EFFECT OF SALT ON PHOTOCYCLE AND ION-PUMPING OF HALORHODOPSIN AND THIRD RHODOPSINLIKE PIGMENT OF *HALOBACTERIUM HALOBIUM*

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ABSTRACT The cytoplasmic membranes of *Halobacterium halobium* contain at least three retinal pigments: bacteriorhodopsin (bR), halorhodopsin (hR), and a third rhodopsinlike pigment (tR). The amplitudes of the phototransient in the photolysis of hR and tR were measured in various salt solutions. Halogen ion (except fluoride) was required to retain the photocycle of hR. Parallels between the amplitude of the phototransient of hR and the magnitude of the photo-induced tetraphenylphosphonium (TPP^+) uptake suggests that hR is a light-driven halogen pump, which supports the hypothesis by Schobert and Lanyi (*J. Biol. Chem.*, 1982, 257:10306–10313). The order of effectiveness of halogen was $Br^- > Cl^- > I^-$. On the other hand, no specific ion was required to retain the photocycle of tR, and tR was concluded to be nonelectrogenic.

INTRODUCTION

The cytoplasmic membranes of *Halobacterium halobium* contain bacteriorhodopsin (bR) which is a light-driven proton pump (1). Matsuno-Yagi and Mukohata (2, 3) isolated an apparently bR-free (bR^-) mutant that exhibited light-induced alkalinization in the medium in contrast to the bR-containing strain. Lindley, MacDonald, and coworkers (4, 5) and Greene and Lanyi (6) suggested the existence of a second light-driven electrogenic pump, which was considered to be a Na^+ pump in earlier studies (4–6). This second pigment was named as halorhodopsin (hR) (7). The presence of hR is exhibited by the uncoupler-enhanced light-dependent H^+ uptake (4–6).

The photocycle of hR was reported by Weber and Bogomolni (8). We examined carefully the photochemical reaction of bR^- mutant by means of flash-photometry, and observed two photocycles (9). Our detailed experiments under various conditions have shown that these two photocycles are attributable to two separate pigments. In other words, the existence of a new pigment in addition of bR and hR in *H. halobium* was suggested, and this pigment was named as the third rhodopsinlike pigment (10).

Spudich and Spudich (11), and Bogomolni and Spudich (12) reported the existence of a new pigment in *H. halobium*. They isolated a mutant that showed no change in membrane potential or in pH upon illumination but possessed a photocycle and photo-taxis (11). The absorption maximum of this pigment was located near 590 nm. The phototransient of the pigment in this mutant was reported to have an absorption maximum at 370 nm with a half-time of decay of ~0.8 s, which is similar to that of the third rhodopsinlike pigment reported by us (9, 10). Thus, it is established that the cytoplasmic membrane of *H. halobium* contains at least three pigments, bR, hR, and the third pigment, which is abbreviated as tR by us (10) and as sR (slow rhodopsin) by Bogomolni and Spudich (12).

The halogen ion-dependent nature of the pigment in bR^- mutant has been reported by Ogurusu et al. (13). They reported that the membrane fragments from their bR^- mutant showed the absorption maximum at 570 nm in the absence of halogen ion and a red-shift of the absorbance by ~10–20 nm when halogen ion was added, although they did not pay any attention to the existence of tR. Schobert and Lanyi (14) reexamined the photo-induced ion transport of the envelope vesicles derived from the strain strictly lacking bR, and found no detectable sodium transport contrary to the previous works (4–6). They demonstrated the primary inward transport of chloride against both electrical and concentration gradients, and have presented the hypothesis that hR is a light-driven chloride pump. Their work, however, did not make reference to the photocycle of the pigment involved in the chloride transport.

In the present paper, both the amplitude of the phototransient of pigments in bR^- mutant and the magnitude of the photo-induced membrane potential are measured in various salt solutions. The amplitude of the phototransient

of hR and the magnitude of the photo-induced membrane potential depend on the halogen (except fluoride) concentration and on the species of halogen ion used. This finding supports the idea that hR is a light-driven halogen pump. On the other hand, the photocycle of tR was not influenced significantly by the ion used, so tR is not electrogenic.

