiment. In a preliminary experiment, almost the same photocurrent was generated at pH 3 using Tris-maleate system or citrate-NaOH system containing 100 mM NaCl (78.3 ± 6.1 fA and 80.3 ± 6.2 fA, respectively) using the same membrane. At pH 10, the Tris-maleate system and the glycine-NaOH system also gave the same photocurrent (143.8 ± 15.6 fA and 137.2 ± 12.4 fA, respectively). Therefore, in order to avoid complicated experimental procedures, we employed the Tris-maleate system over the entire pH range. When the effect of the pH was examined, the solutions of the different pH were exchanged by simple pipetting. The membrane was equilibrated with the solution for 5 min followed by 5 s continuous illumination, a 10 s interval in the dark, 5 s illumination, 5 s interval in the dark, and 5 s illumination. After another 5 min interval in the dark, the same procedure was repeated to ensure the reproducibility. The photoresponse of the same membrane was compared in Figs. 3 and 4, respectively.

A 120 W halogen lamp was used to activate bR. The intensity of the light at the membrane was 8 mW/cm^2.

Atomic force microscopy (AFM) imaging was performed in tapping mode in air with a NanoScope III (Digital Instruments, Santa Barbara, CA) mounted with a K scanner. Micro cantilevers for AC mode (OMCL-AC120TS) were purchased from Olympus (Tokyo, Japan). The cantilever was oscillated at just below its resonance frequency (typically 300 kHz) with a drive amplitude around 350 mV. The samples were scanned at a scan rate of 1–5 Hz. Samples for AFM and electrical measurements were prepared in the same way except that the former preparations were done on the Lumirror film fixed to metal disks with double-sided sticky tape.

3. Results

3.1. Purple membrane adsorption procedure and photoelectric responses

The experimental system is schematically shown in Fig. 1A. The setup is equivalent to a series of a capacitor (C), a battery (E), a resistor (R) and a switch (S) as shown in Fig. 1B [23–25]. The series of the battery (E) and the resistor (R) with a switch (S) in the dashed box correspond to simplified bacteriorhodopsin. Upon energization (light ON), the switch closes. The electric response is a capacitive electric current due to the presence of the capacitor, which corresponds to Lumirror. The specific capacitance of Lumirror was 1180 pF/cm^2 as measured by the electric current response to a rectangular voltage pulse. The electric resistance of the film was too high to be measured precisely (>1000 $G\Omega/cm^2$).

The purple membrane sheets were adsorbed onto the Lumirror film simply by applying an aqueous suspension to the

