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Glu-56 in HtrI is Critical for Phototaxis Signaling in *Halobacterium salinarum*

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Abstract: The attractant (orange light) or repellent (white light) signal is transmitted from SRI (Sensory Rhodopsin I) via protein-protein interaction with its transducer HtrI (Halobacterial Transducer for Sensory Rhodopsin I) which in turn controls a cytoplasmic phospho-transfer pathway that modulates flagella motor switching in *Halobacterium salinarum*. Some mutations in both SRI and HtrI showed an unusual mutant phenotype called inverted signaling, in which the cell produces a repellent response to normally attractant light. Twelve mutations at the Glutamate 56 (E56) position in the second transmembrane helix of HtrI were introduced by site-specific random mutagenesis. Almost all E56 mutants showed orange-light inverted responses in pH and temperature-dependent manners except E56D and E56Y. Except for these two mutants, all mutants accelerated the S_{373} decay compared to wild-type at 18°C. This supported that there is an interaction between SRI and the second transmembrane of HtrI. Also a structural model of HtrI based on the Tar crystal structure and the secondary structure prediction program proposed the E56 residue to be in the middle of the proton channel. The most important observation is that the E56 mutant provides the evidence that this residue is very sensitive for signal relay, which can be explained by the open and closed conformations of the channel (**A** and **R** conformations) in SRI, as was postulated by the unified conformational shuttling model for transport and signaling.

Key words: Sensory rhodopsin, retinal, photoreceptor, signal transduction, inverted response

Halobacterium salinarum is a rod-shaped and extremely halophilic archaebacterium which is found in near to fully saturated saline environment. The cell has a family of four structurally similar membrane proteins to capture light energy by photon-driven electrogenic ion transport and to use light for information to control its motility (phototaxis). Bacteriorhodopsin (BR) and halorhodopsin (HR) function as proton and chloride pumps, respectively, which provide the cells with energy (Jung 2004). Sensory rhodopsin I (SRI) is a phototaxis receptor that uses the energy of an absorbed photon to send attractant or repellent signals to its transducer HtrI via protein-protein interaction. The predicted structure of SRI based on the structure of BR is a seven transmembrane helix protein. All-trans retinal is bound via a Schiff base linkage to lysine residue 205. SRI couples retinal isomerization to activation and phototactic behavior. The sequence of HtrI predicts two hydrophobic transmembrane α-helices near N-terminal that anchor the protein to the membrane (Hoff, 1997). SRI (λmax = 587 nm) and HtrI mediate an attractant response to orange light and thereby enable *H. salinarum* cells to move into the region of illumination where BR and HR are maximally active (Hoff et al., 1996). Also, SRI-HtrI mediates a repellent response to near UV in the presence of orange light (white light). Several mutations in both SRI and HtrI showed an interesting mutant phenotype called inverted signaling, in which the cell produces a repellent response to normally attractant light. The D201N mutant of SRI (Olson et al., 1995), several substitutions (A, S, & Y) of H166 in SRI (Zhang and Spudich, 1997), and E56Q in HtrI (Jung and Spudich, 1996) showed an orange light inverted response. The inverted mutants still showed normal white light 2-photon repellent responses, indicating that these residues were essential for attractant signaling.

The photochemical reactions produced by one-photon and two-photon excitation of SRI have been characterized by kinetic flash spectroscopy. SRI exists in three distinct spectral forms (Hoff, 1997). The SR_{587} (λmax = 587 nm) species is a dark-adapted state and orange light absorption by SR_{587} initiates a series of reactions which produce the long lived S_{373} (λmax = 373 nm) species in < 1 msec. S_{373} thermally returns to SR_{587} with a halftime of ~1 sec,

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completing the SRI photocycle. S_{373} is also photoreactive and converts to a second long-lived (~80 msec) species S^{b}_{510} (λmax = 510 nm). The S_{373} species is the attractant signaling states that, during its transient existence, transmit through HtrI signals to inhibit swimming reversals.

In eubacterial chemotaxis, signal transduction is mediated by reversible phosphorylation of histidine and aspartate residues of signaling proteins (Hoff, 1997). In contrast, in eukaryotic system, signal transduction occurs through the reversible formation of phosphate esters of serine, threonine, and tyrosine residues, although recent evidence indicates an important role for histidine kinase in *Arabidopsis*. The highly conserved signaling domain of about 60 residues of HtrI with chemotaxis transducers probably tightly binds the CheA histidine kinase and modulates its activity during the signaling. The *cheA, cheY, cheB*-like genes and new *cheJ* gene were cloned from *H. salinarum*. $CheA_H$ (the subscript denotes *Halobacterium*) was the first histidine kinase of two-component system from the kingdom Archaea. Therefore, the mechanism of signal transduction in halobacterial phototaxis is similar to the two-component signaling systems of eubacteria.

