Photo-Induced Proton Transport of *Pharaonis* Phoborhodopsin (Sensory Rhodopsin II) Is Ceased by Association with the Transducer

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ABSTRACT Phoborhodopsin (pR; also sensory rhodopsin II, sRII) is a retinoid protein in *Halobacterium salinarum* and works as a receptor of negative phototaxis. *Pharaonis* phoborhodopsin (*p*pR; also *pharaonis* sensory rhodopsin II, *p*sRII) is a corresponding protein of *Natronobacterium pharaonis*. In bacterial membrane, *p*pR forms a complex with its transducer *p*HtrII, and this complex transmits the light signal to the sensory system in the cytoplasm. We expressed *p*HtrII-free *p*pR or *p*pR-*p*HtrII complex in *H. salinarum* Pho81/wr$^-$ cells. Flash-photolysis experiments showed no essential changes between *p*HtrII-free *p*pR and the complex. Using SnO_2 electrode, which works as a sensitive pH electrode, and envelope membrane vesicles, we showed the photo-induced outward proton transport. This membranous proton transport was also shown using membrane vesicles from *Escherichia coli* in which *p*pR was functionally expressed. On the other hand, the proton transport was ceased when *p*pR formed a complex with *p*HtrII. Using membrane sheet, it was shown that the complex undergoes first proton uptake and then release during the photocycle, the same as *p*HtrII-free *p*pR, although the net proton transport ceases. Taking into consideration that the complex of sRII (pR) and its transducer undergoes extracellular proton circulation (J. Sasaki and J. L. Spudich, 1999, *Biophys. J.* 77:2145–2152), we inferred that association with *p*HtrII closes a cytoplasmic channel of *p*pR, which lead to the extracellular proton circulation.

INTRODUCTION

Halobacteria contain four retinal proteins (archaeal rhodopsins), which are bacteriorhodopsin (bR) (reviewed in Lanyi, 1997; Haupts et al., 1999), halorhodopsin (hR) (Matsuno-Yagi and Mukohata, 1977; Lanyi, 1990), sensory rhodopsin I (sRI) (Bogomolni and Spudich, 1982; Tsuda et al. 1982; Hoff et al., 1997), and phoborhodopsin (pR, also called sensory rhodopsin II, sRII) (Takahashi et al., 1985, 1990; Zhang et al., 1996). The former two are light-driven ion pumps; bR works as an outward proton-pump and hR as an inward Cl^- pump. The latter two are photoreceptors of this bacterium. The ground state of sRI (absorption maximum (λ_{max}) of 587 nm) is a receptor-mediating positive phototaxis, whereas its long-lived photo-intermediate (S_{373}, λ_{max} of 373 nm) acts as a receptor of negative phototaxis. pR (or sRII) absorbs maximally 487-nm light and works as a receptor of negative taxis. Each receptor transmits its signals through integral membrane transducer proteins HtrI and HtrII, which are considered to form a signaling complex firmly with respective receptors (Hoff et al., 1997). By these two signaling systems, these bacteria move toward longer wavelength light ($\lambda > 520$ nm) where bR and hR work, while they avoid shorter wavelength light ($\lambda < 520$ nm), which contains harmful near-UV light.

It is reported that *Natronobacterium pharaonis* and *Haloarcula vallismortis* also have pR (sRII)-like proteins (Hirayama et al., 1992; Scharf et al. 1992; Seidel et al. 1995). The protein from *N. pharaonis* is called *pharaonis* phoborhodospin (*p*pR, or *pharaonis* sRII, *p*sRII). Lüttenberg et al. (1998) succeeded in heterologous co-expression of *p*pR (psRII) and *p*HtrII in *H. salinarum* Pho81/W and observed that the transfected cells showed negative phototaxis, suggesting that the complex of *p*pR and *p*HtrII can function even in *H. salinarum* cells.

Interaction of sRI or sRII with their cognate transducers alters the photocycle rate and the proton movement. First, removal of HtrI allows light-driven electrogenic proton translocation by sRI and slows down the photocycling rate (Spudich and Spudich, 1993; Olson and Spudich, 1993; Bogomolni et al. 1994). Although controversial data that the proton transport of sRI was not blocked completely by HtrI had been presented (Haupts et al., 1996), this was reconciled by the explanation that their preparation contained free sRI (Hoff et al. 1997). Second, sRII itself takes up and release protons from and to the extracellular side of the pigment without transmembrane transport, and association with HtrII retards the photocycling rate (Sasaki and Spudich, 1998, 1999). Photo-induced transmembranous proton transport of *p*pR (*p*sRII) has been suggested (Schmies et al., 2000), but the effect of *p*HtrII on *p*pR-proton transport has not been reported. The present communication presents more clear evidence on the photo-induced transmembrane proton transport by *p*HtrII-free *p*pR (*p*sRII) and its cessation by the association with *p*HtrII.