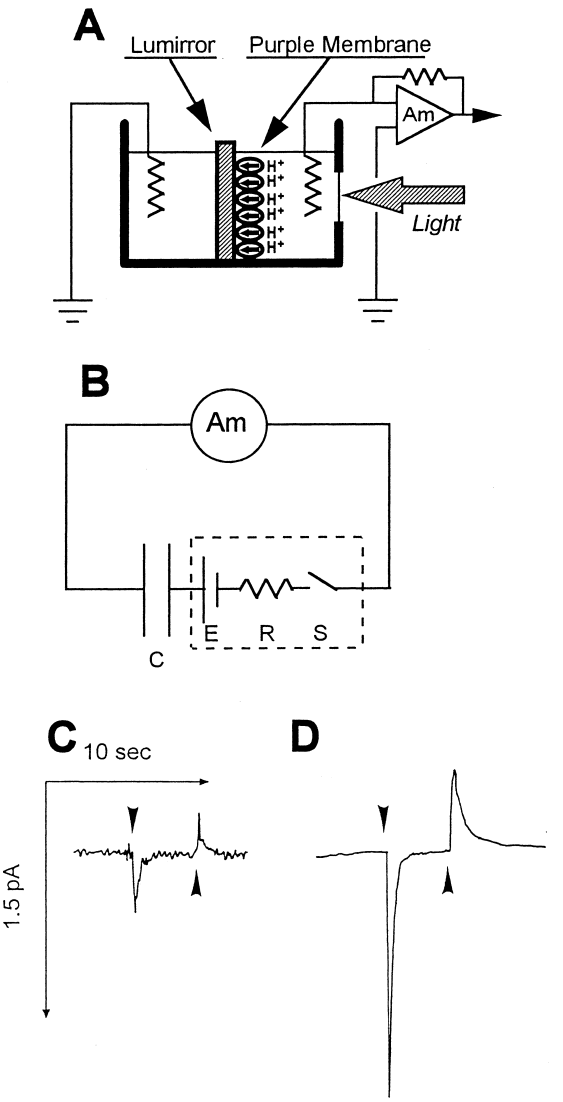

Fig. 1. A: Schematic description of the purple membrane adsorbed Lumirror system. 'Am' stands for a current amplifier. B: Simplified electric circuit for the purple membrane adsorbed Lumirror system. 'C' corresponds to Lumirror. The combination of 'E' (voltage source), 'R' (internal resistance), and 'S' (switch closed by energization) in the dashed box represents a simplified model of bacteriorhodopsin. 'Am' corresponds to a current amplifier. In the present report, the capacitive electric current across the Lumirror film was recorded. C: Photoelectric current just after the adsorption of purple membrane sheets. Downward and upward arrowheads indicate 'ON' and 'OFF' of the light source, respectively. D: As in C after octylglucoside treatment.

film and incubated for 40 min at room temperature. Subsequently, the chambers were filled with buffer solution and the photoelectric response was measured. One example of the capacitive electric current is shown in Fig. 1C. The magnitude of the signal was not stable immediately after adsorption and often increased after repeated exchanges of the buffer. Therefore, we added a wash step using octylglucoside as described in Section 2. As a result of this washing procedure, the magnitude of the photoresponse increased (Fig. 1D) and the reproducibility of the magnitude of the photoresponse was considerably improved. The washing procedure was applied to all subsequent experiments. The direction of the electric current corresponded to the movement of positive charges from the

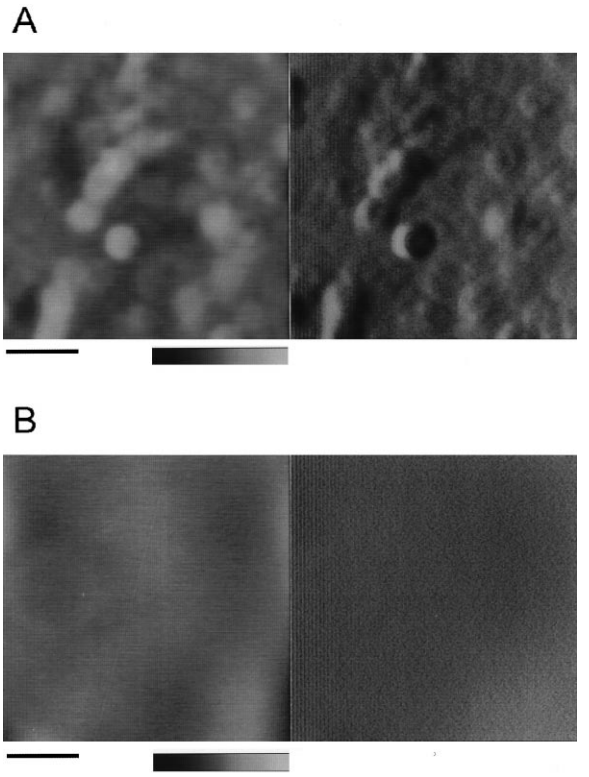

Fig. 2. AFM images of the purple membrane adsorbed to Lumirror. A: Height image (left) and amplitude image (error signal, right) of the purple membrane sheets adsorbed to Lumirror film after washing with octylglucoside. B: Height image (left) and amplitude image (right) of the bare Lumirror film. The film was treated as in A but buffer without purple membranes was used instead of the purple membrane suspension. The height is color-coded (full scale=10 nm), Scale bar=200 nm.

aqueous phase to Lumirror. We suspect that the purple membranes which face extracellular side to Lumirror has slightly higher population and higher stability than those facing their cytoplasmic side to Lumirror.

In order to see how purple membranes are adsorbed on Lumirror film, we investigated the film by AFM. As can be seen in Fig. 2A (right: height image; left: amplitude image), many disks of about 200 nm in diameter were observed when purple membrane sheets were adsorbed to Lumirror film and washed with octylglucoside. As no similar structure was observed in control experiments without purple membrane (Fig. 2B), we concluded that these disks correspond to purple membrane sheets. The film surface seemed to be almost completely covered with purple membranes even after washing with octylglucoside.

3.2. *pH dependence of the photoelectric response by wild type bacteriorhodopsin and the D96N mutant*

One of the advantages of the new system was the quick and simple exchange of the medium in the chamber. Profiting from this system inherent advantage, we examined the pH dependence of the photoelectric response. In Fig. 3, the peak height of the photoelectric current, which corresponds to the initial rate of the charge movement is plotted against the pH of the medium. The pH was decreased from pH 9.4 and changed as indicated by the solid line. One symbol in Fig. 3 is the average of two successive measurements. Deviation of the each measured value from the average was $2.8 \pm 4\%$.

