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Substitution of Pro206 and Ser86 Residues in the Retinal Binding Pocket of *Anabaena* Sensory Rhodopsin is Not Sufficient for Proton Pumping Function

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Abstract *Anabaena* sensory rhodopsin is a seven transmembrane protein that uses all-*trans*/13-*cis* retinal as a chromophore. About 22 residues in the retinal-binding pocket of microbial rhodopsins are conserved and important to control the quality of absorbing light and the function of ion transport or sensory transduction. The absorption maximum is 550 nm in the presence of all-*trans* retinal at dark. Here, we mutated Pro206 to Glu or Asp, of which the residue is conserved as Asp among all other microbial rhodopsins, and the absorption maximum and pKa of the proton acceptor group were measured by absorption spectroscopy at various pHs. *Anabaena* rhodopsin was expressed best in *Escherichia coli* in the absence of extra leader sequence when exogenous all-*trans* retinal was added. The wild-type *Anabaena* rhodopsin showed small absorption maximum changes between pH 4 and 11. In addition, Pro206Asp showed 46 nm blue-shift at pH 7.0. Pro206Glu or Asp may change the contribution to the electron distribution of the retinal that is involved in the major role of color tuning for this pigment. The critical residue Ser86 (Asp 96 position in bacteriorhodopsin: proton donor) for the pumping activity was replaced with Asp, but it did not change the proton pumping activity of *Anabaena* rhodopsin.

Key words: *Anabaena*, rhodopsin, retinal, photoreceptor, sensory transduction, color tuning

A family of four photoactive retinylidene proteins was discovered during 1971-85 in the cytoplasmic membranes of the halophilic archaeon *Halobacterium salinarum*. The first two identified were light-driven ion transporters, bacteriorhodopsin (BR; 18) and halorhodopsin (HR). BR and HR use light energy to transport protons and chloride, respectively, across the cell membrane [19]. The other two are sensory rhodopsins I (SRI) and II (SRII), which are not transport proteins, but rather light sensors. SRI and SRII are attractant and repellent phototaxis receptors for orange and blue-green light that send signals via membrane transducer proteins to the cell's motility apparatus, respectively [10]. The archaeal transport and sensory rhodopsins are well characterized with respect to their atomic structures and functions and are among the best-studied membrane proteins [8, 17, 24, 26, 27].

Recently, genome projects combined with heterologous expression have revealed homologs of the archaeal rhodopsins outside the archaeal domain. An environmental DNA library made from ocean picoplankton uncovered a homologous sequence in an uncultivated proteobacteria species, SAR86 [3]. Heterologous expression of the opsin gene in *Escherichia coli* produced a protein that bound retinal to produce a photoactive pigment that carried out efficient proton transport with a rapid (15 ms) photocycle characteristic of transport rhodopsins [2]. The photocycle rate was confirmed in proteorhodopsin detected in membranes made directly from Monterey Bay picoplankton [3]. This work therefore established the existence of eubacterial transport rhodopsins.

Additional homologous opsin (apoprotein) sequences have also been found in cyanobacteria [7]. A rhodopsin (holoprotein) pigment in cyanobacteria established that sensory rhodopsin is also present in eubacteria [14]. A gene encoding a homolog of the opsin was found via a genome sequencing project of *Anabaena* (*Nostoc*) sp. PCC7120 at Kazusa Institute, Japan. The opsin gene was expressed in *E. coli* and bound all-*trans* to form a pink pigment that had maximum absorbance at 543 nm, which is a mixture of an all-*trans* and 13-*cis* form of retinal in light adapted *Anabaena* rhodopsin [12]. In addition, the absorption maximum of *Anabaena* rhodopsin was reported at 549 nm in dark-adapted form [6].

The *Anabaena* rhodopsin in *E. coli* membrane exhibited a photochemical cycle with an M photointermediate and