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MATERIALS AND METHODS

Membrane preparations from *H. salinarum* cells expressing *p*pR or *p*pR-*p*HtrII complex

H. salinarum strain Pho81/wr$^-$ lacking all of the four archaeal rhodopsins (bR, hR, sRI, and pR(sRII)) as well as the two transducers of HtrI and HtrII

(Perazzona et al., 1996) was used for transformation according to the protocol described previously (Yan et al., 1992). To obtain overexpression, *psopII* and *phtrII* sequences from *N. pharaonis* were cloned under the control of the strong *bop* promoter. The *psopII* and *phtrII/psopII* operon arrangements from *N. pharaonis* were not changed, but the 5′ end of *psopII* and *phtrII* were mutated by polymerase chain reaction to get a *Nde* I restriction site. *phtrII/psopII* or *psopII* was subcloned to pGEM-T Easy vector (Promega, Madison, WI). The *Nde* I and *Pst* I fragments from these subcloned plasmids were ligated to *Nde* I and *Pst* I sites of the pUBP2/*bop* which was the *Escherichia coli/H. salinarum* shuttle expression vector (Blasio and Pfeifer, 1990). Halobacteria transformants expressing plasmid-encoded mevinolin resistance were grown and selected on plates at 37°C on the standard peptone medium with 4 μg of mevinolin (supplied by Sankyo Co., Tokyo, Japan) per ml.

Transformants were grown aerobically in the standard peptone medium in the dark at 37°C, harvested by centrifugation (17500 $\times$ *g* for 10 min at 4°C), and suspended in 4 M NaCl. Right-side-out membrane vesicles were prepared by five-times sonication of the cells for 30 s at 4°C with a power of 180 W at a duty cycle of 50% (UP200H, Kubota, Tokyo, Japan). The ratio of right-side-out membrane vesicles to the total (the sidedness) was checked by the menadion/NADH method (Lanyi and MacDonald, 1979) and was ascertained to be above 85%. Membrane sheets were prepared according to a method of Sasaki and Spudich (1999). Cells were treated by dialysis against 250 mM KCl at 4°C for >12 h, and crude membranes were collected by ultracentrifuge (100,000 $\times$ *g* for 60 min at 4°C), followed by resuspension in 4 M NaCl. According to Sasaki and Spudich (1999), this treatment under low-salt concentration does not dissociate the complex of pR(sRII)-HtrII. The dissociation constant between *p*pR (*p*sRII) and *p*HtrII is reported to be ~100 nM (Engelhard et al., 2000). This strong association together with Sasaki and Spudich's data suggest that the *p*pR-*p*HtrII complex remains in the membrane sheets.

Preparation of inside-out membrane vesicles derived from *E. coli* cells expressing ppR

Expression of *p*pR in *E. coli* membrane was done as previously reported (Shimono et al., 1997). Inside-out vesicles of *E. coli* cells expressing ppR were prepared with a French press according to the standard method (Rosen and Tsuchiya, 1979). At 120 min after induction, cells were harvested by centrifugation at 4°C and washed once with a buffer containing 50 mM Tris-Cl (pH 8.0) and 5 mM $MgCl_2$. Washed cells were suspended in the above buffer (5 ml/g of wet cells) into which DNase (D4527, Sigma Chemical Co., St. Louis, MO) and RNase (R4875, Sigma) were supplemented. This mixture was passed through a French press (Ohtake, Tokyo, Japan) under 800 kg/cm^2, and the passed medium was centrifuged at 8400 $\times$ *g* for 10 min to remove the cell debris. The supernatant was centrifuged further at 180,000 $\times$ *g* for 60 min. The pellet obtained was washed three times with 400 mM NaCl. Experiments of the photo-induced pH change were performed immediately after the preparation.

