The Cytoplasmic Membrane-Proximal Domain of the HtrII Transducer Interacts with the E-F Loop of Photoactivated Natronomonas pharaonis Sensory Rhodopsin II

Chii-Shen Yang, Oleg Sineshchekov*, Elena N. Spudich and John L. Spudich*

Center for Membrane Biology, Department of Biochemistry and Molecular Biology,
University of Texas Health Science Center, Houston TX 77030

*Corresponding author: John.L.Spudich@uth.tmc.edu, telephone 713-500-5473, fax 713-500-0545

Keywords: haloarchaea, phototaxis, phoborhodopsin, fluorescence, FRET, disulfide cross-linking
ABBREVIATIONS

SRII, sensory rhodopsin II from *Natronomonas* (also known as *Natronobacterium*) *pharaonis* (also known as NpSRII and ppR); HtrII, SRII transducer from *N. pharaonis* (also known as NpHtrII); BR, bacteriorhodopsin; SRI, sensory rhodopsin I from *Halobacterium salinarum*; S.D., standard deviation. In this article, the N-terminal fragment of HtrII consisting of residues 1-159 is used exclusively in the *in vitro* measurements. After its initial designation as HtrII<sub>1-159</sub>, we refer to it as “HtrII” for simplicity. Thus, the *in vitro* “SRII-HtrII” complex in this article designates the “NpSRII-NpHtrII<sub>1-159</sub>” complex in more complete notation. The entire HtrII protein for clarity is designated as “full length HtrII”. LY-ia, Lucifer yellow indoacetamide; LY-vs, Lucifer yellow vinyl disulfonate; FRET, Förster (Fluorescence) resonance energy transfer; DDM, n-Dodecyl-β-d-maltopyranoside.
ABSTRACT

The structures of the cytoplasmic loops of the phototaxis receptor sensory rhodopsin II (SRII) and the membrane-proximal cytoplasmic domain of its bound transducer HtrII were examined in the dark and in the light-activated state by fluorescent probes and cysteine cross-linking. Light decreased the accessibility of E-F loop position 154 in the SRII-HtrII complex, but not in free SRII, consistent with HtrII proximity, which was confirmed by tryptophans placed within a 5-residue region identified in the HtrII membrane-proximal domain that exhibited Förster resonance energy transfer (FRET) to a fluorescent probe at position 154 in SRII. The FRET was eliminated in the signaling-deficient HtrII mutant G83F without loss of affinity for SRII. Finally, the presence of SRII and HtrII reciprocally inhibit homodimer disulfide cross-linking reactions in their membrane-proximal domains, showing that each interferes with the others self-interaction in this region. The results demonstrate close proximity between SRII-HtrII in the membrane-proximal domain, and in addition, light-stimulation of the SRII-inhibition of HtrII cross-linking was observed, indicating that the contact is enhanced in the photoactivated complex. A mechanism is proposed in which photoactivation alters the SRII-HtrII interaction in the membrane-proximal region during the signal relay process.
INTRODUCTION

Archaeal sensory rhodopsins I and II (SRI and SRII) are membrane-embedded phototaxis receptors that modulate the motility apparatus of *Halobacterium salinarum* and related haloarchaeal species (1-3). The SRI and SRII proteins transmit signals to their cognate transducer proteins, HtrI and HtrII, respectively, and like their eubacterial counterparts in chemotaxis, the Htr transducers control a histidine-kinase and phosphoregulator protein that modulates motor function.