In order to explain the inverted response, a conformational shuttling model has been proposed as part of a unified mechanism for ion pumping and sensory signaling by archaeal rhodopsins (Jung and Spudich, 1998; Spudich and Lanyi, 1996). Light-dependent interconversions between two protein conformations underlie both ion transport in BR and HR and phototaxis signaling by the sensory rhodopsin I (SRI) of halobacteria (Jung and Spudich, 2004). In BR, the two conformations (open and closed form) facilitate the pumping of a proton unidirectionally across the membrane. An expansion of the cytoplasmic channel is observed during BR_{568} to M_{412} photoconversion (corresponding to SR_{587} to S_{373}) and is implicated in the change of accessibility to the Schiff base from the extracellular to the cytoplasmic membrane surfaces (Spudich and Jung, 2005). Light-induced conformational shuttling between the two conformations occurs in free SRI because it is capable of proton transport and SRI-HtrI complex modulates attractant and repellent signal-transducer activity. This model provides a rationale for the blocking of the cytoplasmic channel of SRI by HtrI (Bogomolni et al., 1994; Spudich and Spudich, 1993), since HtrI in the model monitors the state of SRI by coupling to the channel. A basic assumption of this model is that the SRI/HtrI complex is poised in a metastable equilibrium of the two conformations in the dark, which is easily shifted toward one or the other conformation by several factors - light, pH, temperature, and a single mutation.

Here we introduced 12 mutations at E56 position of HtrI using 32 mixtures of oligonucleotides to reveal that E56 is critical for attractant signal relaying and that the attractant response is influenced by pH and temperature.

MATERIALS AND METHODS

Bacterial strains and plasmids

The plasmid pKJ306 is a shuttle vector that carries ampicillin and mevinolin resistance for selection in *Escherichia coli* and *H. salinarum*, respectively. *E. coli* strains were grown in Luria-Bertani medium at 37°C (Kim, 1999) and halobacterial strains were grown in CM (complex medium) at 37°C (Jung, 1996). The plasmid pKJ306 contains HtrI and SRI (Spudich, et al., 2000) in which SpeI and SacI restriction sites were introduced flanking the *HtrI* gene. Native or mutant forms of HtrI and SRI were expressed from their native promoter by transformation of *H. salinarum* strain Pho81Wr$^-$ with plasmid pKJ306 (Fig. 1) (Jung and Spudich, 1998; Yao and Spudich, 1992). Pho81Wr$^-$ was isolated by screening for the absence of an endogenous restriction system in single colony isolates from Pho81W [BR$^-$, HR$^-$, SRI$^-$, SRII$^-$, HtrI$^-$, and carotenoid-deficient (BR, bacteriorhodopsin; HR, halorhodopsin)]. After selection for motility on swarm plates, *H. salinarum* cells were transformed with polyethylene glycol (PEG) treatment (Cline and Doolittle, 1987), except that PEG was first purified by using AG501-X8 resin (20-50 mesh, Bio-Rad, Hercules, CA). Halobacterial transformants expressing plasmid-encoded mevinolin-resistance were grown on sphaeroplast-regeneration plates at 37°C with 4 μg/mL mevinolin (Lam and Doolittle, 1989).

PCR site-specific random mutagenesis

The plasmid pKJ304 carrying the 758bp SpeI/SacI (HtrI)

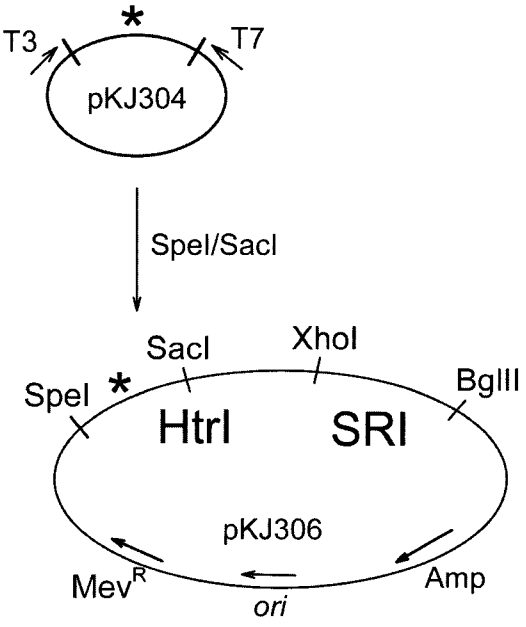


Fig. 1. Site-specific random PCR mutagenesis scheme. Plasmid pKJ304 contains a piece of *HtrI* gene. The plasmid pKJ306 is a shuttle vector for both *E. coli* (ampicillin resistance) and *H. salinarum* (mevinolin resistance).