Flash spectroscopy

Apparatus and procedure were essentially the same as described earlier (Miyazaki et al., 1992)

Measurements of photo-induced pH change

It was previously shown that SnO_2 transparent electrode works well as a very sensitive pH electrode (Iwamoto et al., 1999a). The electromotive force (*emf*) that arises between SnO_2 and a reference electrode is linear against the pH of the medium (data not shown; see Robertson and Lukashev, 1995). The cell was composed as follows: SnO_2/thin layer containing *p*pR envelope vesicles or membrane sheets/4 M NaCl/SnO_2. A schematic illustration of the photo-electrochemical cell was given previously (Fig. 1 of Iwamoto et al., 1999a). In this cell, the sample suspension solution and 4 M NaCl did not contain buffer. This photo-electrochemical cell has the advantage over using a pH-glass electrode in its high sensitivity because of the much lower electric resistance of the system and capability of measurement with a very small sample volume (~40 μl) due to the thin layer of the sample solution adjacent to the flat SnO_2 electrode.

The light source for illumination of the sample was a 300-W xenon arc lamp in combination with an infrared cutoff filter and color and interference filters (HA50, CM500, Y50, KL50, and IRA05; Toshiba, Tokyo, Japan), which provided green light with a maximum intensity at ~500 nm. The intensity was 0.95 kW/m^2 measured with a Kettering radiant power meter (model 4090, Yellow Springs, OH). A pair of SnO_2 electrodes were connected to a potentiostat/galvanostat model 2000 (Toho Technical Research, Tokyo, Japan) operated in a potential measurement mode. For experiments of membrane vesicles, the low-cut electric filter was not used, because the pH change in the medium caused by the illumination was very slow (see Figs. 3 and 4). On the other hand, for membrane sheets, an AC amplifier with a 15-Hz low-cut electric filter (Bioelectric Amplifier MEG-1200, Nihon Koden, Tokyo, Japan) was used as previously (Iwamoto et al., 1999a). The output signals were stored in a digital storage oscilloscope (Hewlett-Packard model 54520C).

RESULTS

The photochemical cycle of *p*pR after a milliseconds time range is as follows (Miyazaki et al., 1992; Chizhov et al., 1998); *p*pR (498 nm) $\rightarrow$ *p*pR$_M$ (390 nm) $\rightarrow$ *p*pR$_O$ (550 nm) $\rightarrow$ *p*pR. We hereafter will denote *p*pR$_M$ and *p*pR$_O$ as M and O intermediates, respectively. Flash-induced light-dark difference spectra where flash light (λ_{max} of 530 nm) was provided to the membrane vesicles of *H. salinarum* are shown in Fig. 1. We used membrane vesicles either from cells expressing *p*HtrII-free *p*pR or from cells co-expressing *p*pR and *p*HtrII. This figure reveals that the complex of *p*pR-*p*HtrII has also the two photo-intermediates that are the same as those of *p*HtrII-free *p*pR. In addition, this figure shows that the association does not change λ_{max} of the *p*pR complex and its photo-intermediates.

Fig. 2 shows the flash-induced absorbance changes at 390, 560, and 500 nm to monitor the change of M, O, and *p*pR, respectively. The pH of the medium was 7.0. Fig. 2 *A* represents data of the membrane vesicles containing *p*HtrII-free *p*pR whereas Fig. 2 *B* represents data when *p*pR and *p*HtrII were co-expressed. This reveals no essential difference of time constants between *p*HtrII-free *p*pR and *p*pR-*p*HtrII complex, as is expected from Fig. 1. No changes were also observed using membrane sheets (data not shown). Flash-photolysis experiments of membrane vesicles were performed at varying pH from 5 to 9. A logarithmic plot of absorbance changes at 390 nm against time was linear, meaning that M decay is mono-phasic (data not shown). The mono-phasic decay of M was reported elsewhere (Miyazaki et al., 1992). Kinetic time constants were estimated and are listed in Table 1. Here, k_1 represents the time constants of M decay and k_2 are that of the O decay. The values estimated from three independent samples were