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110 ms half-life cycle at pH 6.8, and interestingly showed a 13-*cis* photocycle [4, 12, 21]. The *Anabaena* opsin gene and another open reading frame separated by 16 base pairs under the same promoter were found in the genome. This operon was predicted to encode a 261-residue (opsin) and a 125 residue (14 kDa) protein. The rate of the photocycle increased ~20% when the *Anabaena* rhodopsin and the soluble transducer were coexpressed in *E. coli*, indicating physical interaction between the two proteins. The pigments did not exhibit detectable proton transport activity when expressed in *E. coli*, and Asp96, the proton donor of BR, was replaced with Ser86 in *Anabaena* rhodopsin. Generally, in BR, the chromophore all-*trans* retinal is covalently attached via a Schiff base to the ε-amino group of a lysine residue deep in the membrane interior. When the cells are illuminated, all-*trans* retinal bound to bacteriorhodopsin absorbs a photon and undergoes photoisomerization to 13-*cis*-retinal and the restoration to all-*trans*-retinal is accompanied by the outward movements of proton through the plasma membrane. In the dark, the Schiff base is protonated, but photoisomerization of retinal lowers the pKa of this group and it releases its proton to a nearby Asp85 (proton acceptor) residue, triggering a series of proton hops that ultimately result in the release of a proton at the outer surface of the membrane [5]. After the proton has been pumped out, the Schiff base requires a proton from Asp96 (proton donor), which in turn takes up a proton from the cytoplasm. In contrast to the BR case, *Anabaena* rhodopsin has replaced the proton donor to Ser86 residue. This observation is compelling in that *Anabaena* rhodopsin functions as a photosensory receptor in its natural environment, and the 125 residue cytoplasmic soluble protein transduces a signal from the photoreceptor [12], unlike the archaeal sensory rhodopsin that transmits a signal by transmembrane helix-helix interaction with an integral membrane transducer [8].

Here, we report that, on the basis of all criteria available, a newly found *Anabaena* opsin gene encodes the apoprotein of a retinylidene sensory pigment; we also discuss what happens to mutants that have replaced wild type proton donor position (Ser86) to Asp, and the residue involved in the H-bond network between the Schiff base (Pro206) of the retinal-binding pocket, which is replaced from Glu and Asp in *Anabaena* sensory rhodopsin.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli transformants were grown in LB (Luria-Bertani) medium in the presence of ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) at 35°C [9]. The sensory rhodopsin constructs were expressed under the PlacUV5 promoter of pMS107 [12] in *E. coli* strain UT5600. DH5α was used for cloning the operon gene from genomic DNA of *Anabaena* sp. 7120 (provided by James Golden, Texas A&M University, College Station, TX, U.S.A.). *Anabaena* sp. 7120 cells were grown in BG11 [12, 15] medium at 25°C.

Construction of Plasmids Encoding the Retinal Synthase and Various Opsins

Restriction enzymes and T4 DNA ligase were from NEB (MA, U.S.A.) and PFU DNA polymerase was from Vivagen (Sungnam, Korea). Oligonucleotides were purchased from Cosmogentech (Seoul, Korea). Ampicillin, chloramphenicol, and all-*trans* retinal were from Sigma (St. Louis, MO, U.S.A.). Mutants ASR_S86D, ASR_P206D, and ASR_P206E and the ASR_S86D/P206D double mutant were constructed using two-step PCR mutagenesis by a modification of the megaprimer method using the wild-type gene as template [13, 20]. The PCR was performed for 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min with PFU polymerase. The PCR product was purified and digested with NdeI and NotI and introduced into the *E. coli* expression vector under the control of IPTG induction. Point mutations were verified by DNA sequencing of the length of the PCR-generated inserts in the final constructed expression vector. Here, His denotes a tag with six histidine residues attached at the C-terminus of the protein.

Expression of Microbial Sensory Rhodopsins in *E. coli* Cells

ASR mutants were mainly expressed in *E. coli* strain UT5600 using plasmid pKJ900-ASR for proton pumping measurements and *E. coli* strain β-UT using plasmid pKJ900-ASR for Western blot and absorption spectroscopy. The β-UT cell has the plasmid that contains 4 carotenoid biosynthesis genes [16, 28]. Overnight cultures of transformants were diluted 1:100 and grown to 0.4 absorbance units at 600 nm at 35°C. To obtain proteins for proton transport measurements, all-*trans* retinal was added to a final concentration of 5 mM with 0.2% L-arabinose. For measurements of retinal-generated absorption spectra, the apoproteins were induced without the addition of retinal. β-UT Cells were induced with 0.2% L-arabinose and 1 mM IPTG for 3 h at 35°C.

Membrane Vesicle Preparation

The cells containing constructs of various opsins were grown in 500 ml of LB in a 1-l flask with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) at 37°C on a gyratory shaker at 180 rpm for 16 h. Cells were induced at $A_{600\,nm}$=0.4 by adding 1 mM IPTG and 0.2% L-arabinose. After a period of 12 h, the cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.0, containing 150 mM NaCl. Rhodopsin-expressed *E. coli* cells were lysed by sonication at 4°C, followed by low-speed (2,000 ×*g*, 15 min) centrifugation to remove cell debris. Finally, the membranes were sedimented at 100,000 ×*g* for 1 h at 4°C,

and the pellet resuspended in 50 mM Tris (pH 7.0) containing 150 mM NaCl at 4°C. The rhodopsin was extracted from the membranes with gentle shaking in extraction buffer (1% sodium dodecyl maltoside [DM], 150 mM NaCl, 50 mM Tris-buffer [pH 7.0]) for 4–6 h at 4°C. The extracted protein was collected as supernatant after centrifugation at 20,000 ×*g*. Membranes without receptor protein were prepared using the same procedure and used as a control.