Biochemical and spectroscopic studies have shown that there is close interaction between the SR and Htr components of the signaling complex both in the light and in the dark (1); i.e. they are subunits of a molecular complex. Furthermore, chimera experiments demonstrated that the interaction specificities of SRI with HtrI and SRII with HtrII are determined by the transmembrane helices of the Htr subunits (4). Cubic lipid phase crystal x-ray structures of SRII have defined the membrane-embedded and membrane-external portions of the protein’s transmembrane helices and periplasmic and cytoplasmic loops (5,6). Moreover, co-crystallization of SRII with an N-terminal fragment of HtrII containing its two transmembrane segments (TM1 and TM2) and a cytoplasmic extension of TM2 provided an atomic-resolution structure by x-ray diffraction (7). The structure resolved extensive van der Waals and hydrogen-bonding
contacts between HtrII TM2 (and to a lesser extent TM1) and SRII helices F and G within the membrane domain. The presence of the cytoplasmic membrane-proximal domain, comprised of 32 residues beyond Leu82 at the membrane-cytoplasm interface, was necessary for high affinity binding of the HtrII fragment to SRII in detergent micelles used in the crystallization (7,8). The structure of the cytoplasmic extension was not resolved by the crystallographic refinement.

Site-specific mutagenesis suggests that the HtrII membrane-proximal domain is critical for signaling. Cysteine-scanning through the length of TM2 from Ala60 to Ala88 showed that no single residue in the membrane-embedded portion is critical for phototaxis function (9). Also, as reported here, mutation of SRII Tyr199 to Phe or to Ala, which eliminates a known hydrogen bond between the Tyr residue and an Asn residue on TM2 in the membrane domain, does not significantly impair signaling. In contrast, cysteine-substitution of the first cytoplasmic residue (mutants HtrII_G83C and HtrII_G83F) completely eliminated phototaxis (9).

Functional importance of the transducer membrane-proximal domain has been more definitively demonstrated in the SRII transducer HtrI, which has been studied in greater detail with respect to physiological function by mutagenesis and biochemical analysis. A suppressor mutation analysis of randomly mutagenized SRI-HtrI complex revealed a
cluster of functionally important residues ranging from positions 53-96 in HtrI (Asn53 corresponds in HtrII to Leu82 at the membrane/cytoplasm interface), and also 3 residues in the cytoplasmic regions of helices F and G of SRI (10). Also a decreased phototaxis response occurs in HtrI I64C (11). Moreover, cytoplasmic extension of HtrI TM2 by 13 residues is necessary and sufficient for HtrI-dependent properties of SRI in the SRI-HtrI molecular complex, including the HtrI-inhibition of opening of its proton-conducting cytoplasmic channel during the photocycle ((12), Chen and Spudich, submitted). Light-induced tilting of helix F (and to a lesser extent helix G), opens a cytoplasmic channel in BR (13) and this tilting has been shown to occur in SRII both when free and bound to HtrII (14,15), with HtrII inhibiting the proton uptake through the channel as in the SRI-HtrI complex. This conformational change, which involves structural alterations mainly in the cytoplasmic end of helix F and in the E-F loop (13), has been proposed based on several lines of evidence to be responsible for activating the Htr transducers (16).

Our goal in this work was to use fluorescent probe accessibility, FRET, and cysteine cross-linking to test (i) whether physical interaction in the cytoplasmic regions of SRII-HtrII occurs, (ii) whether there are conformational changes in the cytoplasmic E-F
loop of SRII influenced by HtrII during photoactivation, and (iii) to investigate the importance of the HtrII cytoplasmic region for phototaxis signal transduction.

MATERIALS AND METHODS

Reagents: All buffers were purchased from either Fisher (Fairlawn, NJ) or Sigma (St. Louis, MO). Lucifer yellow vinylsulfone (LY-vs), L-Cysteine and NEM (N-ethylmaleimide) were from Sigma and IAEDANS and Lucifer yellow iodoamine (LY-ia) was from Molecular Probes (Eugene, OR). DDM was from Anatrace (Maumee, OH) and the Pfu DNA polymerase used in PCR reactions was from Stratagene (La Jolla, CA).