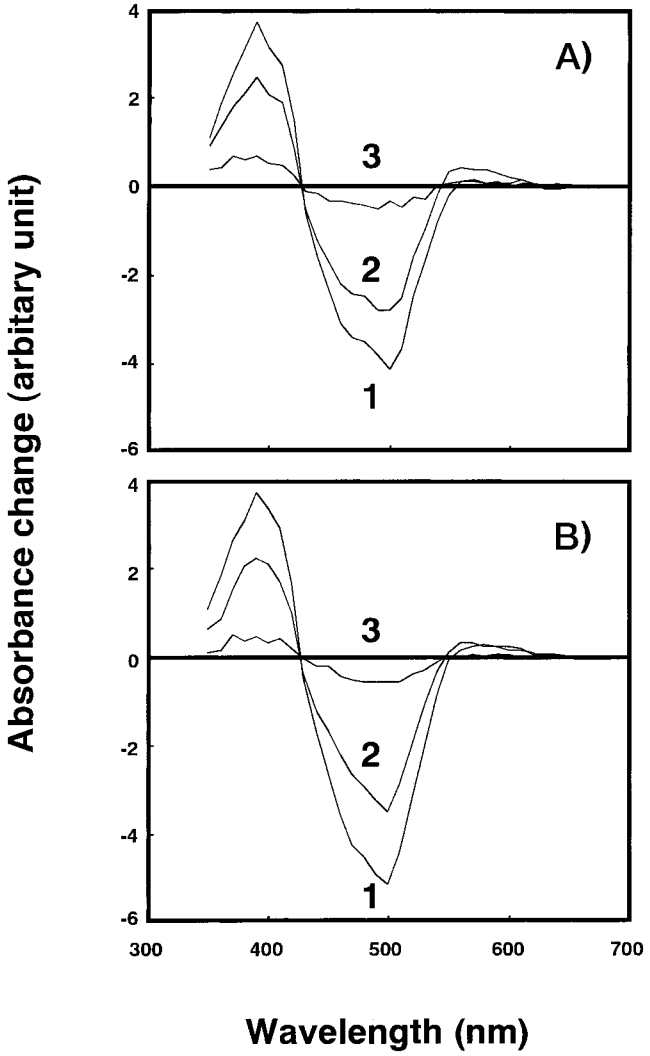

FIGURE 1 Flash-induced difference spectra of membrane vesicles derived from Pho81/wr$^-$ cells being expressed by *p*pR alone (*a*) and being co-expressed by *p*pR-*p*HtrII (*b*). Curves 1–3 of both are spectra of 100 ms (*1*), 1000 ms (*2*), and 5000 ms (*3*) after the flash. The protein concentration was 5 mg/ml, and medium contained 4 M NaCl buffered with 20 mM MOPS-NaOH at pH 7. Temperature was 20°C.

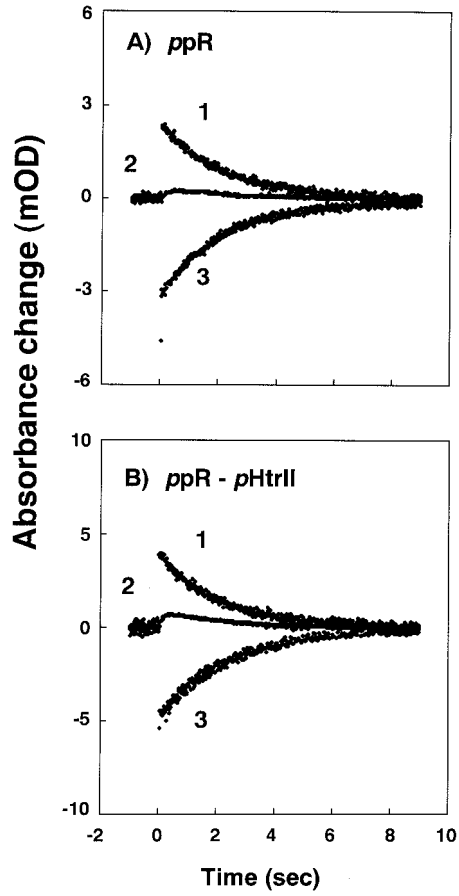

FIGURE 2 Kinetic data after excitation of *p*pR. (*A*) Data of membrane vesicles from cell expressed *p*pR alone; (*B*) Data of the complex of *p*pR-*p*HtrII. For both *A* and *B*, curve 1 depicts the kinetic data observed at 390 nm representing the M decay, curve 2 shows the 560-nm data representing the formation/decay of the O intermediate, and curve 3 represents the recovery of the original *p*pR. Experimental conditions were the same as those in Fig. 1.

averaged, and the variations were less than 5%. According to this table, both k_1 values are identical to each other for all of the pH range. The values of k_2 seem somewhat different between *p*HtrII-free *p*pR and *p*pR-*p*HtrII; however, the difference is very small. Hence we may conclude that the association of *p*pR with its transducer, *p*HtrII does not change the photochemistry or its rate. Wegener et al. (2000) reported also essentially no changes of the photocycling rates using His-tagged *p*sRII (*p*pR) and a truncated transducer that were expressed in *E. coli* cells, although some kinetic constants differed maximally approximately twice.