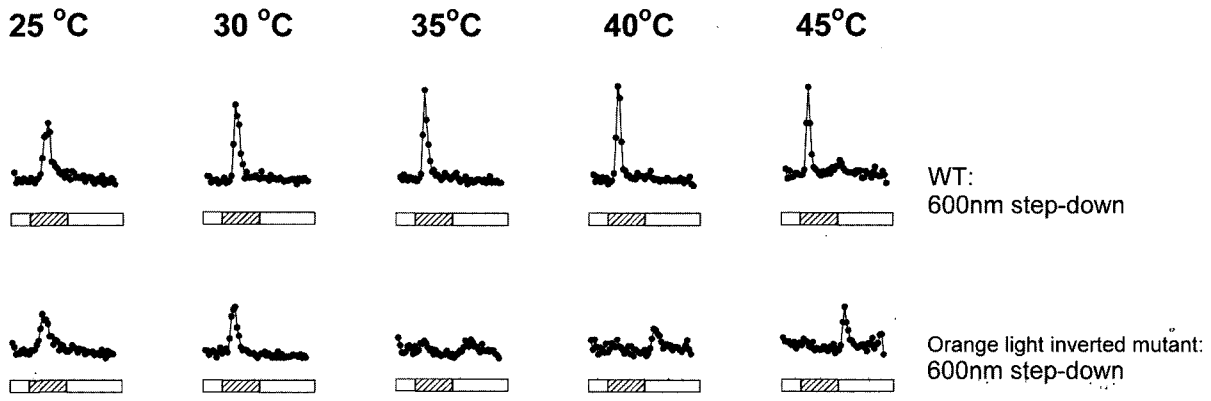


Fig. 2. Phototaxis responses of wild-type and orange-light inverted mutant at temperatures between 25°C and 45°C. The traces represent population reversal-frequency transients collected by computerized motion analysis at pH6.0. Removal of orange light induces the reversal frequency in wild-type, but orange light directly induces reversal in inverted mutant at 40 to 45°C. Lower temperatures convert the orange light inverted phenotype to wild-type. Two second after initiation of data acquisition the cell were exposed to 4-sec removal of orange light in CM. The shades bars are 4-sec dark (removal of orange light at 600 nm).

fragment in pBluescript KS- (Stratagen) were used as templates for site-specific random PCR mutagenesis (Fig. 1). T3 and T7 primers and designed oligonucleotides containing 32 mixtures of E56 mutations were used for the two-step mega primer PCR. The random PCR primer was designed for introducing random mutation on E56 position of HtrI.

54-A A E T V A S I-61 (amino acid sequences)
5-CGCCGCCGAGACCGTCGCCAGCATC-3 (DNA sequences)
(NNS: N=ATGC, S=GC)

The PCR was performed at 30 cycles of 95°C 1 min, 55°C 1 min, 72°C 3 min (Chen and Przybyla, 1994). After the PCR, the mutation changes were confirmed by DNA sequencing and tested for phenotypes. Random library of the mutated *htrI* gene was inserted by replacing wild-type *htrI* gene in the native HtrI/SRI operon on the *H. salinarum* expression vector pKJ306. Restriction enzymes and T4 DNA ligase were from KOSCHEM (Sungnam, Korea) and pfu DNA polymerase was from VIVAGEN (Sungnam, Korea). Oligonucleotides were purchased from Cosmogentech (Seoul, Korea).

Preparation of membrane vesicles

The cells containing wild-type and mutant protein were grown in 200 mL of CM (Complex Medium) in 250 mL flask or 1.8 L in 2 L flask with mevinolin (1 μg/mL) at 37°C on a gyratory shaker at 200 rpm for 5 days (Jung, 1996). Membrane envelope vesicles were prepared by sonication as described (Bogomolni and Spudich, 1995). Membranes were pelleted for 1 hr at 48,000 rpm in a Beckman L3-50 ultracentrifuge and suspended with 1/100 volume of 4 M NaCl/25 mM Tris-HCl, pH 6.8.