Plasmid construction and site-directed mutagenesis: HtrII was expressed using the pCY8 plasmid while SRII was expressed using the pCY9 plasmid, constructed by PCR (17). pCY8 was constructed by using CY4 (9) as template DNA and two primers: a forward primer Nco-004-F, which encodes a NcoI site in the beginning of the HtrII gene and a reverse primer CY8-his6-R contained the first 159 residues of HtrII and added six histidines in the C-terminus followed by a HindIII site. The resulting NcoI-HindIII fragment was ligated with the large fragment of NcoI-HindIII treated pET21d vector (Novagen) to produce pCY8. The pCY9 was constructed by PCR using a pJS005 (18)
as template DNA and a forward primer encoded the beginning of the SRII gene and a reverse primer encoded the last five SRII residues followed by six histidines and a HindIII site. The resulting NcoI-Hind III fragment was ligated with the large fragment of NcoI-HindIII treated pET21d to produce pCY9.

Single-cysteine substitutions or single-tryptophan substitutions in SRII and HtrII were constructed from the pCY8 or pCY9 plasmids by site-directed mutagenesis and each mutant was confirmed by sequencing.

**Protein expression and sample preparation.** BL21(DE3) cells containing SRII and HtrII in were grown to an $A_{600} = 0.4-0.5$ at 37°C before addition of 1mM IPTG (plus 10µM of all-trans retinal for SRII expression). After 3 hours the cells were harvested by centrifugation (6084g, 20 min) and suspended in buffer M (300mM NaCl, 0.1mM PMSF, 50mM MES, pH 6.8). After two freeze-thaw cycles and sonication (Sonifier 250, Branson) power 5, duty cycle 40%, 10 min), the unbroken cells were separated by centrifugation (7093g, 30 min) and the crude membrane was collected by ultra-centrifugation (100,000g, 1 hr at 4°C). The membrane fraction was solubilized in buffer M with 1% DDM (4ml/L culture) overnight at 4°C on a nutator before ultra-centrifugation (100,000g, 20 min at 4°C) to pellet insoluble membranes. The
supernatant was then incubated with Ni-NTA Agarose resin (1ml/L culture, from Qiagen) overnight, applied to a column, which was washed extensively with 20 volumes of buffer (buffer M with 40mM imidazole, 0.1% DDM, with 10mM β-mercaptoethanol added when purifying single-cysteine mutant proteins) and the protein eluted with 8ml of this buffer with 100 and 200mM imidazole.

**Reactivity of SRII cysteine mutants to LY.** Wild type and mutant SRII and HtrII preparations were adjusted to a final concentration of 50µM (1.2 mg/ml for SRII (“Free SRII”) and 0.9 mg/ml for HtrII) and 50µl of SRII was taken and added to 50µl of buffer M or 50µl of buffer M with HtrII (“SRII-HtrII”). The 100µl samples were then treated with 10 x 1-min bath sonication with 1-min intervals followed by 30-min incubation at room temperature for 30min followed by reaction with 50-fold excess of LY (iodoamine form) for 300 min or the same volume of buffer M (control) in the dark or with 500-nm light illumination. Reactions were then quenched by the addition of L-cysteine to a final concentration of 2mM and samples were injected into Slide-A-Lyzers (0.5ml, Pierce) for dialysis against 2.5L of buffer M with 0.1% DDM overnight. Samples were removed and assessed by SDS-polyacrylamide gel electrophoresis and absorption spectroscopy (CARY 4000 UV-Vis Spectrophotometer, VARIAN). The absorption
spectra of unlabeled samples were subtracted from the spectra of corresponding labeled sample. The absorbance corresponding to 100% labeling was calculated from the SRII concentration ($\varepsilon_{498}=40,000 \text{ cm}^{-1} \text{ M}^{-1}$) and $\varepsilon_{430} = 12,400 \text{ cm}^{-1} \text{ M}^{-1}$ for LY.