This observation is in sharp contrast to those of sR-HtrI (sRI-HtrI) and pR-HtrII (sRII-HtrII). In the presence of HtrI the M intermediate of sRI, S_{373} decays to sRI with a half-time of 0.8 s whereas in the absence of HtrI the M decay became very slow (~6 s) at neutral pH. In the HtrII-free sRII (pR), M decays with a half-time of 66 ms and that of O decay is over 1.0 s (Spudich and Spudich, 1993). When sRII (pR) forms the sRII-HtrII (pR-HtrII) complex, the M decay becomes slightly faster and the O decay (170 ms) is greatly accelerated (Sasaki and Spudich 1998). Comparison between these previous and the present observations might lead to the conclusion that even though *p*pR and *p*HtrII are co-expressed in the present experiment, the association does not occur in the membrane. However, this notion is not the case because 1) the co-expressed cells showed negative phototaxis from ~500-nm light (data not shown) as is reported by Lüttenberg et al. (1998) and 2) the membrane transport of proton is inhibited for the membrane vesicles from the co-expressed cells as is shown below.

In our previous paper (Iwamoto et al., 1999a), it was shown by using the transparent SnO_2 electrode that during

TABLE 1 Rate constants of M decay (k_1) and O decay (k_2) of *p*pR and the complex of *p*pR-*p*HtrII under varying pH

	k_1 (s^{-1})					k_2				
	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
*p*pR	5.25	1.78	0.56	0.37	0.22	3.12	4.05	5.21	4.91	4.77
*p*pR-*p*HtrII	5.29	1.75	0.56	0.36	0.21	3.42	4.11	4.78	4.95	4.58

The protein concentrations of *p*pR membrane vesicles were 5 mg/ml. Flash light (λ_{max} = 530 nm) was provided through a cutoff filter (>500 nm) and an interference filter (530 nm, KL53). Buffer solutions of pH 5–9 were used: 20 mM citrate-HCl, 4 M NaCl for pH 5; 20 mM Bis-Tris-HCl, 4 M NaCl for pH 6; 20 mM MOPS-NaOH, 4 M NaCl for pH 7; 20 mM EPPS-NaOH, 4 M NaCl for pH 8; and 20 mM CHES-NaOH, 4 M NaCl for pH 9.

photocycling of *p*pR, proton uptake first occurs coincidentally at the M decay (O formation) followed by proton release at the O decay. Fig. 3 shows the pH change in the medium where inside-out membrane vesicles derived from *p*pR-expressed *E. coli* cells were suspended. The downward deflection indicates the alkalization in the medium. Fig. 3 *a* shows the signal obtained when the medium contains no buffer. Fig. 3 *b* is that in the presence of 100 mM buffer (pH 6.6 of potassium phosphate), meaning that the change cannot be observed in the presence of strong buffer action. Fig. 3 *c* is that in the presence of 1% *n*-dodecyl-β-D-maltoside, which does not inactivate the pigment but destroys the membrane integrity. Fig. 3 *d* is that obtained using membrane vesicles derived from cells into which vector (pET21c) alone was transfected. These data show that *p*pR can transport protons upon illumination from intracellular to extracellular, which is the same direction of bR and HtrI-free sRI (Bogomolni et al., 1994).

Similarly, the photo-induced proton transport was measured using membrane vesicles derived from *H. salinarum* Pho81/wr$^-$ cells in which *p*pR alone was expressed or *p*pR-*p*HtrII was co-expressed. It is noted that the sidedness of *H. salinarum* vesicles is right side out whereas *E. coli* vesicles in Fig. 3 are inverted. Results are presented in Fig. 4, where sheet means the membrane sheet that does not form closed vesicles. This figure clearly shows that *p*pR