Flash photolysis : S_{373} decay measurement

Flash-induced absorption changes were measured with LFT111 (Luzchem) with a minilite Nd-YAG laser (532 nm, 6 ns pulse, 25 mJ, Continuum USA) providing the actinic flash (Fig. 3). The membrane sample was measured in a 1 cm pathlength quartz cuvette. The flashing frequency was 0.08 Hz. Thirty transients for 8 seconds were recorded and averaged for each trace at constant temperature (18°C) with Tetronics. The amplitudes and $t_{1/2}$ values of S_{373} reprotonation were calculated by fitting of single or double exponential using curve fitting programs from SigmaPlot 8.0 (Jandel).

Cell tracking and motion analysis

The swimming behavior of cells was monitored by a computerized cell-tracking system (Wintrack, 2000). Early stationary phase cultures were diluted 1:20 in fresh CM and incubated for 1 hrs at 37°C with agitation. Saturating intensities of photostimuli activating the SRI dark adapted form, SR_{587}, and its photoactive intermediate, S_{373}. Responses to orange light photostimuli was monitored with infrared light (>700 nm). Phototaxis stimuli were delivered through a halogen lamp. Pulse duration was controlled by a Uniblitz electronic shutter.

RESULTS

Isolation of E56 mutants in HtrI

The 32 mixtures of oligonucleotide were used for the production of random library for E56 position (see Materials and Methods). We sequenced 54 plasmids which contained the E56 mutations to check and confirm for the specific mutations (Fig. 1). We finally isolated 12 different E56 mutations (G, A, R, P, N, M, S, K, T, Q, Y, & D). They included several positively charged, negatively charged,

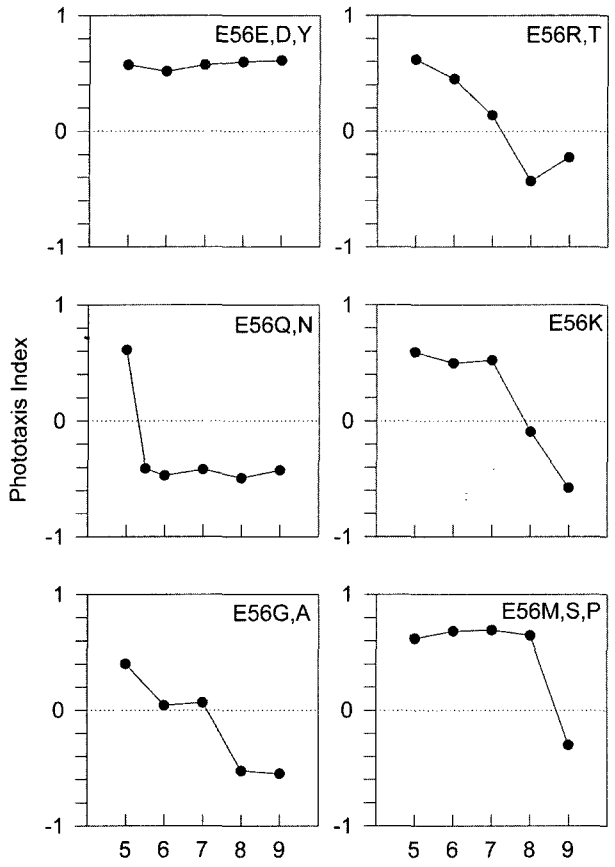


Fig. 3. pH dependency of orange-light response for HtrI mutants at residue position 56. Phototaxis response indices from E56 mutations in HtrI. Phototaxis responses are collected like Fig. 2 and the phototaxis index was calculated from the subtraction of reversals from 2-4 seconds to 6-8 seconds. Positive value indicates attractant responses and negative value indicates repellent responses to orange light (600 nm). Photostimuli were delivered as described in Materials and Methods. E56E is wild-type halobactrerial cells which contains normal SRI/HtrI proteins. Each 12 mutants indicate like this E56D(Asp), Y(Tyr), R(Arg), T(Thr), Q(Gln), N(Asn), K(Lys), G(Gly), A(Ala), M(Met), S(Ser), and P(Pro).

neutral, kink, and bulky groups. Thirty four plasmids had mutations which overlapped and 20 plasmids had no mutations. The halobacterial cells which contained each mutation and wild-type were used for further analysis.