MATERIALS AND METHODS

The "colorless" strain of *H. halobium*, KH-10 (bR^-, hR^+, tR^+, carotenoid$^-$), which was derived from KY-4 (bR^-, hR^+, tR^+, carotenoid$^+$, see reference 15) was used. This strain did not show acidification upon illumination under any condition, and showed light-dependent and uncoupler-enhanced proton uptake, which is characteristic of hR. In addition, the M intermediate of bR was not detected within the accuracy of the present flash-photolysis apparatus. The cells were grown in a peptone medium and envelope vesicles were prepared by sonication method (16). All vesicles (30 to 60 mg protein/ml) were stored in 4 M NaCl at −20°C until use. Protein concentration was assayed by the Lowry method using bovine serum albumin as a standard. The medium was changed to the desired composition (3 M) by osmotic shock method (16). The values of pH in the solution were adjusted to 6.8–7.3 with the corresponding acid and NaOH (or KOH). Because of the solubility, the concentration of Na_2SO_4 and NaF used were 1.5 and 0.9 M, respectively. The chemicals were of analytical grade and used without further purification.

A setup and method of flash spectroscopy were described elsewhere (17). As shown previously (9, 10), the absorbance change at 590 nm caused by flash is biphasic, where the absorption maximum of the pigments (hR and tR) is located. The fast component was assigned to originate from hR (half-time of the change, $T_{1/2}$ = ~10 ms) and the slow, tR($T_{1/2}$ = ~0.8 s). Therefore, analysis of the flash-photolysis data obtained at 590 nm gives the information of both pigments. The depletion in absorbance at 590 nm with time, $\Delta A(t)$, was analyzed by the following equation:

$$\Delta A(t) = -\Delta A_{hR} \exp(-k_{hR}\, t) - \Delta A_{tR} \exp(-k_{tR}\, t)$$

where k_{hR} and k_{tR} stand for the time constants of hR and tR, respectively. In this paper, ΔA_{hR} and ΔA_{hR} denote the "amplitude" of the phototransient of hR and tR, respectively. Note that positive values of the amplitude mean the depletion of absorbance (depletion of the original pigments).

The TPP^+ uptake, indicative of the generation of the interior negative membrane potential, was measured with a TPP^+ electrode (18). Details were described previously (19). The concentration of TPP^+ used was 10 μM. Illumination was provided by a 1-kW projector lamp through 1% $CuSO_4$ water layer (3 cm in length) and a yellow cutoff filter (>520 nm, Toshiba Y52, Tokyo). The temperature of the sample was kept constant by circulating thermostated water around the cuvette. The temperature was 20°C throughout the experiment.

RESULTS AND DISCUSSION

Table I shows the relative amplitude of the phototransients of hR and tR in various salt solutions. Here, the amplitude obtained in 3 M NaCl was chosen as a reference. The first several experiments were performed in various sodium salt solution (3 M). The results show that the presence of Cl^-, Br^- or I^- is required to retain the photocyle of hR, although the amplitude in the presence of I^- is smaller than the others. Due to the low solubility of NaF, the photocycle was examined in 0.9 M NaF and we did not find the photochemical cycle of hR. Next, we examined the effect of cation species. Immediately after the solution of the stock vesicle (4 M NaCl) was exchanged with the desired solution, the relative amplitudes obtained were as follows: 68 for NH_4Cl, 55 for CsCl, 70 for $MgCl_2$, and 67 for $CaCl_2$. For 2 or 3 h, the amplitude changed gradually and reached a constant value. The constant values obtained are listed in the Table.