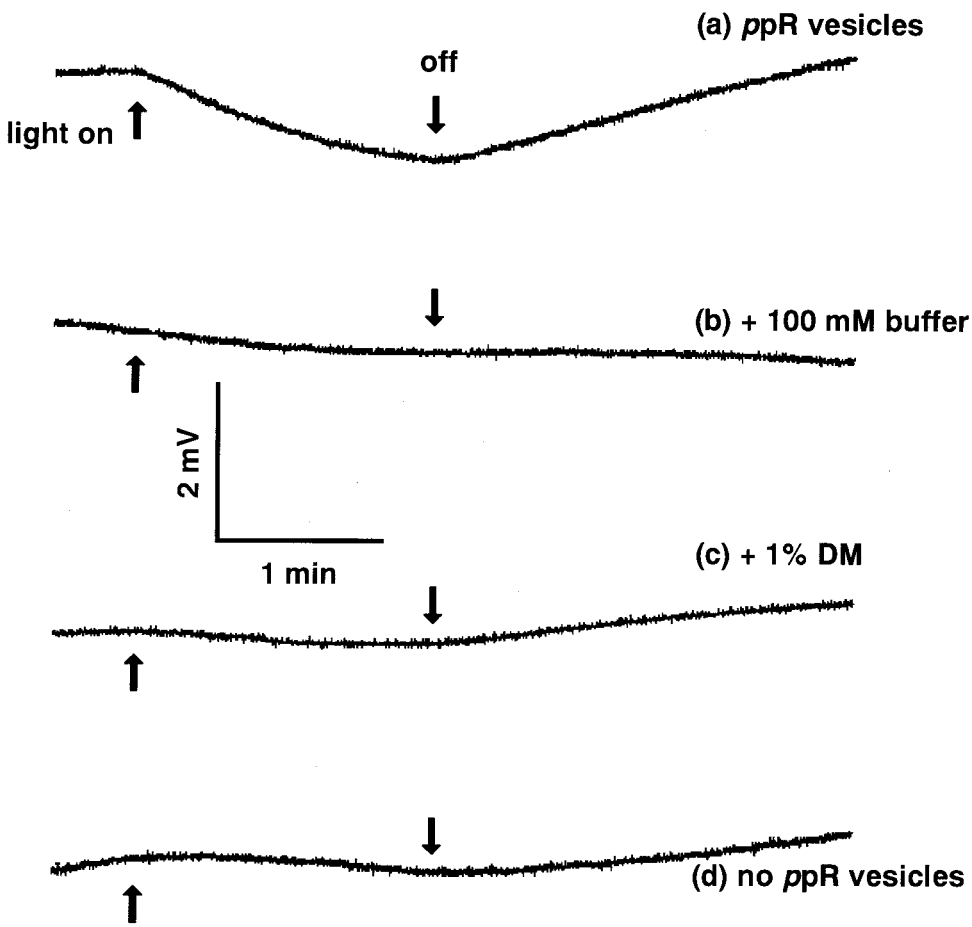

FIGURE 3 Photo-induced transmembrane proton transport by *p*pR using inside-out membrane vesicles from *E. coli* cells expressing *p*pR alone. As is described in text, the signals were picked up by a DC amplifier. The downward deflection means the alkalization in the medium that is equivalent to the proton uptake by the inside-out vesicles. (*a*) Alkalization of *p*pR vesicles; (*b*) Signal obtained in the presence of 100 mM potassium phosphate buffer (pH 6.6); (*c*) Obtained in the presence of 1% dodecyl-β-D-maltoside; (*d*) Signal obtained using membrane vesicles derived from cells into which vector (pET21c) alone was transfected. For all experiments the vesicle concentration was 250 mg/ml and pH was 6.0 ± 0.2.