Temperature and pH effects on phototactic behavior of E56Q mutant

SRI/HtrI mediates an attractant response to an orange light and repellent response to a white light. The wild-type response to orange light (600 nm) is suppression of reversals. When the E56Q mutant in HtrI was expressed with SRI in Pho81Wr$^-$, the cell exhibited an abnormal phototactic response, namely, a large increase in population reversals (Jung and Spudich, 1996). Interestingly, this response of orange light inverted mutants became normal when the temperature was lowered to 25°C (Fig. 2). In order to quantify this attractant and repellent response, a four-sec step-down of orange (600 nm) light in an infrared background response was measured by computerized motion analysis system (Wintrack, 2000). The infrared light used for imaging the halobacterial cells was beyond the absorption range of SRI/HtrI complex. The Pho81 with wild-type SRI/HtrI (E56) showed a peak in reversals induced by the disappearance of the orange light (dark shaded in Fig. 2) at any temperature. The reversals were due to the decay of the intermediates of the orange one-photon cycle, the most predominant of which was S_{373}. The orange light inverted mutants exhibited the opposite behavior. The slight suppression of reversals after the light was turned off, followed by a strong induction of reversals after the orange light returned (bottom 35, 40, and 45°C responses in Fig. 2). At 35°C both the attractant and repellent responses to orange light were observed and at 25°C the orange light response was restored to the attractant response. This suggested that the two conformations in SRI/HtrI shifted from **A** (Attractant) conformation to **R** (Repellent) conformation or *vice versa*. We could also detect transitional state at certain temperatures such as 35°C. Also, D201N in SRI mutant corrected the orange light response only at 25°C (data not shown).

The E56Q mutant exhibited inverted (i.e. repellent) responses at pH 5.5 and above (Fig. 3). However, at pH 5.0 the inversion was restored. First, the phototaxis response was measured at 40°C and phototaxis index was calculate. The wild-type, E56D, and E56Y showed a normal phototaxis responses to the orange light as an attractant. Other mutants showed the orange light response in a pH-dependent manner. The responses of several E56 mutants to the orange light was corrected by low pH 6.0 but still showed the inverted phenotype at high pH (Fig. 3). E56Q and N mutant showed the orange light inverted responses except at pH 5.0. On the other hand, E56M, S, and P mutants showed the orange light inverted responses only at pH 9.0. The cell was only incubated in CM for 30 min. This implied that the outer residues of either SRI or HtrI protein were involved in this response. In order to figure out the relationship between the phototaxis response and the photochemical properties of SRI/HtrI complex, we compared the decay of S_{373} (M intermediate in bacteriorhodopsin) in photocycle of SRI.

Photochemical reactivity of E56 mutant

The membrane of wild-type and residue replacements of E56 position (G, A, R, P, N, M, S, K, T, Q, Y, & D) in the *H. salinarum* phototaxis transducer HtrI was prepared for flash photolysis analysis (see Materials and Methods). Immunoblotting showed that the expression level of the mutant HtrI/SRI complex in cells was at near wild-type levels (data not shown). In previous studies, several mutations in SRI and

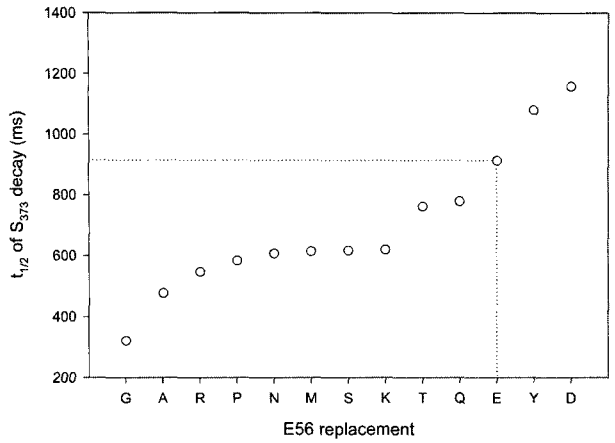
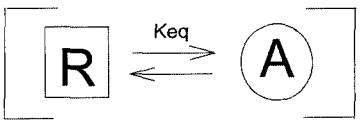


Fig. 4. Photocycle half-lives for HtrI mutants at residue position 56. The $t_{1/2}$ of S_{373} decay in membranes from the halobacterial cells containing the wild-type and E56 mutations. The photocycle was measured at pH 6.8 and 18°C with excitation of halobacterial membrane at 600 nm. Twenty flash photolysis transients were averaged for each determination.