TABLE I
RELATIVE AMPLITUDE OF PHOTOTRANSIENT OF hR AND tR, AND PHOTO-INDUCED MEMBRANE POTENTIAL IN VARIOUS SALT SOLUTIONS

Salt*	hR	tR	Membrane potential‡
NaCl	100§	100§	+
NaBr	117	114	+
NaI	77	66¶	+
NaF	trace	91‖	−
NaAcetate	trace	119	−
NaH_2PO_3	trace	103	−
Na_2SO_4	trace	103	−
$NaNO_3$	trace	116	−
NaSCN	trace	66¶	−
$NaClO_4$	trace	76¶	−
KH_2PO_4	trace	105	−
LiCl	103	91	+
KCl	114	108	+
NH_4Cl	80	79	+
CsCl	68	108	+
$MgCl_2$	116	71	+
$CaCl_2$	92	70	+

*The salt concentration was 3 M (pH 7.0) except Na_2SO_4 (1.5 M) and NaF (0.9 M).
‡+, photo-induced membrane potential was observed when each salt was added in 3 M NaH_2PO_3 or sodium acetate (basal salt solution). −, photo-induced membrane potential was not observed when each salt was added in the basal solution. The actinic illumination was provided through a yellow cut-off filter (>520 nm).
§The absorbance change at 590 nm was observed by flash-photolysis and the amplitude obtained in 3 M NaCl was taken as 100.
‖The amplitude obtained in 0.9 M NaCl was taken as 100.
¶Signals were unstable.

From these results, we can infer that halogen ion except fluoride is required to retain the photocyle of hR. It is noted that the order of effectiveness of halogen ions on the amplitude of the phototransient was $Br^- > Cl^- > I^-$. In the presence of divalent cations, k_{hR} decreased to about half or less. The detailed analysis will be described elsewhere.

On the other hand, the phototransient of tR was observed in all the solutions used, although the amplitude was relatively small for NH_4Cl, $MgCl_2$, and $CaCl_2$. The amplitude did not decrease with time except for I^-, SCN^-, and ClO_4^-. In these typical chaotropic ions, the tR signals were not stable. The time course of the decrease in the amplitude followed approximately a single exponential equation and the half-times of decay were 47, 33, and 119 min for I^-, SCN^-, and ClO_4^-, respectively.

We further examined the effect of Cl^-, Br^-, and I^- on the photocycles of hR and tR. Envelope vesicles were suspended in 3 M NaH_2PO_3, because the vesicles in this

solution did not show any phototransient originating from hR as shown in Table I. Varying concentrations of NaX (X = Cl^-, Br^-, and I^-) were added to the vesicle suspension in 3 M NaH_2PO_3 and the amplitude of the phototransients was measured. Fig. 1 *A* shows the results for hR and Fig. 1 *B*, the results for tR. As the concentration of these halogen ions increased, the amplitude of the phototransient of hR increased until ~0.3 M, and then reached a constant value. The maximum value of the amplitude depended on the halogen ion used (also see Table I). The half-time of decay of the phototransient was not changed by the concentration of X^-. On the other hand, Fig. 1 *B* shows that the amplitude of phototransient of tR was independent of the concentration of Cl^-, Br^-, and I^-. The signal was stable over the concentration range used in Fig. 1 *B*, due maybe to the low concentration of the chaotropic ion. Similar results were obtained when 3 M CH_3COONa was used as a basal medium, but the chloride-dependent, photo-induced TPP^+ uptake was smaller in CH_3COONa than in NaH_2PO_3 medium (see below). Therefore, we used 3 M NaH_2PO_3 as the basal medium in the following experiments.

