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**A light-driven proton pump from *Haloterrigena turkmenica*:  
functional expression in *Escherichia coli* membrane and coupling  
with a H<sup>+</sup> co-transporter**

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**Abbreviations:** DDM, n-dodecyl β-D-maltoside; dR,  
deltarhodopsin from *Haloterrigena* sp. arg-4; HtdR,

deltarhodopsin from *Haloterrigena turkmenica*; bR,  
bacteriorhodopsin from *Halobacterium salinarum*; EmrE, small  
molecular weight multidrug efflux transporter of *E. coli*; ppR,  
phoborhodopsin (sensory rhodopsin II) from *Natronomonas  
pharaonis*;

## Abstract

A gene encoding putative retinal protein was cloned from *Haloterrigena turkmenica* (JCM9743). The deduced amino acid sequence was most closely related to that of deltarhodopsin, which functions as a light-driven H<sup>+</sup> pump and was identified in a novel strain *Haloterrigena* sp. arg-4 (J. Mol. Biol. 285:163-174 (1999), GenBank accession no. **AB009620**). Thus, we called the present protein *H. turkmenica* deltarhodopsin (HtdR) in this report. Differing from the *Halobacterium salinarum* bacteriorhodopsin (bR), functional expression of HtdR was achieved in *Escherichia coli* membrane with a high yield of 10 ~ 15 mg protein/liter culture. The photocycle of purified HtdR was similar to that of bR. The photo-induced electrogenic proton pumping activity of HtdR was verified. We co-expressed both HtdR and EmrE, a proton-coupled multi-drug efflux transporter in *E. coli.*, and the cells successfully extruded ethidium, a substrate of EmrE, on illumination.

**Keywords:** archaeal rhodopsin; bacteriorhodopsin; light-driven  
proton pump; EmrE; photocycle; SMR; deltarhodopsin;  
archaerhodopsin; multi-drug resistance transporter

## Introduction

Bacteriorhodopsin (bR) expressed in purple membrane of *Halobacterium salinarum* is an outward light-driven proton pump [1,2]. Upon illumination, bR is excited and relaxed to the original bR via a variety of photo-intermediates. During this linear and cycling photochemical reaction (photocycle), bR transports one proton from the cytoplasm to the extracellular medium. In addition to bR, three other kinds of the retinal proteins (archaeal rhodopsins) are expressed in the same strain *H. salinarum* [3], and they are structurally similar to one another; they have seven transmembrane helices (A-G) with a chromophore (all-*trans* retinal) bound to a lysine residue on helix G via a protonated Schiff base. In addition, photo-excitation undergoes a photocycle for all archaeal rhodopsins. These three distinctly related retinal proteins are called halorhodopsin [4], sensory rhodopsin (also called sensory rhodopsin I) [5,6] and phoborhodopsin (also called sensory rhodopsin II) [7-10], which are a light-driven inward

Cl<sup>-</sup> pump, a sensor for both positive (toward red light) and negative (from blue light) phototaxis, and a sensor for negative (from blue-green light) phototaxis, respectively.

Mukohata and collaborators isolated a bacterium from an Argentine salt flat, which was named *Haloterrigena* sp. arg-4. Of interest, from this strain, Ihara et al. discovered a new retinal protein [11, 12], which conserves completely the amino acid residues essential for light-driven proton pumping, but the homology with bR has as low as 53%. This protein was named deltarhodopsin (abbreviated as dR, GenBank accession no. **AB009620**). In addition, Mukohata and his colleagues [11] discovered another class of light-driven pumps such as archaerhodopsin (aR) and cruxrhodopsin (cR) having low homology with bR and dR.

In this report, we cloned and sequenced a dR homologue from *Haloterrigena turkmenica* (JCM9743) and call this protein *H. turkmenica* dR (abbreviated as HtdR, hereafter). The sequence

showed only two differences (described later) in comparison with dR from *arg-4*.

Several attempts have been made for large-scale functional expression of bR in *Escherichia coli* membrane, but those experiments have remained unsuccessful. In trials by Khorana and collaborators, the expressed bR distributed in the membrane, but an additional treatment was necessary to obtain a proper conformation [13, 14]. The first functional expression of an archaeal rhodopsin in *E. coli* membranes was achieved by Shimono et al. against phoborhodopsin (sensory rhodopsin II) from *Natronomonas pharaonis* (ppR) [15]. Using a similar expression system, Hohenfeld et al. attempted a functional expression of bR, but the expression amount was very low and the bR obtained showed somewhat unusual photocycle [16].