Purification of His-Tagged Rhodopsin

The proteins containing 6 histidine residues at the C-terminus were purified separately by using Ni^{2+}-NTA agarose beads (Qiagen, Valencia, CA, U.S.A.). The *E. coli* membrane containing *Anabaena* rhodopsin was solubilized in 1% DM with 150 mM NaCl, 10 mM imidazole, and 50 mM Tris, pH 7.0, and incubated with beads at 4°C for 16 h. The protein-bound beads were washed with 50 mM imidazole and eluted by 250 mM imidazole, 0.1% DM, and 50 mM Tris-buffer.

Western Analysis

Cell pellets harvested from the 500-ml cultures were washed once with buffer (50 mM Tris, 150 mM NaCl, pH 7.0) and centrifuged at 4,000 ×*g* for 15 min at 4°C. Cells were disrupted by sonication, unbroken and large debris were removed by low-speed centrifugation, and cell membranes were collected by ultracentrifugation at 100,000 ×*g* for 1 h. The membranes were resuspended with buffer (50 mM Tris, 150 mM NaCl, pH 7.0).

Cell lysates and membrane preparations containing 20 μg of protein were subjected to SDS-PAGE and Western analysis. Anti 6× His monoclonal antibody at a 1:3,000 dilution was used as the primary antibody, and an anti-mouse IgG HRP conjugate at a 1:5,000 dilution was used at the secondary antibody. Reactive bands were visualized using ECL Western blot detection reagents.

Proton Pumping Measurements

For the measurement of proton pumping of cells, the *E. coli* cells with expressed *Anabaena* sensory rhodopsins were pelleted at 5,000 rpm for 15 min at 4°C and washed twice with unbuffered solution (10 mM NaCl, 10 mM $MgSO_4 \cdot 7HO_2$, 100 mM $CaCl_2$) at room temperature [29]. Samples were illuminated with 100 Watts/m^2 at 500±20 nm using a 150-Watt tungsten halogen lamp in combination with wide-band interference and heat-protecting ($CuSO_4$ and glass) filters. The pH was monitored with a computerized Horiba F51 pH meter.

Absorption Spectroscopy

Difference spectroscopy was used to measure the retinal-generated absorption in *E. coli* membranes. After adding