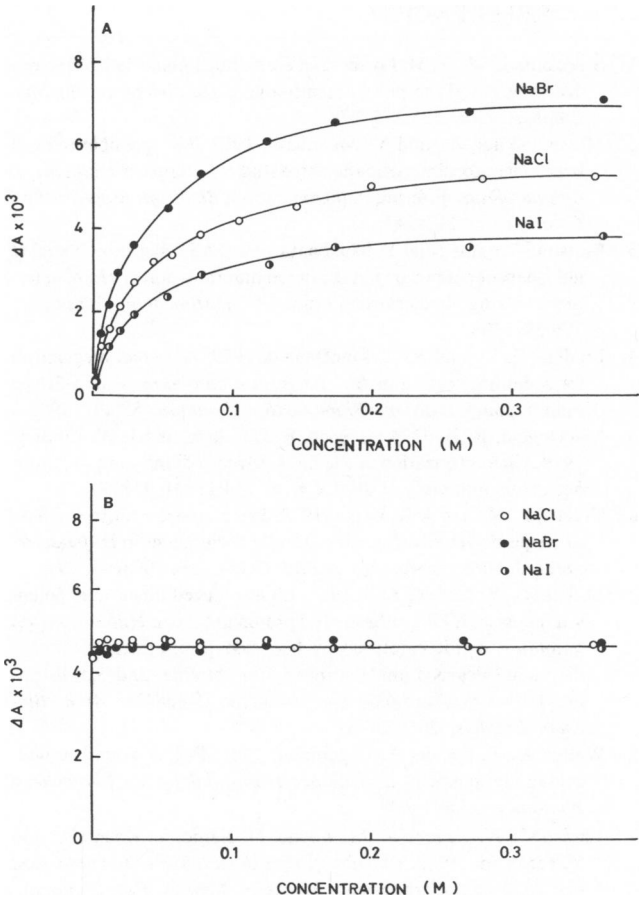

FIGURE 1 Relationship between the amplitude of absorbance change of the phototransients (hR in *A* and tR in *B*) and the concentration of halogen. The measuring wavelength was 590 nm and the definition of the amplitude; see Materials and Methods for further comments on *A*. ●, NaBr; ○, NaCl; ◑, NaI. Note that the increase in ΔA is the same as the increase in the depletion of the absorbance at 590 nm (depletion of original pigment) by flash light (>620 nm). The basal solution was 3 M NaH_2PO_3 buffered with H_3PO_3 at pH 7.0. Protein concentration was 2.5 mg/ml.

In Fig. 2, the reciprocal of the amplitude of the phototransient of hR is plotted against the reciprocal of the halogen ion concentration and linear relationship is obtained. From this figure, the maximum amplitude of absorbance change was 4.26×10^{-3} for NaI, 5.57×10^{-3} for NaCl, and 7.11×10^{-3} for NaBr. The concentration giving the half of the maximum was 35 mM for NaI, 34 mM for NaCl, and 32 mM for NaBr. Schobert and Lanyi (14) described that the photo-induced membrane potential across the envelope vesicles of bR^- mutant depended on the Cl^- concentration and that the half maximum concentration was 30–40 mM, which is consistent with the present result. They stated, however, that I^- was ineffective in producing the photo-induced membrane potential because they failed to observe it in the presence of I^-. Fig. 3 shows the photo-induced TPP^+ uptake, indicative of the generation of interior negative membrane potential under the same condition as shown in Fig. 1. We observed a small but steady photo-induced TPP^+ uptake in the presence of NaI. The possible reason why the generation of membrane potential is so small in the presence of I^- is that the membrane becomes leaky. This notion is supported by the results obtained in the presence of NaBr. Below 0.04 M, the photo-induced TPP^+ uptake observed in the presence of Br^- was larger than that in the presence of Cl^-. This is consistent with the result of flash photolysis, which reveals that the amplitude of phototransient in Br^- is larger than that of Cl^-. As the concentration of Br^- increased, the TPP^+ uptake became small although the amplitude of the phototransient of hR continued to increase. These facts indicate that high concentration of Br^- destroys the integrity of the membrane.

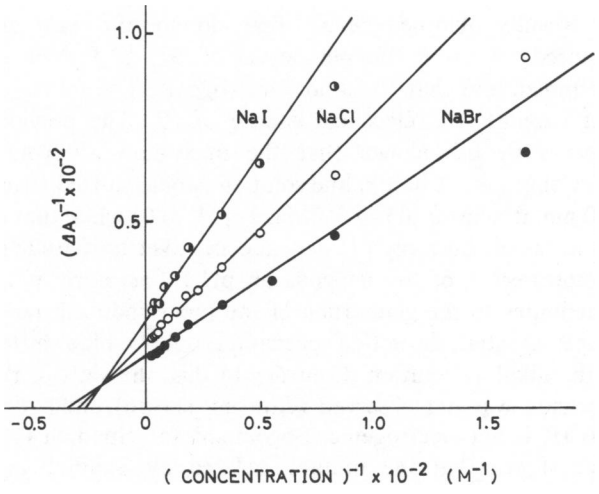

FIGURE 2 Double reciprocal plot of the amount of hR measured by the absorbance change at 590 nm against the concentration of halogen ion. Experimental conditions and notations were the same as in Fig. 1.