1) light	2 photon (white light)	1 photon (orange light)
2) pH	low pH	high pH
3) temperature	low temp. (25°C)	high temp. (45°C)

Fig. 5. The factors affect the two conformational states in dark. The conformational equilibrium of a wild-type signaling complex and the signaling complex of an orange light inverted mutant is proposed at the relative bias of the dark equilibrium toward R form (repellent form) or toward A form (attractant form) of the WT signaling determines either the response will be attractant or repellent. The range of values in which A and R are both present in sufficient amounts in the dark equilibrium mixture to yield wild-type behavior is placed at the center. The one photon reaction (orange light response), high pH and high temperature shifts the equilibrium toward A form, causing an attractant response. The two-photon reaction, lower pH, and lower temperature shift the equilibrium back toward R form.

HtrI were shown to alter the half-life of the S_{373} intermediate (Jung and Spudich, 1996; Jung and Spudich, 1998) but the relation between the inverted orange light response and the rate of S_{373} decay has not been explained. Half-times from single exponential decay fits are shown for each trace (Fig. 4). The $t_{1/2}$ of S_{373} decay of wild-type was about 900 ms at 18°C. Almost all E56 mutants showed a fast S_{373} decay rate and orange-light inverted responses. On the other hand, E56D and E56Y showed a slow photocycle compared to the wild-type (Fig. 4) and the cells showed the attractant responses to 600 nm photostimuli at pH 5.0 to 9.0 at 40°C (Fig. 3). The most accelerated S_{373} half-life was demonstrated in E56G mutation and the most retarded in E56Y mutation. This effect is interpreted to mean that this residue is critical for coupling HtrI to SRI photochemistry.

DISCUSSION

A model has been proposed in which the SRI/HtrI complex is poised in a metastable equilibrium between two conformations that are easily shifted by changes in light, pH and temperature, and single mutations (Fig. 5). Therefore, several replacements of E56 of HtrI showed the pH-dependent orange light responses (Fig. 3). The interconvertability of the two signaling conformations can explain the response of orange light inverted mutants becoming normal when pH (Jung and Spudich, 1996) or temperature is lowered (Fig. 2). A prediction of the two-conformation shuttling aspect of the model has been confirmed by a suppressor study (Jung and Spudich, 1998). That is the degree of suppression in the dark equilibrium was determined from several suppressor mutations by orange and white light responses.

If the mutations affect the rate-limiting step of spectral transition, it is possible to detect the changes in rate of transition between the spectral intermediates or new photo-intermediates in the SRI photocycle. Flash-induced spectrophotometry involves the measurement of spectral transitions, and it provides information about kinetics from formation and decay of photo-intermediates, structural changes in the protein after light stimulus, and the molecular nature of the interaction sites. We detected different S_{373} decay and the mutants which showed that the fast photocycle gives a orange light inverted response (repellent response). It might be that inverted signaling is due to the fast rate of decay of signaling conformation (S_{373}) which are defined in terms of the structure at local interacting regions between SRI and HtrI.

Two conformations of BR have been directly detected by cryoelectron microscopy (Grigorieff et al., 1996; Subramaniam et al., 1993). Helix F of BR is observed to be tilted outward and opening the structure on the cytoplasmic side in the M_{412} intermediate (corresponding to the S_{373} intermediate of SRI) (Brown et al., 1995). A similar opening structure has been found by X-ray diffraction of the D85N-D96N double mutant in the dark and D85N at alkaline pH. The channel in SRI that is closed by HtrI interaction is functionally similar to the cytoplasmic channel of BR (Spudich, 1994). The rate of decay of S_{373} is pH-dependent in the absence of HtrI but not in the presence of HtrI. It indicates that a proton-conducting path exists between the protonated Schiff base nitrogen and the extramembranous environment in the transducer-free receptor, and transducer binding blocks this path (Olson and Spudich, 1993). Probably, E56 of HtrI mutation twists the helix TM2 and

HtrI is not able to block the channel completely because the rate of S_{373} decay showed partial pH-dependence (Fig. 4) and phototaxis responses were delayed and reduced when the pH_{ext} was high (Fig. 3). This is a piece of evidence that the open and closed conformations of the channel are the **A** and **R** conformation, as proposed. All these results support the existence of two conformations within SRI/HtrI and that this is related to phototaxis signaling.

The detailed kinetic analysis of protein-protein interactions in signal transduction process and phosphorylation activity assay will be performed to understand more about the molecular basis of phototaxis responses in living cells which underlies the increase in the level of phosphorylation *in vitro* causing reverse swimming behavior *in vivo*.

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