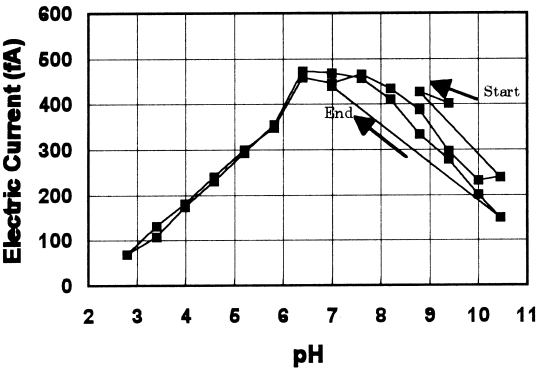

Fig. 3. pH dependence of the photoelectric current of wild type bR. The peak height of the photoelectric current was measured at various pH after 5 s dark intervals. The pH was first decreased from pH 9.4 as indicated by the arrow 'Start'. Then, it was changed following the solid line, finally to pH 7 as indicated by the arrow 'End'. One symbol represents the average of two successive measurements.

Then, we compared the photoelectric responses in case of wild type bR (Fig. 4A) and its D96N mutant (Fig. 4B) over a wide pH range after different dark intervals. As described in Section 2, 5 s illumination was carried out after 5 min, 10 s or 5 s intervals in the dark. Each column in Fig. 4 corresponds to one time interval. In the case of wild type bR, the peak height of the photoelectric current did not alter with respect to the

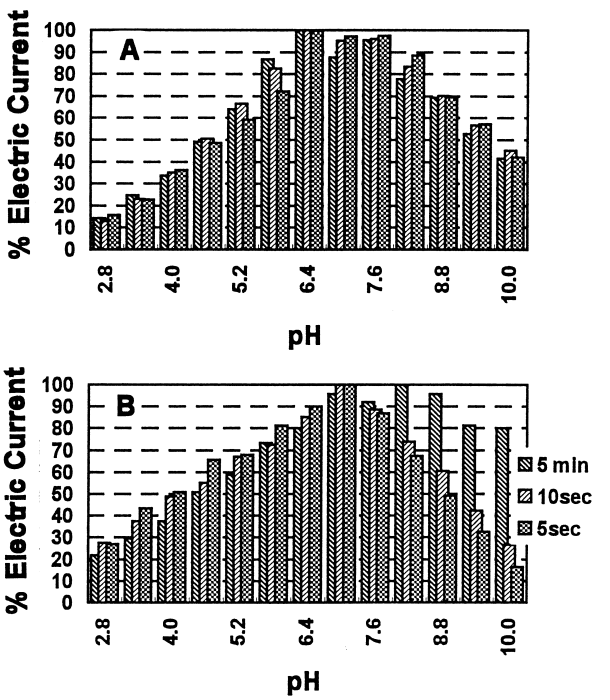

Fig. 4. pH dependence of the photoelectric current of wild type bR and its D96N mutant after different dark interval times. The peak current was measured after a dark period of 5 min, 10 s, and 5 s, respectively. For every dark period, the data were expressed relative to their maximum values. A: Wild type bR. 100% corresponds to 560, 500, 470 fA after a dark period of 5 min, 10 s, and 5 s, respectively. B: The D96N mutant. 100% corresponds to 150, 120, 100 fA after a dark period of 5 min, 10 s, and 5 s, respectively.

length of the dark period. This means that wild type bR returns to its initial state within 5 s in the dark at any pH. Further, a broad pH optimum was observed around 7 (Fig. 4A). Half of the maximum of the peak height was measured around pH 4.6 and 9.4.

In contrast, the D96N mutant showed considerably higher peaks after 5 min dark interval than after 10 or 5 s intervals at pH > 7 (Fig. 4B). This means that the D96N mutant does not return to its initial state within 10 s in the dark at pH > 7. Above pH 7, the peak height after a 5 min dark interval was relatively insensitive to pH, whereas the peak height after a 10 s or 5 s dark period had a steep pH dependence. At pH 10, it was only 20% of the maximum in case of the D96N mutant compared to 40% of the maximum in the wild type after short intervals. Below pH 7, the half maximum of the peak height was around pH 4.6. At pH 2.8, it was about 25% of the maximum compared to 15% of the maximum for the wild type.