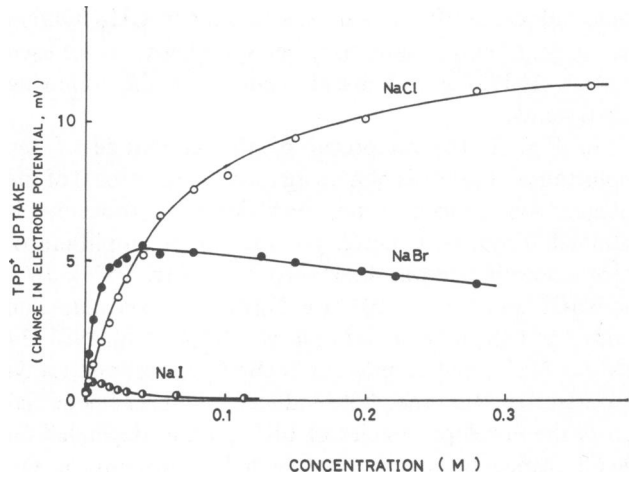

FIGURE 3 Light induced TPP^+-uptake as a function of the concentration of halogen ion in the medium. The ordinate represents the change in TPP^+ electrode (in millivolts). The increase in the change in TPP^+ electrode potential corresponds to the increase of the interior negative membrane potential. Experimental conditions and notations are the same as in Fig. 1. The intensity of actinic light (>520 nm) was 800 W/m^2.

To find out which ion is indispensable for generation of the photo-induced membrane potential, various salts were added to the vesicles in 3 M NaH_2PO_3, and photo-induced TPP^+ uptake was measured. Results obtained have been listed in the right-most column of Table I, where + indicates the successful observation of the photo-induced membrane potential in the presence of the salt concerned and – indicates the failure in observation. We obtained a good parallel between the amplitude of the phototransient of hR and the magnitude of the photo-induced membrane potential. This fact suggests that hR might be a light-driven halogen (except for fluoride) pump. Our data support the hypothesis of Schobert and Lanyi (14) that hR is a light-driven Cl^- pump. In addition, the present paper identifies that the pigment having faster photocycle in the bR^- mutant is halorhodopsin.

Results obtained reveal that no specific ions are required to retain the photocycle of tR, as far as we examined, and that tR is not electrogenic. The following fact supports the electrical silence of tR. The previous paper (10) has shown that the maximum absorption wavelength of tR in alkaline solution is blue shifted (from 580 nm at neutral pH to 550 nm at pH 10.0) while that of hR does not show any pH-dependence. (The amplitude of phototransient of hR depends on pH.) Therefore, if tR contributes to the generation of the photo-induced membrane potential, the action spectrum should be blue shifted in the alkaline solution. Contrary to this, the shift of the spectrum was not observed (data not shown), indicating that tR is not electrogenic. Bogomolni and Spudich (12) have shown that the mutant isolated by Spudich and Spudich (11) has only one pigment which exhibits slow photocycle ($T_{1/2}$ = 0.8 s), and that on illumination neither membrane potential nor pH change was generated. Considering the agreement of the maximum wavelength of the phototransient (9), the decay time (9), and the nonelectrogenic character, the pigment in their mutant might be the same as tR reported previously by us (10).

The results obtained show that the halogen ion (except fluoride) is required to retain the photocyle of hR. The explanation for this observation is as follows: There are two forms of halorhodopsin and the following equilibrium is held: $hR_o + X^- \rightleftharpoons hR\text{-}X$, where X^-, hR_o, and hR-X represent halogen ion, the halogen-free, and halogen-bound form, respectively. The hR-X form shows the photocycle having the phototransient absorbing at 490 nm. On the other hand, the hR_o form does not show any phototransient at the wavelengths studied (400–700 nm) with the time resolution of 1 ms. However, it is open for further study whether hR_o has its own photocycle or not.

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