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REVIEW

An Inward Proton Transport Using Anabaena Sensory Rhodopsin

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ATP is synthesized by an enzyme that utilizes proton motive force and thus nature creates various proton pumps. The best understood proton pump is bacteriorhodopsin (BR), an outward-directed light-driven proton pump in *Halobacterium salinarum*. Many archaeal and eubacterial rhodopsins are now known to show similar proton transport activity. Proton pumps must have a specific mechanism to exclude transport in the reverse direction to maintain a proton gradient, and in the case of BR, a highly hydrophobic cytoplasmic domain may constitute such machinery. Although an inward proton pump has neither been created naturally nor artificially, we recently reported that an inward-directed proton transport can be engineered from a bacterial rhodopsin by a single amino acid replacement. *Anabaena* sensory rhodopsin (ASR) is a photochromic sensor in freshwater cyanobacteria, possessing little proton transport activity. When we replace Asp217 at the cytoplasmic domain (distance ~15 Å from the retinal chromophore) to Glu, ASR is converted into an inward proton transport, driven by absorption of a single photon. FTIR spectra clearly show an increased proton affinity for Glu217, which presumably controls the unusual directionality opposite to normal proton pumps.

***Keywords*:** microbial rhodopsin, photochromism, proton pump, retinal, FTIR

ATP is synthesized by an enzyme that utilizes proton motive force and thus nature creates various proton pumps. The best understood proton pump is bacteriorhodopsin (BR), an outward-directed light-driven proton pump in *Halobacterium salinarum* (Haupts *et al*., 1999; Lanyi, 2000; Kandori, 2006). Many archaeal and eubacterial rhodopsins are now known to show similar outward proton pump activity, whereas an inward proton pump has been never been created. Proton pumps must have a specific mechanism to exclude transport in the reverse direction to maintain a proton gradient, and in the case of BR, a highly hydrophobic cytoplasmic domain may constitute such machinery. In BR, a retinal chromophore is located at the center of the membrane, and the hydrophobicity is different between the cytoplasmic and extracellular domains (Luecke *et al.*, 1999). The cytoplasmic domain is highly hydrophobic, whereas the extracellular domain is composed of charged and polar amino acids that form a hydrogen-bonding network. Figure 1A shows the presence of 7-8 water molecules in the extracellular domain, but only 2 water molecules in the cytoplasmic domain. Such an asymmetric hydrogen-bonded network could be the reason of unidirectional proton transport in BR, where the proton transfer to the extracellular side occurs in 10^{-5} sec, followed by reprotonation through a transiently formed proton pathway in the cytoplasmic domain on a slower timescale (10^{-4}-10^{-3} sec) (Lanyi, 2000; Kandori, 2006). We previously converted BR into an inward chloride-ion pump (Sasaki *et al.*, 1995), but an inward proton pump has never been created. It may be difficult to design an inward proton pump from "normal" outward proton pumps.

Anabaena sensory rhodopsin (ASR) is an archaeal-type rhodopsin found in *Anabaena* (*Nostoc*) sp. PCC7120, a freshwater cyanobacterium. ASR does not show proton pump activity, and as it forms a single operon with a soluble protein of 14 kDa (Jung *et al*., 2003), it has been suggested that ASR is a photochromic sensor activating the 14 kDa transducer protein at the cytoplasmic surface (Vogeley *et al.*, 2004). The X-ray crystallographic structure of ASR has a similar α-helical arrangement to that of BR (Vogeley *et al.*, 2004; Sineshchekov *et al.*, 2005), but a very different hydrogen-bonded network. Figure 1B shows that in ASR both extracellular and cytoplasmic domains contain 5 water molecules, and form hydrogen-bonded networks. Consistent with the hydrogen-bonded network in the cytoplasmic domain, an unusual proton transfer has been found in this protein. Shi *et al*. (2006) reported reverse proton transfer, from the Schiff base to Asp217 in the cytoplasmic domain, whereas Sineshchekov *et al*. (2006) reported that the direction of this proton transfer is dependent on