**Fluorescent probe labeling for FRET measurements.** Labeling of SRII_S154C with IAEDANS was carried out by treating SRII_S154C protein (in buffer M) at room temperature for 16 hours at a molar ratio of protein:IAEDANS = 1:30. Excess IAEDANS was removed by overnight dialysis against 250 volumes of buffer M with 0.1% DDM. For each single-tryptophan mutant FRET measurement, three cuvettes were used: Cuvette #1 contained IAEDANS-SRII-HtrII with a molecular ratio of 1:1 and cuvettes #2 and #3 contain the same concentration of IAEDANS-SRII and of HtrII, respectively, as in cuvette #1. Emission spectra from 300nm-520nm with excitation at 280nm were then measured. The emission change at 336nm and 472nm were calculated by subtracting the sum of emission spectra from cuvettes #2 and #3 from that of cuvette #1. The final values of emission changes for each mutant was determined by subtracting that observed at position 121 as background.
**Photocycle measurements.** Nd-YAG laser-flash absorbance changes were acquired on a laboratory-constructed flash spectrometer as described in the companion article (Chen & Spudich, submitted).

**Fluorescent probe labeling at S154-SRII for SRII-HtrII interaction measurements.**

Labeling of SRII_S154C with (vinyl sulfone) was carried out by incubating SRII_S154C protein (in buffer M) at room temperature overnight at a molar ratio of protein:LY = 1:50. After excess LY was removed by overnight dialysis against 250 volumes of buffer M with 0.1% DDM, the concentration of labeled sample was determined. For fluorescence measurements (Hitachi F2000), all of the cuvettes were prepared to contain a final concentration 4µM of LY labeled SRII and different concentrations (0, 2.4, 4.4, 22.2 and 45µM) of wild type or G83F-HtrII. After a one minute on and off of total of 10 minutes of ultrasonic bath (with ice), all sample were allowed to sit in room temperature for 30 minutes before fluorescence emission at 520nm (430nm as excitation wavelength) readings were taken for each sample.

**Disulfide bond formation assay.** Cross-linking reactions were carried out as previously described (9). All SRII and HtrII samples were adjusted to 10µM with
buffer M containing 0.1% DDM and cross-linking reactions were initialized with 3mM 1,10-phenanthroline (in ethanol) and 1.5mM CuSO$_4$ for 3 min before terminated by stop solution (2% SDS, 50mM NEM and 5mM EDTA). Photoactivation group were subject to continuously illumination with 500nm light for 3 min before and during the reaction. All samples were then analyzed with 15% SDS-PAGE.

**Solvent-accessible surface area calculations**

Solvent-accessible surface areas ($\AA^2$) were calculated by using MolMol 2K.2 for Linux (19) in SRII (PDB id: 1JGJ and 1H2S). The probing size (solvent radius) was set to be 1.4 Å.

**Software**

Curve-fitting was performed with Prism 4.0 (Graphpad) and Origin Pro 7.0 (OriginLab).

Protein sequence analysis used DNA Star V5.05 (Lasergen).

**RESULTS**

*Light decreases the accessibility of E-F loop position 154 in the SRII-HtrII complex,*

*but not in free SRII.* As a first method to examine the effect of association with HtrII
and of photoactivation on SRII cytoplasmic loop structure, we measured fluorescent probe accessibilities of Cys residues introduced at 8 cytoplasmic surface residues (1 on the A-B, 1 on the C-D, and 6 on the E-F loop) of SRII. Reactivities of the single-cysteine SRII mutants were measured in HtrII-free SRII and HtrII-complexed SRII in the dark and in the light. The accessibilities in the dark of the residues on the A-B and C-D loops were unaffected by the presence of HtrII, whereas HtrII reduced the accessibility at 3 positions on the E-F loop (151, 154, and 158), position 154 most strongly (Figure 1). Surface area calculations from the crystal structures of HtrII-free SRII (Luecke et al., 2001) and SRII bound to a 114-residue HtrII N-terminal fragment (Gordiley et al., 2002) predict nearly equal or greater accessibility in the HtrII-bound SRII at these 3 positions (134Å², 96Å², and 47Å² for free SRII at positions 151, 154, and 158, respectively, and 155Å², 92Å², and 49Å² for the same positions in HtrII-complexed SRII). Therefore, the reduced accessibilities suggest an effect of the unseen presence of HtrII residues in this region (i.e. residues present but unresolved in the crystal structure of the complex), which inhibit access to the probe.