4. Discussion

4.1. Experimental system for the measurement of the electrogenicity of ion pumps

Many methods have been reported for measurements of the electrogenic processes mediated by membrane proteins. These include direct measurements of electric signals using giant cells or oocytes by electrophysiological methods [26–29]; electric measurements using gel systems which contained oriented light driven ion pumps [30,31]; electric measurements using artificial planar bilayers with transmembranously reconstituted ion pumps [32–34] and the system where membrane fragments (or vesicles) were adsorbed on various supports [1–18,35]. While the transmembranous reconstitution systems, which allowed to control the electrochemical potential gradient of transported ions, provided information on the energetics [22,34,36,37], membrane adsorbed systems which allowed the measurement of the capacitive current or the voltage generation, gave insights into the accompanying molecular mechanisms by virtue of their high time resolution [7–13]. However, as stated in the introduction, some problems in the latter systems were caused by the characteristics of the support materials. As is shown in Fig. 1A,B, the support material in such systems behaves like a simple capacitor which transduces the charge movement by an ion pump protein to a capacitive current or the generation of a potential [23–25]. Therefore, a substrate with high specific capacitance and low specific conductance, which is mechanically stable and does not respond to changes of the light, the pH or the ionic concentration, is an ideal solid support in such systems. With respect to these criteria, we have tested many materials. Unfortunately, all the metal substrates tested responded to light or solutes in the buffer even in the absence of ion pump proteins and stable recording was difficult due to low electric resistance. Films or filters impregnated with lipids sometimes showed irreproducible artifacts such as oscillatory or periodic noises. Finally, a thin polymer film (Lumirror, Toray Co., Japan) which was originally designed for film condensers fulfilled all the requirements for a solid support in the membrane (vesicle) adsorbed system. Lumirror is mechanically very strong, chemically inert, has a very high electric resistance, and a relatively high capacitance compared to other polymer films tested. However, Lumirror (900 nm thick) is still much thicker than black lipid films or folded bilayers (less than 10 nm) and the capacitive electric current per constant area is much smaller than in the system with the lipid membranes. Nevertheless, the handling of a large area membrane is easier using Lumirror and we could obtain an electric response similar to those with lipid membranes with considerably higher stability.

Another advantage in the present method is the washing with octylglucoside. This process was possible due to the stability of Lumirror. In the experiments without the octylglucoside washing, the magnitude of the response was relatively small and usually increased upon exchange of the buffer in the compartment of the purple membrane adsorbed side (Fig. 1C). We suspect that many purple membrane sheets were loosely adsorbed onto Lumirror with nearly random orientations just after adsorption. It is likely that the loosely adsorbed fraction was removed by the buffer exchanges whereas purple membrane sheets which tightly contacted the extracellular side to Lumirror film preferentially remained, which might have lead to larger photoelectric responses. Washing with octylglucoside seemed to remove the loosely bound fraction more efficiently. After this procedure, the magnitude of the photoresponse became significantly larger and the reproducibility of the magnitude of the photoresponse was improved (Figs. 1D and 3). The result obtained by AFM imaging (Fig. 2) supports the above interpretation. Although it is not clear what kind of interaction stabilizes the purple membrane sheets on Lumirror and the orientation of the purple membrane sheets is not completely controlled, purple membrane sheets were adsorbed tightly after the octylglucoside treatment. Surprisingly, the same electric response was obtained when the original buffer was filled again after removing the buffer and leaving the membrane for 40 min in the atmosphere. The orientation of the purple membranes seems nearly random and only a slight imbalance of the orientation seems to cause the observed net electric current. As the light intensity was not strong enough to make maximum photoresponse and the amount of the purple membranes could not be precisely determined, we cannot estimate the extent of the orientation precisely. However, if we assume that 80% of the surface of Lumirror was covered by purple membranes, light intensity was at saturation level and that the presence of Lumirror did not attenuate the electric current (though unlikely), the expected magnitude of the electric current generated by perfectly oriented purple membranes is more than 100 μA (assuming that the area occupied by a bR protomer is 1100 $Å^2$ and one turnover of the photocycle takes 10 ms), which is more than 10^7 times greater than the observed electric current. Yet the possible interference caused by the randomly adsorbed purple membranes would not affect the observed pH dependence as their population is constant.