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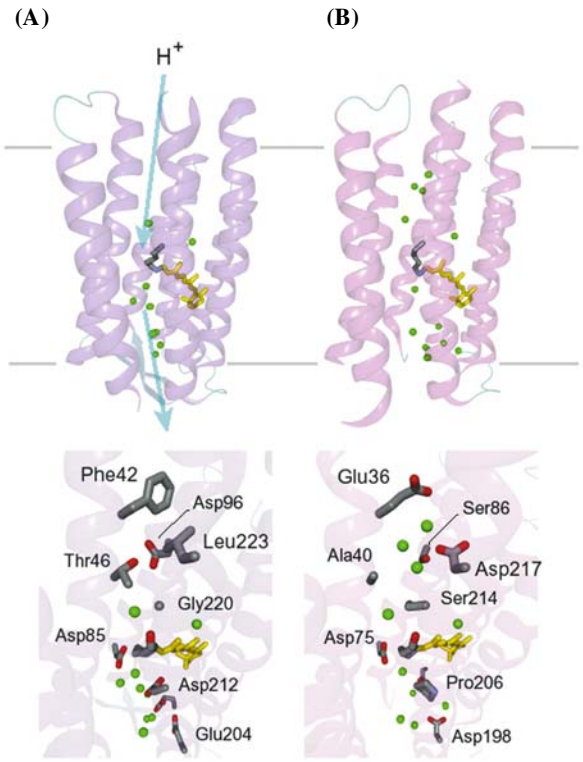

Fig. 1. X-ray crystallographic structures of BR (A) and ASR (B). Top and bottom panels represent views from the membrane plane and the cytoplasmic side, respectively. In the top panel, top and bottom regions correspond to the cytoplasmic and extracellular sides, respectively. The retinal chromophore is colored yellow, and green spheres represent internal water molecules. BR (A) is a light-driven outward proton pump, where Asp85 accepts a proton from the Schiff base and Asp96 donates a proton to the Schiff base. No proton pump activity has been reported for ASR. ASR (B) is a sensor protein that activates a soluble protein at the cytoplasmic surface, and it was reported that the Schiff base proton is transferred to Asp217 in the cytoplasmic region. Polar amino acids as well as five water molecules in the cytoplasmic region, characteristic for ASR, which must be advantageous for primary proton transfer that may be important for activation of the soluble transducer protein. This is an experimental basis of designing an inward proton transport in the present study. This figure is reproduced from Kawanabe *et al.* (2009).

C-terminus truncation. Therefore, proton conduction in ASR remains still unclear, although proton conductivity toward the cytoplasmic domain appears possible. This suggests the potential to design an inward proton transport out of ASR, and indeed we have achieved this. A single amino acid replacement of Asp217 to Glu confers inward proton transport activity to ASR. In the present short review, we first summarize our spectroscopic studies of ASR, followed by the creation of inward proton transport.

Photochemical properties of ASR

As a possible photochromic sensor, structural features of the retinal chromophore in ASR are important (Kawanabe and Kandori, 2009). Microbial rhodopsins accommodate all-trans, 15-anti (AT) and 13-cis, 15-syn (13C) forms of the retinal Schiff base in the dark (Kandori, 2006). For example, BR possesses more 13C than AT in the dark, but the latter only shows the proton-pump activity. In archaeal sensor proteins, SRI and SRII, AT form is only responsible for activation of each transducer protein. These results implicated the functionally important state to be only AT form in microbial rhodopsins. What is about the case in ASR?

It is known that light causes photoisomerization of a single double bond at position C13=C14, indicating that the primary K intermediates of AT (AT-ASR_K) and 13C (13C-ASR_K) forms contain 13-cis, 15-anti, and 13-trans, 15-syn chromophore, respectively. We first applied low-temperature FTIR spectroscopy to AT-ASR, and compared the difference spectra at 77 K with those of BR (Furutani *et al.*, 2005a). The AT-ASR_K minus AT-ASR difference spectra show that the retinal isomerizes from the all-*trans* to the distorted 13-*cis* form like BR. The N-D stretching of the Schiff base was observed at 2,163(-) and 2,125(-) cm^{-1}, while the O-D stretchings of water molecules were observed in the >2,500 cm^{-1} region. These results indicate that the protonated Schiff base forms a strong hydrogen bond with a water molecule, which is connected to Asp75 with a weak hydrogen bond. This result with ASR supports the working hypothesis by our group about the strong correlation between the proton pump activity and the existence of strongly hydrogen bonded water molecules in microbial rhodopsins (Furutani *et al.*, 2005b; Shibata *et al*., 2005, 2007; Kandori, 2006, 2010).