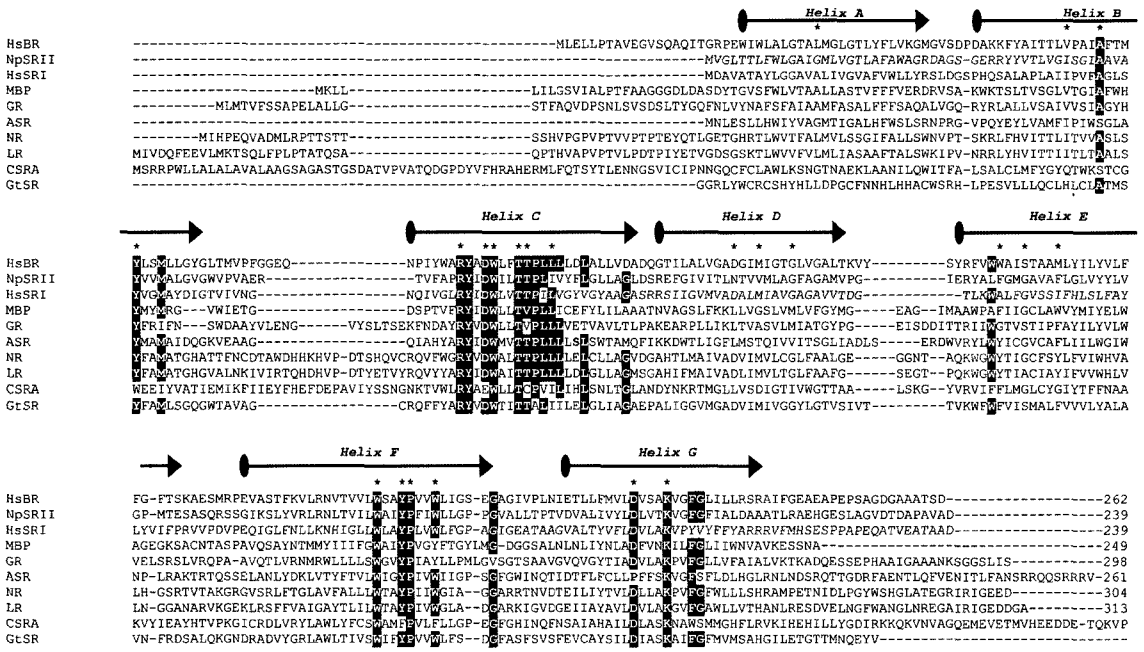

Fig. 1. Sequence alignment of microbial rhodopsins.
Primary sequence comparison between opsins from *Halobacterium salinarum*, *Haloterrigena*, *Natronomonas pharaonis (NpSRII)*, *Anabaena*, *Guillardia*, and *Gloeobacter*. Conserved residues are marked in black boxes (HsBR, *H. salinarum* Bacteriorhodopsin; NpSRII, *Natronomonas pharaonis* Sensory rhodopsin II; HsSRI, *H. salinarum* Sensory Rhodopsin I; MBP, Monterey Bay Proteorhodopsin; GR, *Gloeobacter* Rhodopsin; ASR, *Anabaena* Sensory Rhodopsin; NR, *Neurospora* Rhodopsin; LR, *Leptosphaeria* Rhodopsin; CSRA, *Chlamydomonas* Sensory Rhodopsin A; GtSR, *Guillardia theta* Sensory Rhodopsin). The mutation sites (S86 and P206) are in helix C and F. The residues KWG in helix E are conserved mostly among fungal rhodopsins.

the retinal solutions, the spectra were recorded with a Shimadzu 2450 spectrophotometer every 3 min, after which no further absorption changes occurred. The λ_{max} at different pH values was measured according to the reconstitution spectra of the membranes with *Anabaena* of the wild-type and mutants. The position of the absorption maxima of the mixtures of alkaline and acidic forms at different pH values did not depend linearly on the relative concentrations of the two forms because of different structures and half-bands of their spectra.

RESULTS

Various Rhodopsin Production in the Presence of Internal and External Retinals

A lot of archaeal-type rhodopsins were recently identified (Fig. 1) from three domains of life, Archaea, Eubacteria, and Eukarya [25]. Here, we showed several microbial rhodopsins that have been studied for ion transport and sensory function. The seven transmembrane helices were aligned based on amino acid residue sequence homology. In order to understand the function of these microbial rhodopsins, we were trying to overexpress these seven transmembrane proteins in *E. coli*. The first successful expression of microbial rhodopsin was done by Dr. Kamo's group [22]. We expressed various opsins in *E. coli* to which all-*trans* retinal had been added externally or was internally synthesized, and purified the pigment in non-denaturing detergent (DM) using Ni^{2+}-NTA resin. We observed a reproducible effect on rhodopsin *in vivo*, when it was coexpressed with retinal synthase or added external retinal in *E. coli*. The coexpression of mouse retinal synthase does not much enhance the production of microbial rhodopsins, which was confirmed by Western immunoblot (Fig. 2). In our construct, a sequence of encoding six histidine residues at the C-terminus was included to provide an immunoreactive epitope. Western analysis indicated that all of the mutant ASRs were expressed, and the expression level of mutants differed from that of wild-type (increased in P206E mutant and decreased in S86D, P206D/S86D mutant; data not shown).

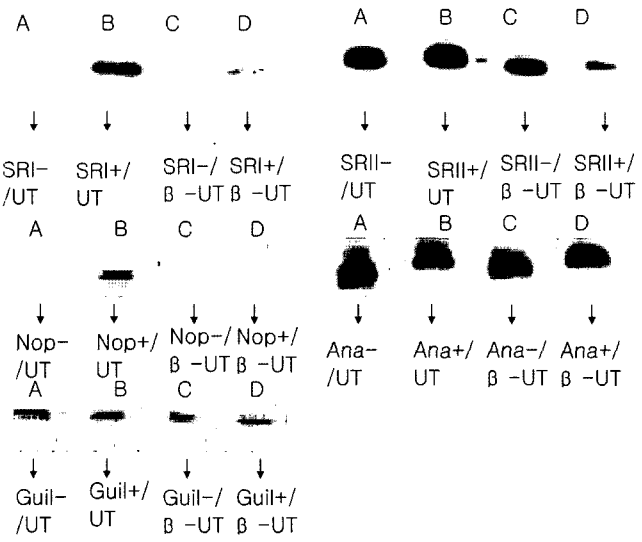

Fig. 2. Western blot analysis of various his-tagged microbial rhodopsins.