Probe accessibilities of the single-cysteine SRII mutants were then measured under 500-nm illumination in free SRII and HtrII-complexed SRII. In free SRII, none of the positions exhibit significant changes in accessibility upon photoactivation (Figure 2),
confirming the logic of this approach since the residues were selected for the property of protruding outward into the cytoplasm in the crystal structure and therefore should be relatively insensitive to structural perturbations. However, in the presence of HtrII, photoactivation caused a decrease of 40% labeling efficiency in residue S154C in SRII and smaller but significant changes in S158C and L159C (Figure 2). We conclude that there is an influence of HtrII on the light-induced structural change of SRII or on its consequence in the E-F loop at position 154, and to a lesser extent positions 158 and 159. S154C shows the largest decrease of labeling efficiency in the dark as well as the largest HtrII-dependent effect of light activation.

These results strongly suggest that HtrII residues are physically near Ser154 in the dark and the residues interact with Ser154 upon light activation of the receptor, further hindering probe-access. To test this possibility further and to localize the region of interaction in HtrII we examined whether Trp residues introduced into the membrane-proximal domain would be within energy-transferring distances from a fluorescent probe attached to SRII_S154C.

*Tryptophans placed within a short region in the HtrII membrane-proximal domain exhibit Förster resonance energy transfer to a fluorescent probe at position 154 in SRII.*
We constructed a series of single Trp substitutions at HtrII residue positions 87-95, and at 121. HtrII_{1-159} does not contain native Trp residue, hence the substitutions result in single-Trp HtrII mutants. The 87-95 region was chosen based on its projected location at a similar distance from the membrane as Ser154 in SRII (assuming an $\alpha$-helical extension of TM2 roughly perpendicular to the membrane), and position 121 was selected as a negative control since it is expected to be well beyond interaction distances from the SRII cytoplasmic domain. In complex with SRII_S154C labeled with IAEDANS (Fig. 3), the single Trp mutants exhibited energy transfer from HtrII positions 91-95 with a peak at position 93. No energy transfer was observed from positions 87-89 nor from the Trp at 121. Our interpretation is that the region near Ser154 of SRII interacts with the 91-95 region of HtrII.

FRET is eliminated by the signaling-deficient HtrII mutant G83F without loss of affinity for SRII. To test for a relationship of the FRET-detected proximity of SRII and HtrII to function, we repeated the FRET measurements with the function-deficient mutant HtrII_G83F. FRET is eliminated in this mutant (Fig. 3), and therefore it became critical to determine whether the binding affinity, presumably determined by the extensive interactions between the SRII and HtrII transmembrane domains, was altered by the
mutation.

To evaluate the SRII-HtrII binding constant in 0.1% DDM as used in the FRET assays, we applied two different methods. The first was based on an assay developed by Kamo and coworkers (20), which demonstrated that the photocycle of SRII_D75N was affected by the binding of HtrII and thus, flash photolysis of SRII_D75N can be used to determine its affinity for HtrII. The kinetics of the flash-induced absorption change at 570 nm in free SRII_D75N and that of SRII_D75N complexed with HtrII (Fig. 4A) shows that association with transducer resulted in changes in both the relative amplitudes and the rates of kinetic components in the photocycle of the SRII. We used the full amplitude of the differential signal (Fig. 4A, insert) to assess the extent of binding. Based on the concentration-dependence effect of HtrII on the photocycle of SRII_D75N (Figure 4B), the Kd was determined to be 0.35 µM under our conditions. Similar measurement with HtrII_G83F produced a Kd of 0.22µM. These values are close to the affinities measured in previous reports (8,20), and show that the G83F mutation does not significantly inhibit HtrII binding to SRII_D75N.