We extended the low-temperature spectroscopic study at 77 K to 13C-ASR (Kawanabe *et al.*, 2006). HPLC analysis revealed that light-adapted ASR with light >560 nm at 4°C possesses 78% 13C-ASR, while dark-adapted ASR has AT-ASR predominantly (97%). Then, we established the illumination

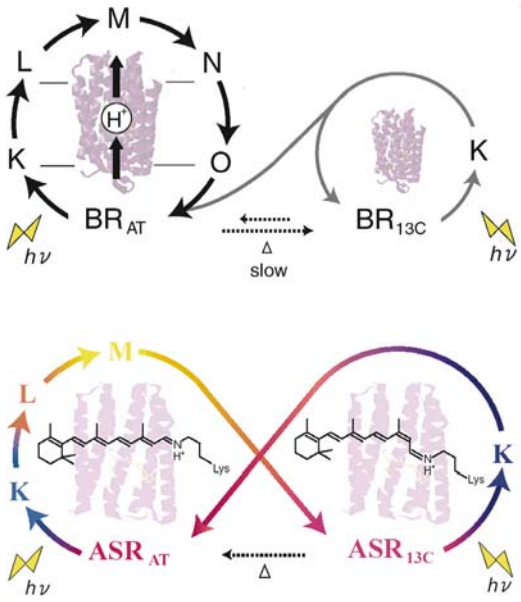

Fig. 2. The photoreaction of ASR. Both isomers (AT-ASR and 13C-ASR) convert 100% yield to another isomer, respectively. This figure is reproduced from TOC of Kawanabe *et al.* (2007).

conditions to measure the difference spectra between 13C-ASR and its K state without subtracting the difference between AT-ASR and its K state. Spectral comparison between 13C-ASR and AT-ASR provided useful information on structure and structural changes upon retinal photoisomerization in ASR. In particular, previous X-ray crystallographic study of ASR reported the same protein structure for 13C-ASR and AT-ASR, whereas the FTIR study revealed that protein structural changes upon retinal photoisomerization were significantly different between 13C-ASR and AT-ASR. The differences were seen for HOOP modes of the retinal chromophore, amide I, cysteine S-H stretch, the Schiff base N-D stretch, and water O-D stretch modes. These must trigger different global protein structural changes in each photoreaction leading to the observed photochromic behavior.

ASR has been believed to function as a photoreceptor for chromatic adaptation. In this case, branching reactions, from AT-ASR to 13C-ASR and from 13C-ASR to AT-ASR (Fig, 2), are favorable for ASR, but they are in striking contrast to what is known for microbial rhodopsins. Ideally, the conversion ratios should be unity for photochromic reactions, but this is exactly the opposite of the properties of pump rhodopsins, such as BR. X-ray crystal structures reported similar chromophore structures and protein environments for ASR_{AT} (Vogeley *et al.*, 2004) and BR_{AT} (Luecke *et al.*, 1999). Do photochromic reactions indeed take place for AT-ASR and 13C-ASR? In the paper of 2007, we determined the branching ratios for AT-ASR and 13C-ASR by means of low-temperature UV-visible spectroscopy (Kawanabe *et al.*, 2007). Surprisingly, the obtained branching ratios were unity, indicating that the photoreactions of AT-ASR and 13C-ASR are completely photochromic. The complete photochromic reactions are highly advantageous for the chromatic sensor function of ASR.

We then applied low-temperature FTIR spectroscopy at 170 K to the dark-adapted ASR that has predominantly AT-ASR (97%) (Kawanabe *et al.*, 2008). The obtained AT-ASR_L minus AT-ASR spectra were similar between the full-length and C-terminally truncated ASR, implying similar protein structural changes for the L state. The AT-ASR_L minus AT-ASR spectra were essentially similar to those of BR, but a unique spectral feature was observed in the carboxylic C=O stretching region. The bands at 1,722 (+) and 1,703 (-) cm^{-1} were observed at pH 5, which was reduced at pH 7 and disappeared at pH 9. The mutation study successfully assigned the bands to the C=O stretch of Glu36. Interestingly, Glu36 is located at the cytoplasmic side, and the distance from the retinal Schiff base is about 20 Å. We also observed pH-dependent frequency change of a water stretching vibration, which is located near Glu36.