E. coli β/UT5600 strain was constructed with transformation of pORANGE plasmid for β-carotene synthesis into *E. coli* UT5600 strain. The pKJ900 plasmid contains various opsin genes and mouse dioxygenase gene, which could produce all-*trans* retinal from β-carotene. WT and rhodopsin with an included leader sequence gene was inserted into the pKJ900 and transformed into *E. coli* β/UT for protein expression. Immunoblot analysis of membrane proteins from *E. coli* UT5600 strain expressing tagged rhodopsins of several different plasmids was performed with the anti-His antibody. The membrane proteins were separated by 15% SDS-PAGE, transferred onto a PVDF membrane and probed with antibodies against the 6× His-tag (His). Lanes A, B contain exogenous retinal system; lanes C, D contain endogenous retinal biosynthesis; lanes A, C contain each rhodopsin; lanes B, D contain each rhodopsin including the 18 leader sequence, ATGGGTAAATTATTACTGATATTAGGTAGTGCTATTGCACTTCCATCATTTGCT (MGKLLLILGSAIALPSFA) from BPR. Each rhodopsin is SRI, SRII, NR, ASR, and GtSR.

Furthermore, the expression level was increased in the presence of a *N*-terminal 18 amino acids leader sequence from BPR. In the case of *Anabaena* rhodopsin, the protein expression level was maximum using external retinal in the absence of leader sequence (Fig. 2).

Absorption Spectra and Determined pKa Value of the ASR

Anabaena rhodopsin (ASR) protein exhibits high identity in other proteins. The Schiff base proton acceptor in ASR is the same as that of proton pumps, but the proton donor (D96 in BR) is replaced with a serine residue. ASR has the presence of a proline (P206) one helix turn before the Schiff base lysine (K210), replacing the aspartyl residue found in all other known microbial rhodopsins. We expressed the wild-type and all the mutant *Anabaena* rhodopsins with a 6-histidinyl tag in *E. coli* and purified the rhodopsins in dodecylmaltopyranoside using Ni^{2+}-NTA resin. In former data, the absorption spectra of the purified wild-type ASR protein exhibited a typical rhodopsin bandwidth (~100-nm half bandwidth) and here we have a single peak at 545 nm (Fig. 3).

We showed the absorption spectrum of PR as the reference to proton pumping rhodopsin (Fig. 3). Fig. 2 and 3 show visible absorption spectra of WT ASR, and P206D, S86D, S86S/P206D mutant ASR measured at room temperature. Absorption spectra obtained from S86D mutant at pH 7.0 were similar to that of wild type ASR. However, the absorption maximum of P206D was blue-shifted compared with that of wild-type. We tried to get pH-titration curves from wild-type and mutant ASRs in dodecylmaltopyranoside (DM) to determine the pKa of the spectral transition. The spectral changes were very small between pH 4.0 to 11.0 in wild-

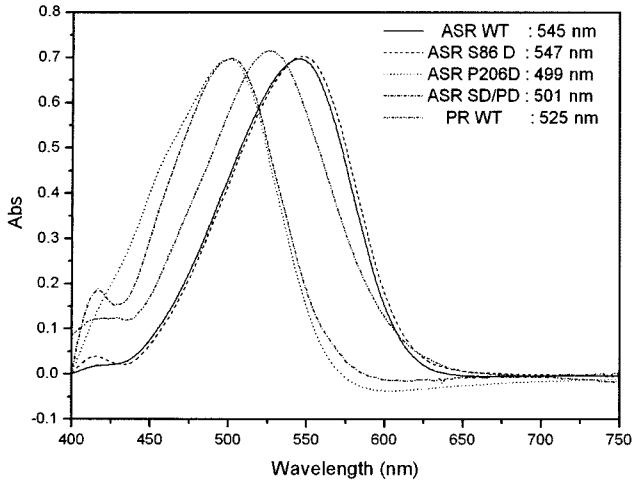

Fig. 3. Absorption spectra of WT and mutant ASRs.
Absorption spectra from 400 nm to 750 nm of purified C-terminal His-tagged wild-type and site-directed mutants of *Anabaena* sensory rhodopsin. The absorption of purified wild-type ASR exhibits a typical rhodopsin bandwidth and a single maximum peak at 545 nm and several mutants also exhibit a pattern like the wild-type. However, each mutant shows a different absorption maximum from each other. P206D and S86D/P206D mutants in the retinal-binding pocket shift the absorption maximum to the blue.

type ASR, like xanthorhodopsin [1, 11], and P206D did not show any spectral changes between pH 4.0 and pH 11.0 (Fig. 4).