The most attractive point of this paper is the success of a high level functional expression of HtdR in *E. coli* membranes (10 ~ 15 mg/liter culture). The purified HtdR showed essentially same photocycle with bR. Illumination extrudes protons to

generate the interior-negative proton electrochemical potential, which can drive EmrE [17], a proton-coupled exporter of lipophilic toxic cations, such as ethidium.

## **Materials and Methods**

### *Bacterial strains*

*E. coli* JM109 was used as host for DNA manipulation. The high-level expression of HtdR or bR was carried out in BL21 (DE3). The expression and purification procedure of HtdR and bR were essentially the same as those for ppR [18, 19]. For co-expression of HtdR and EmrE, AS1 (provided by Dr. Kawagishi, Nagoya Univ.) was used. AS1 lacks AcrA, a major multi-drug efflux transporter in *E. coli*. The cells were grown in 2 x YT medium supplemented by ampicillin (final concentration of 50 µg/ml). *H. turkmenica* strain JCM9743 was obtained from the Japan Collection of Microorganisms (RIKEN, Japan) and grown in JCM medium 168. The growth of *H. salinarum* strain S9 and the

isolation of purple membrane were made by the standard procedure [20].

#### *Plasmids for high-level expression of HtdR and bR*

To isolate the HtdR gene, PCR was employed on *H. turkmenica* genomic DNA. Two PCR primers were designed referring to a sequence reported by Kamekura et al. [21], in which an *Nde* I site was engineered at the initiation codon in the sense primer, and the termination codon was omitted instead of designing an additional *Xho* I site in the antisense primer. Sense and antisense primers were 5'-catatgtggttacgctgctctag-3' and 5'-ctcgagggtcggggcagccgctcggcg-3', respectively. An amplified 0.8 kbp fragment was cloned on a pGEM-T easy plasmid (Promega), and DNA sequences were determined by a standard procedure (377 DNA sequencer, Applied Biosystems). Among 10 single colonies examined, a sequence of 3 clones was exactly identical to one another (plasmid pGEM-HtdR).

To construct a high-expression vector of HtdR, a 756 bp *Nde* I/*Xho* I fragment from pGEM-HtdR was inserted into suitable sites on pET21c (Novagen). The obtained plasmid was named pET-HtdRHis. By the similar procedure, a plasmid encoding bR was constructed (pET-bRHis). bR gene was obtained from the genomic DNA of *H. salinarum*. These expression plasmids result HtdR and bR having additional amino acids in those C-terminuses (-LEHHHHHH).

#### *Plasmid for co-expression of HtdR and EmrE*

In the construction of a pACYC-based expression plasmid, we first cloned a 464 bp *lacZ* $\alpha$  fragment of pBluescript II SK+(460-934) into the *Bam* HI site on pACYC184 for an easier detection of the insertion of HtdR on the pACYC plasmid. The resulting pACYC184Z $\alpha$  showed a standard  $\alpha$ -complementation in *lacZ* $\Delta$ M15 background, and provided us a convenient selection. In order to change the promoter from *T7* to *lac*, the HtdR gene on pET-HtdRHis was fused to *lac* promoter of pGEM-T plasmid by

a PCR-based ligation using primers common to both. The resulting 1093 bp PCR product was blunted with a Blunting Kit (TaKaRa, Japan), and inserted into the *Sma* I site of the pACYC184Z $\alpha$  plasmid. The nucleotide sequence of the HtdR coding region was verified, and the plasmid was termed pACT2.

The EmrE expression vector pRA337 was easily derived from pGEM-T, having the *emrE* gene amplified by PCR under *lac* promoter. When the plasmid was introduced into *E. coli* strain AS1, the cell growth was heavily suppressed even in the absence of IPTG, so that the *Sph* I/*Psh* AI digested *lacI*<sup>q</sup> fragment from pET32b+ (Novagen) was blunted and inserted into the blunted *Sph* I site of the plasmid.

#### *Co-expression of HtdR and EmrE*

For the construction of a co-expression system of HtdR and EmrE, AS1 cells harboring pACT2 were used as competent cells and pRA337 was introduced by the heat-shock method. The selection markers were ampicillin and chloramphenicol. AS1 cells

harboring both plasmid were grown to an  $OD_{660}$  of 0.3, followed by the addition of both IPTG (final 0.5 mM) and all-*trans* retinal (10  $\mu$ M). After 2-hour incubation, the cells were harvested. An efflux of ethidium from *E. coli* cells was measured using a fluorescence spectrometer model F-4500 (Hitachi), according to Neyfakh [22].