For the structural analysis by low-temperature FTIR spectroscopy, we prepared many mutant proteins. It should be noted that they include the Glu-to-Asp and Asp-to-Glu mutations, which are not commonly used in biochemical and functional analysis. Glu and Asp are normally mutated into Gln and Asn, respectively, to examine the effect of the negative change at carboxylate. On the other hand, as carboxylic C=O stretching vibrations appear at different frequencies between Glu and Asp, the Glu-to-Asp and Asp-to-Glu mutations are useful to identify which carboxylate in protein contributes to the signal (Furutani *et al.*, 2006; Shibata *et al.*, 2006; Kitade *et al.*, 2009; Hashimoto *et al.*, 2010). Interestingly, during such attempt, we accidentally created an ASR protein that transports protons inwardly.

Engineering an inward proton transport from ASR

As shown above, ASR exhibits unique photoreaction properties. They are very different from those of other archaeal-type rhodopsins, and optimized for the photochromism sensor. The X-ray crystal structure of ASR reported the similar protein architecture characteristic of archaeal-type rhodopsins. Why is such functional optimization achieved in ASR? This is still a question that should be answered in future. Nevertheless, we suggest an important role of internal water molecules, particularly (i) at the Schiff base, and (ii) in the cytoplasmic side.

Like BR, ASR has a bridged water molecule between the Schiff base and Asp75. However, we showed the absence of strongly hydrogen-bonded water molecules in ASR, unlike BR (Furutani *et al.*, 2005a). The reason for the lack of strongly hydrogen bonded water molecules in ASR was explained by the difference in the geometry of the hydrogen bond. Such weak hydrogen bond of the Schiff base and Asp75 is presumably correlated with no proton transfer from the Schiff base to Asp75 (Bergo *et al.*, 2006). In other microbial rhodopsins such as BR, the coupling between the Schiff base (proton donor) and the counterion (proton acceptor) is strong, and

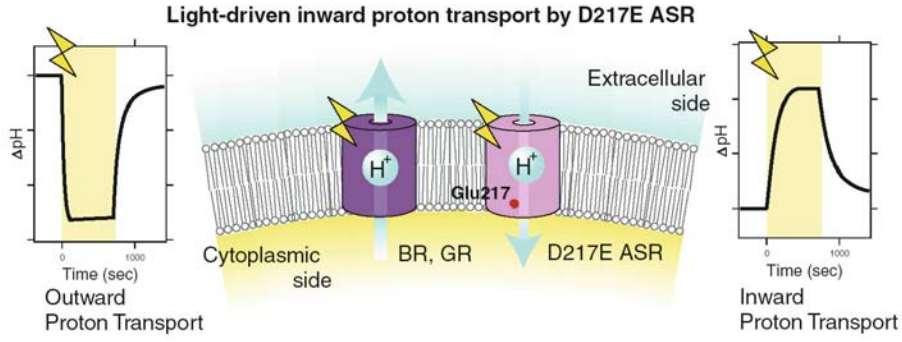

Fig. 3. Light-driven proton transport activity in sphaeroplast vesicles containing GR or ASR. Positive signal corresponds to a decrease in pH (inward proton transport). This figure is reproduced from TOC of Kawanabe *et al.* (2009).

the Schiff base proton is transferred to the counterion. In contrast, the Schiff base is deprotonated in AT-ASR$_M$ like other microbial rhodopsins, whereas the proton acceptor is weakly coupled to the donor. Shi *et al*. (2006) reported a proton transfer from the Schiff base to Asp217 in the cytoplasmic domain, whereas Sineshchekov *et al*. (2006) reported that the direction of this proton transfer is dependent on C-terminus truncation. These results show the proton conductivity toward the cytoplasmic domain in ASR, suggesting the potential to design an inward proton transport out of ASR. The X-ray crystallographic structure of ASR (Fig. 1B) supports this idea, because both extracellular and cytoplasmic domains possess water-containing hydrogen-bonded networks. But then, how can be inward proton transport created? Such machinery must have a specific mechanism to exclude transport in the reverse direction to maintain a proton gradient, as well as the case in BR.