Measure of Proton Pumping

ASR did not exhibit detectable proton transport activity when expressed in *E. coli*. We supposed that differences in which contact with the retinal between ASR and proton pumps are important, and we constructed the ASR mutant protein that changed the position 86 (proton donor) and 206 by a two-step mega primer PCR method. It has been reported that the *Anabaena* rhodopsin cannot transport proton in *E. coli* membrane. If the position 86 or 206 is critical for proton transport, a significant proton pumping should be detected in these mutant proteins. We used proteorhodopsin as a control to show activity in *E. coli* sphaeroplast. Proton ejection was evident as a light-induced pH decrease in cell containing the light-driven proton pump proteorhodopsin in *E. coli* sphaeroplast. Cells containing P206D, S86D, and P206D/S86D mutants did not eject proton like the wild-type ASR (Fig. 5). In *E. coli* membrane, proton flux is caused by preexisting electrochemical potential of the energized *E. coli* cell. The CCCP(m-

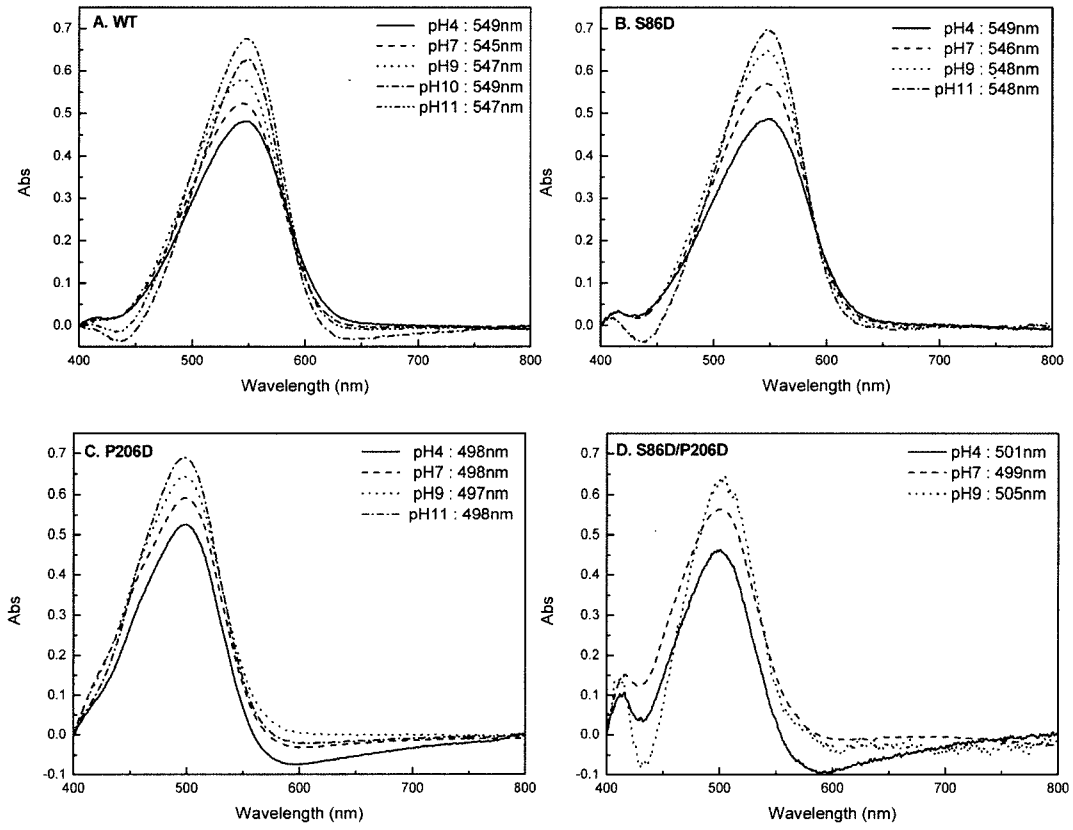

Fig. 4. Absorption spectra of various mutant rhodopsins at different pHs.
Absorption spectra from 400 nm to 800 nm of purified C-terminal His-tagged wild-type and mutants of *Anabaena* sensory rhodopsin. The absorption spectrum of purified ASR was measured at different pHs. The changes of each absorption maxima were less than 4 nm. To better observe the peak wavelength from the spectra, we measured different proteins quantitatively.

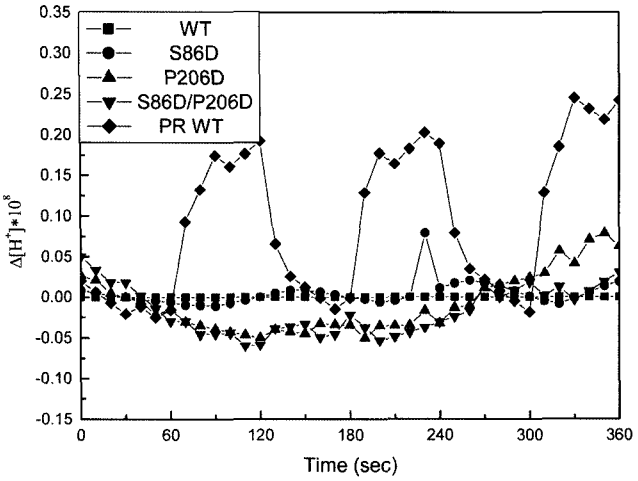

Fig. 5. Light-induced proton pumping of WT and mutant ASRs in vesicles.
Proteorhodopsin (MBP) is a control for the proton pumping activity. The light was illuminated every 60 s in the presence of 440 nm cutoff filter. For dark adaptation, the protein was kept in the dark for 2 min. The pumping activity was measured by pH changes in the sphaeroplast suspension of each mutant using a pH electrode.