*pH titration, flash-photolysis and light-induced proton transport*

pKa of the counterion of protonated Schiff base was determined from absorption changes at 615 nm. For the pH titration, a six-mixed buffer (citric acid, MES, HEPES, MOPS, CHES and CAPS, each at 10 mM concentration) was used, whose advantage is the equal buffer strength in the wide pH range. Absorption spectra were obtained using a V-560 UV/VIS spectrophotometer (JASCO). The retinal composition of HtdR was measured by HPLC [23]. The apparatus and procedure of flash-photolysis were described elsewhere [24]. Photo-induced proton transport across inverted

*E. coli* membrane vesicles was observed using a transparent SnO<sub>2</sub> electrode [25]. The membrane vesicles were prepared through a French press (1,200 kg/cm<sup>2</sup>, Ohtake).

### **Results and Discussion**

The differences in the amino acid sequences of a putative proton pump between that from the sp. arg-4 [11, 26] and the present result from JCM9743 were found at only two positions: at the 3<sup>rd</sup> position, Cys (arg-4) changed to Tyr (JCM9743), and at 166<sup>th</sup> position, Glu (arg-4) to Lys (JCM9743). Here, the number starts from the first methionine residue. Kamekura et al. [21] reported that a genome in an extreme halophile strain, HT (later deposited to JCM, the strain used in this study), encoded a bR-like protein, but this protein is not expressed in HT cells. The differences in the amino acid sequence between Kamekura's and ours are: Phe changed to Ser at the 18<sup>th</sup> position, Val to Tyr at the 34<sup>th</sup>, Asn to Asp at the 81<sup>st</sup>, Pro to Arg at the 90<sup>th</sup> (corresponding to the 82<sup>nd</sup> of bR), Phe to Ile at the 143<sup>rd</sup>, and

Asp to Gly at the 229<sup>th</sup>, where the preceding amino acid is that obtained by Kamekura et al. Our results from *H. turkmenica* JCM9743 revealed the presence of the arginine residue corresponding to R82<sup>bR</sup> (Arg at 82<sup>nd</sup> position of bR). It is noted, however, that our present result does not completely rule out the presence of the gene reported by Kamekura et al. [21]. The possibility still remains that there exists the other gene reported by them. This Arg-residue is a super conserved residue among microbial rhodopsins and is considered to be one of important residues for the pumping. Therefore, an interesting question arises as to the role of the arginine residue, since the protein they expressed in *H. salinarum* strain L33 cells displayed the typical photocycle characteristic of bR. Therefore, mutation of the arginine residue at the 90<sup>th</sup> position to proline may be an interesting next experiment.

Shimono et al. [15] succeeded in expressing ppR functionally in the *E. coli* cell membrane using an expression vector derived from pET21c (Novagen). In the present work, we

constructed similar vectors for HtdR (pET-HtdRHis) and bR (pET-bRHis) to express them in *E. coli* membrane. The inset of Fig. 1 shows pellets of *E. coli* BL21(DE3) cells harboring pET21mock, pET-bRHis and pET-HtdRHis from left to right, respectively. It is noted that the pellet derived from pET-HtdRHis has reddish color and that others did not, suggesting a high-level expression of HtdR. After solubilization of the membrane fraction with 1.0% n-dodecyl  $\beta$ -D-maltoside (DDM) and 1-hr incubation, bR became colored and showed photo-sensitivity. These suggest that the folding of bR was not valid in the membrane, and during the incubation the proper folding occurs *in vitro*. On the other hand, HtdR may undergo valid folding in *E. coli* membranes, and as shown later HtdR in the *E. coli* membrane can transport protons on illumination. The origin of this difference in folding between bR and HtdR remains to be elucidated in a future study.