Since the above measurement used SRII_D75N whereas the FRET measurements used SRII_S154C, we applied a second method using the fluorescent probe lucifer yellow
vinyl sulfone (LY) attached to SRII_S154C as a fluorescent reporter group for the binding of HtrII. LY has been used successfully in several studies to report protein conformational changes (21) or protein association (22). LY-labeled SRII_S154C was divided into several different aliquots with equal volume and different concentrations of HtrII (Fig. 4C) or HtrII_G83F (Fig. 4D) were added. Both wild type HtrII and HtrII_G83F cause an increase in the LY fluorescence signal and the saturation curves are identical, confirming that the mutation does not inhibit binding. Therefore the loss of FRET between SRII_S154C-IAEDANS and Trp residues in HtrII_G83F is not due to loss of association and results instead from a change in HtrII structure evidently distancing the protein in the cytoplasmic region from SRII.

**SRII and HtrII reciprocally inhibit disulfide cross-linking reactions in their membrane-proximal domains.** Two additional experiments were designed to test for physical interaction in the membrane-proximal domains of SRII-HtrII. First, disulfide cross-linking reactions of SRII_S150C and SRII_S154C were carried out with or without HtrII (Figure 5A). The cross-linking efficiency at S150C was not affected by the presence of HtrII, whereas the cross-linking in S154C was abolished by association with HtrII. This result supports the fluorescent probe labeling data at S150 and S154, only
the latter of which showed a significant decrease in labeling when complexed with HtrII, and provide further evidence for proximity of SRII and HtrII in the membrane-proximal region.

The converse experiment was performed to test whether SRII would inhibit disulfide cross-linking of the mono-Cys mutant HtrII_L90C. The presence of SRII reduced the cross-linking efficiency of L90C and it was further decreased when SRII was photoactivated (Figure 5B). These results further indicate the existence of close proximity between SRII-HtrII in the membrane-proximal domain, and in addition, suggest that the contact is enhanced when the receptor is photoactivated, in agreement with the accessibility data.

DISCUSSION

The results above (i) provide compelling evidence for close proximity of E-F cytoplasmic loop residue Ser154 in SRII to residues 91-95 in the HtrII membrane proximal domain, (ii) show that SRII photoactivation alters the interaction in this region, and (iii) correlate the loss of interaction with the loss of function in HtrII mutant G83F. The results suggest a model in which the crystallographically demonstrated (7) tight interactions between SRII and HtrII membrane-embedded domains hold the cytoplasmic
domains of the receptor and transducer in close juxtaposition, and SRII photoactivation, which is believed to cause an outward displacement of helix F (14,16) that would in turn be expected to alter the structure of the E-F loop, alters the interaction in the membrane-proximal region during the signal relay process (Figure 6a).

Site-directed spin-labeling measurements revealed a light-induced transient decrease of nitroxide mobility in SRII S158C-R1 which faces HtrII TM2 (where R1 indicates the spin label attached to the Cys residue) and an increase in mobility of the probe attached at L159C, which faces away from TM2 on helix F. These results were interpreted as a movement of the helix toward TM2 of HtrII during activation (14,15). This movement fits well with the model suggested here. Ser154 is 2 helical turns below Ser158 facing in the same direction and the HtrII 91-95 region is on the cytoplasmic extension of TM2. Assuming a similar structural change in SRII as in light-activated BR, the proposed motion of Ser154 toward HtrII 91-95 residues would entail tilting of helix F from Pro175 in the membrane-embedded portion of SRII, and would require the Ser158/Leu159 motion.

Cysteine-scanning through the length of TM2 from Ala60 to Ala88 showed that no single residue in the membrane-embedded SRI-HtrI interface is critical for phototaxis function (9). Furthermore mutation of SRII Tyr199 to Phe or to Ala, which eliminates a
known hydrogen bond between the Tyr residue and an Asn residue on TM2 in the membrane domain, does not significantly impair phototaxis signaling assessed by computerized cell tracking (data not shown). In contrast to this permissive property of the SRI-HtrI interface within the bilayer, mutation of HtrII Gly83 to Cys or Phe causes a total loss of phototaxis responses in *H. salinarum* (9). Gly83 is the first residue beyond the membrane-cytoplasm interface in HtrII and begins the sequence Gly-Gly-Asp-Thr, which protein structure analysis (Figure 6A) predicts to be a turn and a flexible region. Also, a second turn and flexible region is predicted at positions 101-104, which would serve to bring the two post-turn helices of the HtrII monomers together in the coiled coil dimer motif expected from sequence algorithms for HtrII (11) and observed in the homologous Tsr transducer (23). In the model (Figure 6B), elimination of the turn at the membrane-cytoplasm interface by mutation of Gly83 would result in loss of the proper interaction in the cytoplasmic 90-95 position stretch and hence account for loss of phototaxis signaling.