chloro-carbonylcyanide-phenylhydrazine) dissipates proton gradients across membranes. We examined proton flux in

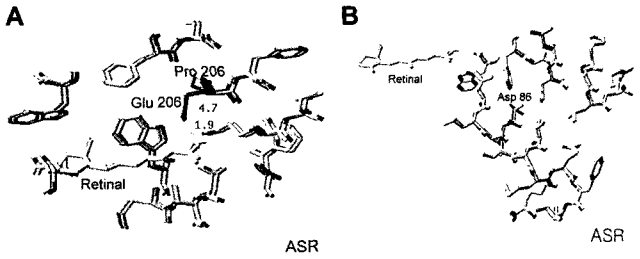

Fig. 7. 3D prediction of Pro206Glu and Ser86Asp mutants.
X-ray crystallographic structure models of ASR (PDB entry 1XIO). Hydrogen bonding network showed using a Swiss-Pdb viewer. Top and bottom regions correspond to extracellular and cytoplasmic sides, respectively. Each mutant residue is introduced, fitted by an energy minimized program, and overlapped with wild-type ASR structure. Yellow molecule is retinal and the numbers are the hydrogen bond distances in angstroms. Pro206 Glu mutant structure showed that the negative charged side chain changed the environment of retinal; however, the Ser86Asp model structure showed that this residue was located far from the retinal.

E. coli cells with CCCP, and the result was similar to others (data not shown).

Absorption Spectra of Retinal-Reconstituted ASR Membrane from *E. coli*

All-*trans* retinal was added to hydroxylamine bleached membrane, which was prepared from wild-type and P206E

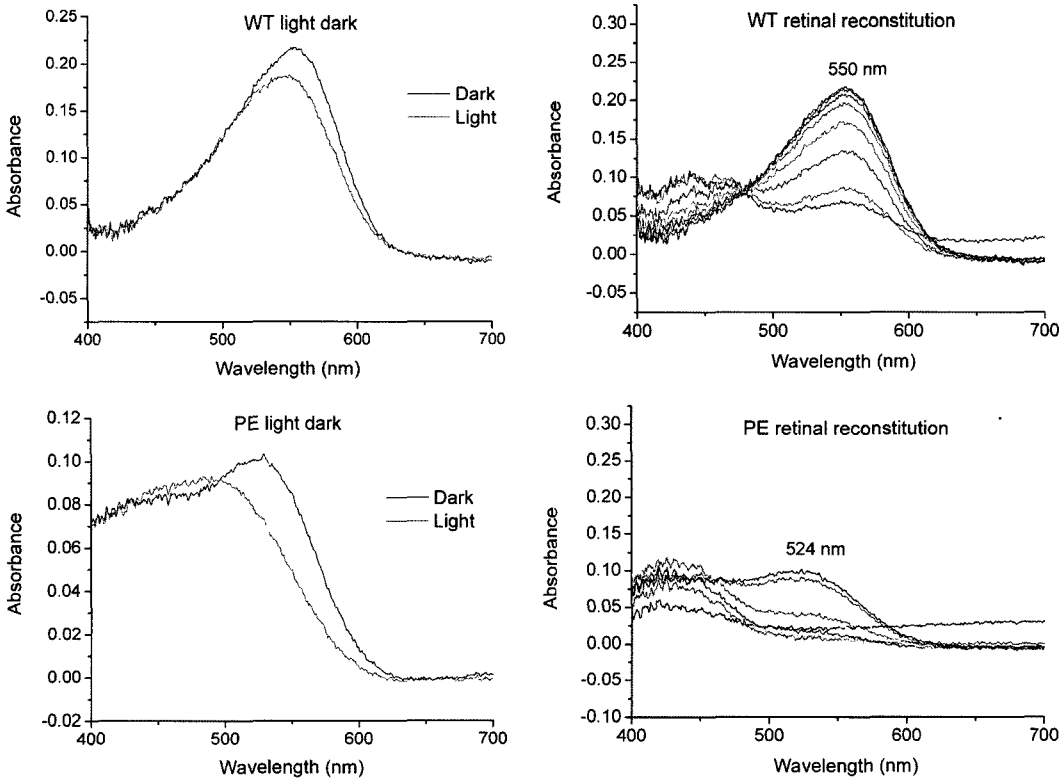

Fig. 6. Absorption spectra of retinal-reconstituted membrane from WT ASR and P206E.
All-*trans* retinal was added to a 3 ml hydroxylamine bleached membrane from UT5600. After the reconstitution was completed in the dark, light was illuminated to observe the light-adapted form. A time series of spectra is shown for reconstitution of all-*trans* retinal incorporated into WT ASR and its mutant membrane.