Figure 3 shows schematic illustration of proton transport by microbial rhodopsins. For normal outward proton pumps such as BR and *Gloeobacter* rhodopsin (GR) found in a primitive cyanobacterium (Miranda *et al.*, 2009; Hashimoto *et al.*, 2010), light causes transient pH decrease, which diminishes by addition of a protonophore CCCP. The pH change was entirely different for D217E ASR, resulting in alkaline pH of the medium upon light illumination. For proton transport measurement, we prepared sphaeroplast vesicles by removing the cell wall by lysozyme treatment. The observation of no transport after addition of CCCP and in the absence of retinal clearly demonstrates that the D217E mutant functions as a light-driven inward proton transport (Kawanabe *et al.*, 2009). The observed inward proton transport can be straightforwardly interpreted as a light-driven inward proton pump. Nevertheless, an inward cation "pump" has to be carefully defined in sphaeroplast vesicles, because the interior of the cell is negatively charged. An ion pump is a protein that transports ions against the electro-chemical potential (up-hill reaction), whereas the observed inward proton transport may be driven along the potential gradient (down-hill reaction). Since the electrochemical potential for protons in the present sphaeroplast vesicles is unclear, we describe "inward proton transport", not "inward proton pump", in this article. The inward proton transport activity was also observed for the WT ASR, but the pump activity was much less than D217E ASR. The proton transport activity was negligible for D217N ASR. It should be noted that light-driven inward proton transport associated with two-photon reactions was reported for the D85N mutant of BR in films attached to planar lipid bilayers (Tittor *et al.*, 1994), where the molecular mechanism has not been well established. It appears that absorption of a second photon by the deprotonated Schiff base is necessary for the inward proton pump. In the present case, the proton transport activity shows a linear relationship with light intensities, indicating that the inward proton transport in D217E ASR is driven by a single-photon reaction (Kawanabe *et al.*, 2009). The action spectrum for proton transport activity, measured with monochromatic light, resembles the absorption spectrum of D217E ASR. The initial slope for the inward proton transport in D217E ASR was 15.1±4.0 H^+/protein/min (Kawanabe *et al.*, 2009), which is about half of that in GR and 15-times smaller than that in BR (Mogi *et al.*, 1988).

What determines the inward proton transport activity of D217E ASR? Aspartate and glutamate have similar properties, but the proton transport activity was much higher for glutamate. The absorption spectra of WT and D217E are similar and HPLC analysis showed that the all-*trans* form of retinal is dominant in both dark-adapted samples. The similarity of D217E to WT in the unphotolyzed state is reasonable because Asp217 is located at about 15 Å from the retinal chromophore (Fig. 1B). The M intermediate state is similarly formed for D217E, whose decay was also similar to the case for WT. Light-induced AT-ASR$_M$ minus AT-ASR difference FTIR spectra are also similar between WT and D217E, but a remarkable difference is seen at 1760-1700 cm^{-1}, the characteristic frequency region of protonated carboxylic acids (Kawanabe *et al.*, 2009). The spectrum of WT exhibits a broad positive band at 1740-1700 cm^{-1}, and Shi *et al*. (2006) interpreted this feature as evidence for protonation of Asp217 in the M intermediate. A stronger positive peak is observed at 1713 cm^{-1} for D217E, which is down-shifted in D_2O, suggesting that the Schiff base proton is transferred to Glu217 in the M intermediate. When we normalize the amount of the photoconverted AT-ASR$_M$, protonation of Glu217 is about 10-times larger than that of Asp217 in WT (Kawanabe *et al.*, 2009). This is completely coincident with the proton pump activity, and we concluded that proton affinity at position 217 is correlated with the inward proton transport activity. In the FTIR measurements, detergent-solubilized ASR molecules are reconstituted into PC liposomes. Therefore, there is no electrochemical gradient for the ASR sample, being in contrast to those in sphaeroplast vesicles. The FTIR results show that protons are transferred to Glu217 under such conditions, being coincident with the proton transport activity of D217E ASR. This observation suggests that D217E ASR is an inward proton pump. However, the active transport has to be measured for the direct evidence of inward proton pump.

On the basis of the FTIR data, the mechanism of the inward proton transport in D217E ASR can be explained as follows. M formation accompanies deprotonation of the Schiff base, and Glu217 acts as a proton acceptor in D217E ASR. It was suggested that pKa of Glu217 is lower than 6.5 in the unphotolyzed state, while being higher than 8.0 in M. In BR, the proton acceptor is Asp85 in the extracellular side, and the Schiff base nitrogen interacts with the side-chain oxygen (distance 4.4 Å (1)) through a strongly hydrogen-bonded water in the unphotolyzed state (Fig. 1A) (Luecke *et al.*, 1999). Although ASR has a negatively charged Asp75 at 3.5 Å (Vogeley *et al.*, 2004), the Schiff base proton is transferred to Glu217 that is far distant (~15 Å) (Fig. 1B). This suggests that the accessibility of the Schiff base just before releasing the proton is toward the cytoplasmic side in ASR, while being toward the extracellular side in BR. This may suggest the important role of Asp212 in BR, the second negative charge in the Schiff base region, which is replaced by Pro in ASR (Fig. 1).