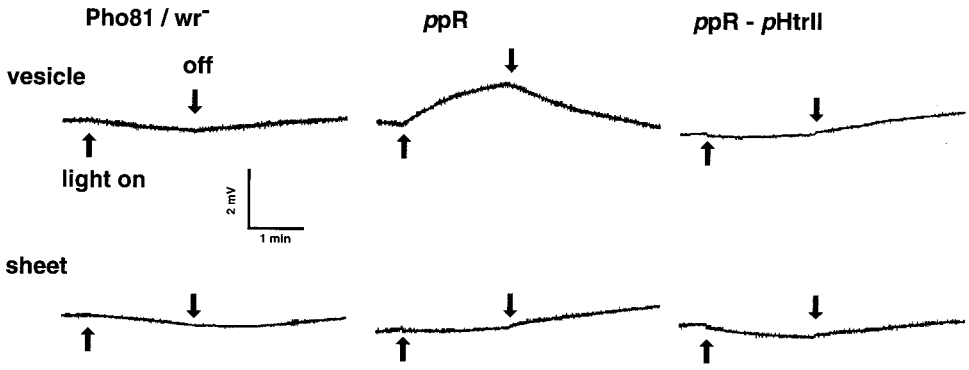

FIGURE 4 Photo-induced proton movement by membrane vesicles (*upper row*) and by sheet (*lower row*), both of which were derived from *H. salinarum* Pho81/wr$^-$ cells. The sheet means the membrane sheets that do not form closed vesicles (see text). The downward deflection means the alkalization in the medium, as in Fig. 3. The rightmost data of each row show the pH change using cells that do not express *p*pR. The center data show those using cells expressing *p*pR alone. The *p*pR concentrations were adjusted to be constant at 26 μmol/L where the molar extinction coefficients of *p*pR and of *p*pR-*p*HtrII were taken as 48,000 M^{-1} cm^{-1}. Before and after the experiments, the integrity of vesicles, especially for *p*pR-*p*HtrII vesicles, was checked by the menadion-NADH method (see text). The protein concentration of both vesicles and sheet from nonexpressed Pho81/wr$^-$ cells was 200 mg/ml, and pH was 6.6 $\pm$ 0.2.

expressed in *H. salinarum* also can transport protons upon illumination and the complex of *p*pR-*p*HtrII fails to transport. It is noted that examination of the ratio for right-side-out vesicles (measurements of menadion-NADH oxidoreductase, activity which locates the inner half of membrane) also checks the integrity of membrane vesicles. The vesicles derived from co-expressed cells also showed a high ratio of right-side-out (>85%). This rules out the possibility that no photo-induced proton transport by *p*pR-*p*HtrII might be due to the incomplete formation of closed vesicles.

Careful inspection of the signal traces reveals a very small downward deflection observed in sheets as well as in the membrane vesicle of *p*pR-*p*HtrII on illumination, although it is not seen for cells that do not express *p*pR. To examine whether this deflection is a noise or a signal, the sensitivity of the apparatus should be increased. Unfortunately, because of the baseline drift and small S/N ratio, the increase in the sensitivity was difficult. On the other hand, this may be achievable when an AC-coupling amplifier is used (Iwamoto et al., 1999a) instead of the DC amplifier employed in Figs. 3 and 4. For the experiments of membrane vesicles of Figs. 3 and 4, the generation of the proton gradient is not rapid and the change in the pH was not fast enough to be recorded by an AC-coupling amplifier. In contrast, when a sheet is used, an AC-coupling amplifier (low-cut filter of 15 Hz) is applicable because of the rapid pH change in the medium. Data are shown in Fig. 5 presenting the photo-induced pH change of a membrane sheet of Pho81/wr$^-$ cells that were expressed by *p*pR alone or co-expressed by *p*pR-*p*HtrII. This figure indicates that on illumination the proton uptake first occurs followed by the proton release for both preparations. The off-response seems to be different between those of *p*HtrII-free *p*pR and *p*pR-*p*HtrII complex, which might be related to the observation shown in Table 1; the O decay rate seems to be affected by the association although the difference is very small. Despite this, we may consider that there is no essential difference of proton uptake and release between *p*HtrII-free *p*pR and *p*pR-pHtrII. This indicates that *p*pR-*p*HtrII first takes up the proton and later releases it, although the trans-membranous proton transport does not occur, imply-

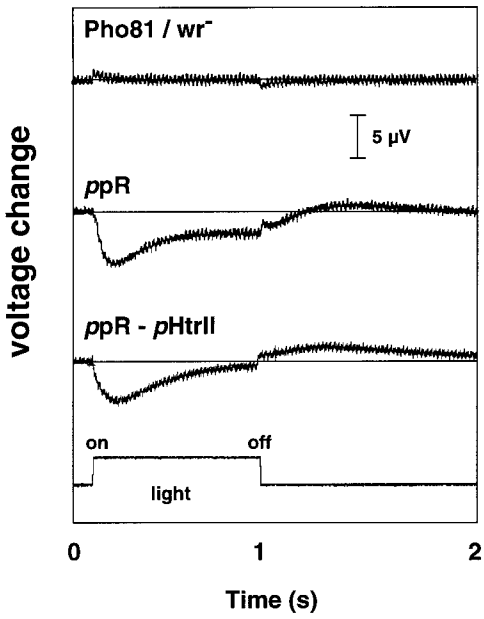

FIGURE 5 Photo-induced pH changes by membrane sheets from *H. salinarum* Pho81/wr$^-$ cells. The signals were amplified with an AC-coupling amplifier using a 15-Hz low-cut electric filter, and this method was different from that of Figs. 3 and 4. The *p*pR concentrations were adjusted to be constant at 26 μmol/L. Protein concentration of the uppermost data was 180 mg/ml, and pH was 6.6.

ing that a non-electrogenic proton circulation occurs for the *p*pR-*p*HtrII complex.