Several lines of evidence (14-16) indicate SRII undergoes a light-induced conformational change similar to that of BR, which undergoes a rotation and outward tilting of helix F and associated movement of the E-F loop (24-26). The results from Ser154 labeling reported here confirm the movement of the E-F loop upon SRII
photoactivation. For BR the conformational change has been resolved by cryoelectron crystallography (13), and most of the movement occurs in the cytoplasmic region when comparing the “closed” and “open” states (Figure 6B). Since SRII has a very similar structure to that of BR, in Figure 6B SRII in the SRII-HtrII complex structure was replaced with the structure of the BR “open” state to visualize possible interaction with HtrII during the SRII conformational change. The conformational changes in helix F and G and the E-F loop are substantial in the cytoplasmic region, and are therefore likely to induce movement of the 91-95 region of HtrII shown here to be in close proximity to the E-F loop.

A stretch of 5 residues on the HtrI membrane-proximal domain (positions 62-66) blocks or prevents opening of the cytoplasmic proton-conducting channel during the photocycle of transducer-free SRI (Chen and Spudich, submitted). It is very likely that SRI undergoes a similar conformational movement of helix F and the E-F loop as do BR and SRII. Aligning HtrI and HtrII shows that positions 62-66 in HtrI correspond to positions 91-95 in HtrII, the interacting 5-residue region revealed in our FRET analysis of the SRII-HtrII complex. The evidence for receptor interaction in the corresponding 5-residue stretch in HtrI obtained by a completely different method in the companion article (Chen and Spudich, submitted) further strengthens the conclusions here, and
argues for a similar coupling between SRI and HtrI as we observe for SRII and HtrII.

Conformational change in the cytoplasmic E-F loop upon light-activation appears to be a common feature among seven transmembrane photoreceptors. In BR, conformational change in helix F and the E-F loop occur in the proton pumping cycle (26-28), while in bovine rhodopsin, the E-F loop is involved in G protein activation (28-31). In this report, changes in the E-F loop of SRII upon association of transducer are demonstrated and this change is enhanced upon photoactivation. Furthermore the data provide compelling evidence for physical coupling of the SRII E-F loop to membrane-proximal residues in HtrII.
ACKNOWLEDGEMENT

This research was supported by National Institutes of Health grant R37GM27750 and the Robert A. Welch Foundation.

FIGURE LEGENDS

Figure 1. Effects of HtrII-1-159-binding on labeling efficiencies in SRII. Lucifer yellow iodoacetamide (LY-ia, dipotassium salt, shown in Figure 2) was used for labeling single-cysteine SRII mutants alone (shaded bars) or mutants complexed with HtrII (textured shaded bars) in the dark. Length of error bars are 1 S.D. for 2-4 independent measurements. In subsequent figure legends “HtrII-1-159” is designated “HtrII”.

Figure 2. Light-induced labeling efficiency changes in free SRII and SRII-HtrII complex. Photoactivation-dependent labeling efficiency changes in SRII (unshaded bars) and SRII-HtrII complex (textured filled bars) were calculated as % change from the dark values (Figure 1). Length of error bars are 1 S.D. for 2-4 independent measurements. Chemical structure of LY-ia used for labeling in Figures 1 and 2.
Figure 3. FRET measurements of single-Trp HtrII mutants to SRII_S154C-IAEDANS. The changes in Trp emission (336 nm, black bars) and IAEDANS emission (472 nm, gray bars) are shown. For each single-Trp mutant FRET measurement, three cuvettes were used: Cuvette #1 contained SRII-HtrII-IAEDANS with a molecular ratio of 1:1 and cuvettes #2 and #3 contained the same concentration of either SRII-IAEDANS or HtrII, respectively, as cuvette #1. Emission spectra from 300nm-520nm with excitation at 280nm were then measured. The emission changes at 336 nm and 472 nm were calculated by subtracting the sum of emission spectra from cuvettes #2 and #3 from that of cuvette #1. The final values of emission changes for each mutant were determined by subtracting -3.45 for 336nm and -1.14 for 472nm, the value observed for position 121 taken as background. G83F-T92W and G83F-L93W indicate double mutants which combine the function-deficient mutation G83F in HtrII with either T92W or L93W, respectively.