Using Ni-chelate chromatography, HtdR was isolated, as was the same as that for ppR [18, 19], and the yield was as much

as 10 ~ 15 mg protein/liter culture. The absorption maximum ( $\lambda_{\max}$ ) of HtdR was 547 nm (see the spectrum shown in Fig. 1), which is blue-shifted from that of bR embedded in purple membranes (~570 nm). However, this wavelength does not necessarily mean that in *E. coli* membrane or that in the native host, since the solubilization might change the maximum wavelength. The blue-shift of bR spectrum upon solubilization of purple membrane is well-known. In fact, the  $\lambda_{\max}$  of purified bR from *E. coli* by the same procedure for HtdR was 553 nm. Another possible reason of the change in the  $\lambda_{\max}$  of *salinarum* bR from that in purple membrane might be the strongly bound lipids. The retinal composition of HtdR was examined: the ratio of all-*trans* to 13-*cis* was 5 : 1 in a dark-adapted state and 93 : 1 (6% of unidentified) in a light-adapted state. In acidic media, the absorption maximum shifted to 615 nm, and the pKa of Asp-93 (the counterion of protonated Schiff base corresponding to Asp-85<sup>bR</sup>) was determined to be 2.2 from this spectrum shift (Fig. 2). The

bi-polar CD (circular dichroism) spectra were not found only with a positive band.

A laser flash photolysis measurements were performed for the purified HtdR, bR from *E. coli* and purple membrane from *H. salinarum*. The purified HtdR showed essentially same photocycle with the purple membrane. In Fig.3, panels of the upper row show the transient absorption changes at three selected wavelengths that are representative of the formation and decay of characteristic intermediates (upper two traces), and depletion and recovery of initial ground states (lower traces). The panels of the middle and the lower rows show the difference spectra of the first (middle) and second (lower) halves of the respective photocycle. In all three samples, the decay of K (above 620 nm) and L (around 450 - 620 nm), and formation of M intermediates (below 450 nm) are observed in the first halves of the photocycle, and then the decay of M and formation of O (above 620 nm) intermediates and recovery to the respective ground state are observed in the second halves. As for

K-intermediates, in this time domain (5  $\mu$ s ~), only the last parts of the decay were observable. The accumulation of O, characteristic intermediate in the end of photocycle, may become evident in the acidic medium.

All characteristic intermediates in the purple membrane photocycle were fully observable in both purified HtdR and bR. However, the kinetics of the bR has distinct difference; M intermediate shows accelerated formation and prolonged decay (see 400 nm trace of the upper panel in Fig.3). This difference in the kinetics is similar closely to a previous report on the effects of solubilization of purple membrane [27]. Thus, the purified bR appears to take the similar feature with solubilized bR from purple membrane. The kinetic difference should be originated from a perturbed conformation of bR in the solubilized state. On the other hand, HtdR also has a somewhat accelerated and prolonged decay of M intermediate, but its photocycle kinetics is much close to that of purple membrane.

Even in the solubilized state, HtdR may maintain the conformation similar to that under the membrane environment.

HtdR fully conserves amino acid residues essential for the proton-pumping activity of bR, which correspond to R90 (R82<sup>bR</sup>), D93 (D85<sup>bR</sup>), D104 (D96<sup>bR</sup>), E202 (E194<sup>bR</sup>) and E213 (E204<sup>bR</sup>). To verify the photo-induced electrogenic proton-pumping activity, three independent experiments were performed. First, we measured the proton uptake and release from HtdR using SnO<sub>2</sub> transparent electrode [25]. On illumination, first proton release from the DDM-solubilized HtdR occurred, followed by the proton uptake during the photocycle (data not shown). This result is the same as that in the case of bR. Secondly, we observed the photo-induced uptake of tetraphenylphosphonium cation (TPP<sup>+</sup>) by starved *E. coli* cells (data not shown), implying the generation of inside-negative membrane potential [28]. Thirdly, as shown in Fig. 4, we observed the photo-induced proton uptake by inverted *E. coli* membrane vesicles using SnO<sub>2</sub> electrodes [25]. The negative change of the signal corresponds

to an increase in pH of the vesicle suspension (i.e., the proton uptake into the vesicles). Unfortunately, the present electrochemical cell of SnO<sub>2</sub> cannot calibrate the extent of proton-movement, but one must note that no change was observed for the membrane vesicles derived from cells not-expressing HtdR.