DISCUSSION

The present data show that *p*HtrII-free *p*pR can transport protons on illumination and that this does not occur in the *p*pR-*p*HtrII complex. Sasaki and Spudich (1999) revealed that the proton circulation occurs on illumination for sRII-HtrII in *H. salinarum*, and the uptake and release are from and to the extracellular side. Because both *p*pR (*p*sRII) in *N. pharaonis* and sRII (pR) in *H. salinarum* are similar proteins acting as negative photoreceptors, it may be concluded that the complex of *p*pR-*p*HtrII also undergoes extracellular proton circulation.

The alkalization observed in the membrane sheet of Fig. 4 (also see the enlarged signal shown in Fig. 5 using an AC-coupling amplifier) might be interpreted as follows. The photo-steady complex may contains mainly O intermediate because the O decay is rate determining. The O intermediate is a proton-uptake intermediate because proton uptake occurs at the O formation (M decay). The downward shift (alkalization of the medium), hence, is observed. It is noted that a similar shift is observed for vesicles containing *p*pR-*p*HtrII. When we accept the above conclusion of extracellular proton circulation, this change is conceivable because envelope vesicles are right side out. To prove this further, the inverted membrane vesicle would be useful, which unfortunately is not obtainable.

As assumed above, when the complex of *p*pR and *p*HtrII forms, photo-induced extracellular proton circulation may happen, implying that the cytoplasmic channel (CP) of *p*pR may be closed by the association with its cognate transducer. This has been proposed by Spudich and his colleagues for sRII (pR) and sRI (sR) (Spudich, 1998; Sasaki and Spudich, 2000). Because of the photo-induced proton transport of *p*pR whose direction is the same as that of bR, we might consider that at the M decay of *p*pR, the proton may come from the cytoplasmic space through the CP to the deprotonated Schiff base, whereas after the association with *p*HtrII, the proton may come from the opposite direction, i.e., through extracellular channel (EC). On the basis of this notion we can hardly answer a question of why the association of *p*pR with *p*HtrII does not change the M decay rate although the pathway of proton uptake becomes different. One possible consideration may be that even for *p*HtrII-free *p*pR, the proton comes from either channel to the deprotonated Schiff base at the M decay, but the energy barrier of the proton transport through the EC is much smaller than that of the CP. Hence, with large probability, the proton comes through the EC (resulting in proton circulation as in sRII described by Sasaki and Spudich (1999)), but there is still probability of the movement through the CP. This leads to the conclusion that the observed M decay rate is determined mainly from the rate at which the proton comes through the EC, and the transmembrane proton transport is contributed from the proton movement through the CP. Therefore, the close of the CP does not change the M decay rate but ceases the proton transport.

The proton transport activity of *p*HtrII-free *p*pR is weak. Bamberg, Engelhard, and their colleagues (Schmies et al. 2000) reported the photo-induced electric current through a black membrane with which *p*pR was associated. They observed very small steady current, but addition of azide increased greatly the current. This observation is not understandable in terms of the turnover kinetics because the overall photocycling rate is not changed by azide although M decay is accelerated (Takao et al., 1998). Hence, they proposed a two-photon process theory that hypothesizes the necessity of the O-intermediate irradiation (Schmies et al., 2000). On the other hand, if we consider that azide decreases the energy barrier for the proton permeation through hydrophobic CP, the increase in the net photo-induced electric current may be easily understood. In this regard, it is worthwhile to note that a double mutant of F86D/L40T (the location of which is in CP) showed rapid M decay (Iwamoto et al., 1999b) and that investigating whether association of this mutant with *p*HtrII changes the M decay rate will be an interesting additional investigation.

Note added in proof

After submission of the present manuscript, Bamberg et al. expressed *p*pR (*p*sRII) or *p*pR + *p*HtrII in oocyte membrane and found the cessation of a photo-induced electric current by the association of *p*pR with *p*HtrII (G. Schmies, M. Engelhard, P. G. Wood, G. Nagel, and E. Bamberg, Ninth International Conference on Retinal Proteins, Sept. 14–19, 2000, Szeged, Hungary).

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