Lumirror was found to be a possible substrate for AFM. The physical strength of the film makes the preparation and the imaging of the sample much easier compared to lipid layers. Although the flatness of the film surface is not so good as mica, gold or any other atomically flat surfaces, the advantage of using Lumirror is that we can assay the activity of the purple membrane directly on this film. As mentioned above, the purple membrane was still active 40 min after the removal of the buffer, which strongly suggests that the purple membranes observed with AFM were active. We also tried to image the purple membrane-adsorbed Lumirror before wash-

ing with octylglucoside but we could not obtain a stable AFM image. This result also supports our interpretation that the purple membranes were unstably piled up on the film before the octylglucoside washing.

So far, many AFM images of purple membranes have been reported. But most of those preparations were made on mica and it was impossible to assay the activity of the membranes. Our system will open a way to observe active membrane protein with AFM, which has not been achieved yet in AFM protein imaging.

4.2. pH dependence of the photoelectric current of wild type bR and the D96N mutant

Previously, we reported the ΔpH dependence of the steady state electric current by wild type bR [37]. Because of the small signal and the large noise, the pH range examined was limited from 5 to 9 and the ΔpH imposed was 2 units at maximum. It was reported that the rate of proton translocation from the neutral side (pH 7) to the acidic side (pH 5) was at least 60% of the rate without proton gradient and that from the basic side (pH 9) to the neutral side (pH 7) was at least 80% of that of the rate without proton gradient, respectively. The data presented here are consistent with the previous ones, the capacitive peak current at pH 5.2 had about 60% and the one at pH 8.8 had about 70% of the maximum activity (Fig. 4A). The pH or ΔpH profile is thought to reflect the protonation/deprotonation states (pK_a) of the key residues accepting and releasing protons. At high pH, the residues taking up protons (D96 [9,38] and possibly other residues [39,40] at the cytoplasmic surface of bR) are unable to capture a proton and at low pH, the residues for releasing protons (D85 and E204 [41] and possibly other residues at the extracellular surface of bR [40]) are unable to release protons. The pK_as of D96 and D85 are estimated to be about 11 [42–45] and 2.2 [41] in unphotolysed bR, respectively. The pK_a of E204 in unphotolysed bR is estimated to be 9.4 and it decreases to 4.7 after formation of the M intermediate facilitating proton release to the extracellular side [41].

In the D96N mutant, the photoelectric responses after a 10 s dark interval were considerably lower than those after a 5 min intervals at $pH > 7$ (Fig. 4B). This indicated that the mutant protein does not return to its initial state within 10 s in the dark at $pH > 7$. It is consistent with the accepted fact that the decay of the M intermediate, which corresponds to the reprotonation of the deprotonated Schiff base, is considerably slowed down in this mutant [9]. In other words, the smaller photoelectric response after the 5 or 10 s interval is due to the incomplete reprotonation of the Schiff base in the absence of the proton donor, D96. However, the fact that the peak height after the 5 min interval was quite insensitive to the pH above pH 7 compared to the wild type requires some additional explanation. After a 5 min interval, the relative peak height in the D96N mutant at pH 10 (80%) was much higher than that of the wild type (50%). This means that the absence of D96 apparently causes highly efficient proton transfer at high pH. There are two possible interpretations for this result.

As the reprotonation of the deprotonated Schiff base is significantly slow in D96N mutant, the reprotonation step does not contribute to the initial rate of the charge movement above pH 7. Therefore, the observed electric current by the D96N mutant is mostly due to proton transfer from the Schiff base to D85 above pH 7. This step is relatively insensitive to the medium pH because of the extremely high pK_a of the Schiff base and the low pK_a of D85 in the unphotolysed state. In case of the wild type bR, the observed electric current is the sum of the proton transfer from the Schiff base to D85 and from D96 to the deprotonated Schiff base. Whereas the former is insensitive to medium pH above pH 7 as in the D96N mutant, the contribution of the latter transfer becomes smaller at higher pH, resulting in an apparent decrease of the electric current. Second, we would like to mention that in the case of the wild type, a fraction of the protons released from the Schiff base might migrate to the deprotonated D96 at high pH, as well as to D85, whereas in the case of the D96N mutant, there is no proton acceptor on the cytoplasmic side and all the protons released from the Schiff base move to D85. This could also result in a higher efficiency of the proton transfer in the D96N mutant than in the wild type at high pH. Although experimental evidence is lacking, the results suggest the possibility that the direction of proton transfer from the Schiff base is, at least partially, influenced by the protonation/deprotonation state of the surrounding proton acceptor groups. Further precise studies are required to prove (or disprove) the latter possibility.

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