mutant ASR enriched *E. coli* membrane. The absorption spectrum was monitored every 5 min. In 5 min, the peaks at 550 nm (WT) and 524 nm (P206E) bands were evident and increased during the incubation. After the illumination of the white light, the absorption maximum was shifted to 540 nm in the WT and 500 nm in the P206E mutant. It seems like a 13-*cis* form of retinal in the ASR protein and the return of the 13-*cis* retinal to all-*trans* was slow, so we could detect this one of the equilibrium form [23].

DISCUSSION

Unlike other microbial rhodopsins, the *Anabaena* rhodopsin has Ser86 and Pro206 residues, which are uncharged and not able to accept protons from the environment. They are expected to play a key role for the pumping function of the rhodopsin because those are well-conserved residues among other rhodopsins. We mutated these residues to aspartic acid, which are negatively charged and capable of accepting and donating protons. However, we could not detect any proton pumping activity from these mutants. It is suggested that these residues are critical for proton pumping but other factors are also important for restoring the pumping activity. For example, it may need a H-bond network between the extracellular side to the Schiff base, global conformation for accessibility to water, or helices arrangements, etc.

The *Anabaena* opsin was expressed with a 6-histidine tag in *E. coli* and all-*trans* retinal had been added. The absorption maximum of all-*trans* retinal in ethanol is 381 nm. The electron density of retinal is equally distributed at this state, and it can absorb energy at the maximum level. Wild-type *Anabaena* rhodopsin was purified in a pink pigment and the colors of the purified mutated proteins were lighter than the wild-type, in order of Ser86Asp, Pro206Asp, and Ser86Asp/Pro206Asp double mutant. The mutant Ser86Asp was slightly red-shifted relative to the wild-type. The red-shift of absorption spectrum means an increase in the wavelength and a decrease in the energy level of absorbed light. The absorbed energy level gets lower with the decrease of the stability of retinal's electron distribution state. Asp86 residue, a charged amino acid, as a substitute for Ser86, an uncharged but polar amino acid, destabilizes electron distribution of retinal. Pro206Asp revealed a blue-shift of 46 nm relative to the wild-type. Replacing the uncharged Pro206 to Asp, a charged amino acid, changes the electron distribution of rhodopsin's microenvironment (Fig. 7), and stabilizes the retinal electron state relative to that of the wild-type. On the other hand, the special characteristic of *Anabaena* rhodopsin is interconversion between all-*trans* and 13-*cis* retinal (6s) conformation. When we added all-*trans* retinal, we observed λmax at 524 nm in Pro206Glu mutants. However, we could observe 499 nm in the Pro206Asp mutants red-shifted form and it did not return to the 524 nm state (dark-adapted state). It is possible that retinal was converted to the 13-*cis* form in the Pro206Asp mutant after the illumination (Fig. 6). In order to test whether the minor side arm length might affect the proton pumping or color tuning, we created Pro206Glu and characterized the properties. It was not much different from the Pro206Asp mutant. In comparison with Ser86Asp's red-shift of 2 nm, the Pro206Asp showed a blue-shift of 46 nm. It means that the 206 position plays a critical role in determining the electron distribution of opsin-bound retinal. The proline, a nonpolar amino acid, affects considerably the distribution of retinal electrons because it does not have a charged group and twists the alpha helix.

The absorption spectra of the wild-type and three mutated *Anabaena* rhodopsins were measured at various pHs. It did not show much change between pH 4.0 and pH 11.0. like xanthorhodopsin [1, 11] and *Gloeobacter* rhodopsin. The spectrum of Pro206Asp also did not reveal detectable change with increased pH value. That is, the Asp 206th residue was not affected by the external pH alteration in spite of its facility to donate or accept protons rather than the Pro206.

We introduced the Ser86Asp/Pro206Asp double mutant like other proton pumping rhodopsins. According to the results (Fig. 5), the proton pumping function cannot be reconstructed by the simple replacement of two amino acid residues, ser86 and Pro206 to aspartic acid. Theoretically, the proton pumping is possible at a pH value above 11.0, but the retinal detaches from rhodopsin at strong alkaline condition so the measurement is impractical.

The physiological role of *Anabaena* rhodopsin still awaits characterization. Based on our results, it is not involved in proton pumping activity but in sensory function in the *Anabaena* cell.

Acknowledgments

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