Since HtdR has the activity of the photo-induced electrogenic outward proton-pump in *E. coli* cells, the coupling of a transporter with HtdR was attempted. Illumination can drive secondary transporters that utilize the proton gradient across the membrane. EmrE belongs to the SMR family and expels structurally non-related toxic compounds from *E. coli* cells in exchange for protons [17]. We co-expressed HtdR and EmrE in *E. coli* membrane (strain AS1 as a host cell), anticipating that illumination would extrude ethidium, one of substrates of EmrE. The ethidium remaining in the cells was measured by its fluorescence. As seen in Fig. 5, the illumination-activated efflux of ethidium was observed only in the case of the

co-expression of HtdR and EmrE. Dissipation of the proton electrochemical potential difference by addition of uncoupler, CCCP, prevented the ethidium efflux. Inset in the figure shows the comparison of the extrusion activity with the HtdR spectrum, meaning that the driving force for the efflux originates from the activation of HtdR. The activity was defined as an initial slope of the obtained curves in the plot of ethidium remaining in the cell against time.

These confirm the proton-pumping activity of HtdR, and it is worth noting that this is accomplished because HtdR can be expressed in a functional form in *E. coli* membranes. Why does HtdR have a functional form in *E. coli* membranes while bR does not? This is an interesting question to be studied in future. Since light is easy to switch on or off, and almost all molecules may be activated at once, the present system may be useful tool for the investigation of the transport mechanism of EmrE or other transporters if their gene is incorporated into the pUC-derived plasmid; for example, it is easy to change the

strength of the driving force by changing the light intensity with no trace of chemicals. Another possible use of this co-expression may be an investigation of the efflux mechanism through the transporter aiming to clarify the uptake mechanism; the removal of illumination immediately may start efflux from the compartment where the substrate has accumulated due to light. In fact, investigation of the efflux mechanism gave us an important clue as to the uptake mechanism through PEPT1 ( $H^+$ /oligo-peptide co-transporter) [29]. In addition, this system may become a useful tool for studies on membrane-potential-dependent phenomena.

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## Figure Legends

**Fig. 1.** Absorption spectrum of purified HtdR from *E. coli* membranes. The medium contained 400 mM NaCl and 0.1% DDM adjusted at pH 7.0 using 10 mM 6-mixed buffer (see Materials and Methods), and temperature was 25 °C. The inset shows the pellets from *E. coli* (BL21(DE3)) cells harboring only vacant plasmid pET21c (left), the cells expressing bR (middle) and HtdR (right). The color of the pellets on the right is different from the others, suggesting the large-scale functional expression of HtdR.

**Fig. 2.** Spectroscopic pH titration of HtdR to determine pKa of Asp-93, the counterion of the protonated Schiff base. By protonation of the counterion, the absorption maximum shifts from 547 to 615 nm. This spectrum shifts were shown as the inset. The pKa value, 2.2, was determined by a nonlinear fitting for the absorption change at 615 nm. The experimental condition is the same as that in Fig. 1.

Fig. 3. Flash induced transient absorption changes at typical wavelengths (upper row) and the time-resolved difference spectra (middle and lower rows) of purified HtdR (left), bR (middle) and purple membrane (right). For the difference spectra, the data were acquired every 10 nm, and the spectra picked up in a logarithmic time scale are shown. Buffer solutions were 10 mM six-mixed buffer at pH 7.0 (see Materials and Methods) containing 400 mM NaCl. For HtdR and bR, the solutions were supplemented with 0.1% DDM. All measurements were at 25 °C.

**Fig. 4.** Photo-induced proton movement of inverted *E. coli* vesicles expressing (a) and not-expressing HtdR (b). The downward deflection means the alkalization of medium, indicating the proton-uptake (details see [25]). Note that the vesicle is inside-out, and then this result means the photo-induced proton transfer from cytoplasm to extracellular space, as is in bR.

**Fig. 5.** Photo-induced ethidium extrusion from *E. coli* cells in which HtdR and EmrE are co-expressed. The lines from the top represent the data obtained under the following conditions: top, HtdR +  $\Delta$ EmrE; second,  $\Delta$ HtdR +  $\Delta$ EmrE (" $\Delta$ " means non-expression of the protein); third,  $\Delta$ HtdR + EmrE; forth, the addition of 1  $\mu$ M CCCP to the complete system (the bottom curve) showing the abolishment of the extrusion; bottom, the complete system of HtdR + EmrE. The difference from the top to third may be negligible, indicating that both of HtdR and EmrE are indispensable for the photo-induced ethidium extrusion. The inset indicates the approximate coincidence of the ethidium efflux rate with the HtdR absorption spectrum when the actinic light is provided through an interference filter. Note that this is achievable because the light-driven proton pump, HtdR, is functionally expressed in *E. coli* membrane.