We infer that the Schiff base proton is transferred both inwardly and outwardly upon M formation in ASR, because Asp75 does not act as the proton acceptor (Bergo *et al.*, 2006; Shi *et al.*, 2006). This view is also consistent with the previous photocurrent results (Shineshchekov *et al.*, 2006), though the effect of truncation was not reproduced in the present study.

The proton acceptor at the extracellular side is unclear, and it is possible that the Schiff base proton is released into the extracellular aqueous phase. The proton acceptor at the cytoplasmic side is Asp217, but the small FTIR signal of WT suggests that the proton is not fully occupied by Asp217. Hydrogen-bonding network in the cytoplasmic domain may accept the Schiff base proton (Kawanabe *et al.*, 2009), or it may be released into the cytoplasmic aqueous phase. During the relaxation process, the Schiff base is reprotonated from the same sides of release in WT. In contrast, the strong proton affinity of Glu217, not Asp217, presumably yields reprotonation from the extracellular side in D217E ASR. Thus, ASR does not possess the specific switch mechanism of the BR-like outward proton pump (Haupts *et al.*, 1999; Lanyi, 2000; Herzfeld and Lansing, 2002; Lórenz-Fonfría and Kandori, 2009), but the clear inward proton transport was observed.

For BR-like proton pumps, outward vectoriality is particularly important to create a proton gradient. It has been inferred that the ancestral rhodopsin functioned as a light-driven outward proton pump (Sharma *et al.*, 2006). In fact, the archaeal-type photosensors, SRI and SRII, pump protons outwardly in the absence of transmembrane transducer protein (Bogomolni *et al.*, 1994; Jung *et al*., 1996; Sudo *et al.*, 2001). During evolution, conversion into inward proton transport must be strongly prohibited, because it is dangerous for survival. Then, why can ASR be easily converted into an inward proton transport? We infer that ASR became a light sensor that activates a soluble transducer protein, and the hydrogen-bonding network in the cytoplasmic domain and its changes must be important for the activation. It is the symmetrical hydrogen-bonded network from the Schiff base (Fig. 1B) that allowed creation of an inward proton transport by a single amino acid replacement.

Conclusion

We created a light-driven inward proton transport from ASR, a bacterial photochromic sensor protein, by a single amino acid replacement. The native ASR has only small proton transport activity, but a mutation of Asp217 to Glu creates an inward proton transport. FTIR spectroscopy clearly detects the protonation signal of Glu217, but little for Asp217 in WT ASR. The strong proton affinity of the acceptor in the cytoplasmic side appears to force proton uptake from the extracellular side after the Schiff base deprotonation, even though the carboxylate is 15 Å distant from the retinal Schiff base. The newly designed inward proton transport may be useful as an application tool in cell biology. The recently found channelrhodopsin, with a light-activated cation channel (Nagel *et al.*, 2002), allowed numerous applications in neurobiology, because transport of cations can be triggered by light (Boyden *et al.*, 2005). Currently, channelrhodopsin and halorhodopsin, a light-driven inward chloride pump, are used as neuroengineering tools to investigate neural circuit function (Zhang *et al.*, 2007). Outward proton have been also applied for optical neural silencing (Chow *et al.*, 2010). The newly designed inward proton transport (D217E ASR) could provide another kind of active control of electrochemical potential in cells by light in contrast to channelrhodopsin of a purely passive nature. Another application of this protein may be in the field of acidosis-induced cell death. Intracellular pH is precisely regulated around 7.2 by various transporters, which may be changed by acidification around tumor cells (6.9-7.0). Thus, tumor metabolism and pH-control systems have been targets for novel anticancer therapies (Pouyssegur *et al.*, 2006). Acidification of cells by light using an inward proton transport will be useful in the research field. For these applications, a more efficient proton transport may be required, because the current efficiency of D217E ASR is 20-times lower than that of BR, and an additional mutation study is in progress.

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