Figure 4. Binding affinity of SRII-HtrII in 0.1% DDM. A. Flash-induced absorption change of SRII_D75N with and without HtrII bound. B. The concentration-dependence of the differential signal from A. on HtrII concentration. Inset: \( \Delta(\Delta A) \) signal amplitude versus HtrII concentration linear saturation curve. Data were fit
with the Hill function (shown) to determine $K_d$ and $n$. C. Binding of HtrII to SRII measured by the fluorescent probe lucifer yellow vinyl sulfone (LY, insert)-modified SRII at residue S154C. The fluorescence increases in the SRII_S154C-LY upon addition of HtrII (left) or HtrII_G83F (D).

**Figure 5.** Disulfide cross-linking reactions in single-cysteine SRII mutants alone and complexed with HtrII. Membrane proteins separated by 15% SDS-PAGE. XL, 3mM 1,10-phenanthroline and 1.5mM CuSO$_4$ added; HtrII, HtrII$_{1-159}$. A. The cross-linking reactions of membranes containing SRII_150C or SRII_S154C with and without HtrII and/or Cu-phenanthroline. Reactions carried for 3 min at 24°C in 500-nm light before terminated by addition of stop solution [2% SDS, 50mM NEM (N-ethylmaleimide) and 5mM EDTA]. B. The cross-linking reactions of membranes containing HtrII_L90C with and without SRII and/or Cu-phenanthroline. Reactions carried for 3 min at 24°C in the dark or 500-nm light, as indicated before stop solution was added.

**Figure 6.** Model of the cytoplasmic domain in HtrII and the conformational change in model SRII. A. Residues 1-159 of HtrII were analyzed with algorithms
identified at the right of the figure. Circle a indicate residues 49-52 as the turn structure predicted by the Chou-Fasman method, and is confirmed by the crystallographic structure (7). Flexible regions and possible turns are also predicted by the algorithm at circle b (residues 83-86) and circle c (residues 101-104). **B.** Closed state (green, pdb id: 1FBK) and open state (yellow, pdb id: 1FBB) structures of BR were substituted for the structure of SRII in the SRII-HtrII complex (pdb id: 1H2S). The proposed position of residues 83-110 of SRII is indicated in purple.
Figures

Figure 1:
Figure 2:

Light-induced labeling efficiency changes in SRII and SRII-HtrII complex (%)

SRII mutation

Chemical structure of the ligand.
Figure 3:

![Graph showing relative fluorescence change by HtrII mutation](http://www.jbc.org/)
Figure 4:

A. 

B. 

C. 

D. 

\[ y = \frac{x^n}{K_d^n + x^n} \] 

\( K_d = 0.35 \, \mu M \) 

\( n = 1.09 \)
Figure 5

A.

| SRII S150C | SRII S154C |
|------------|------------|
| 500nm      | 500nm      |
| SRII       | SRII       |
| XL         | XL         |
| + HtrII    | + HtrII    |

B.

| HtrII L90C |
|------------|
| Dark       | 500nm      |
| SRII       | HtrII      |
| XL         | XL         |
| + HtrII    | + HtrII    |

- SRII dimer
- HtrII dimer
- SRII monomer
- HtrII monomer
Figure 6:

B.
References

1. Hoff, W. D., Jung, K. H., and Spudich, J. L. (1997) *Annu Rev Biophys Biomol Struct* 26, 223-258

2. Klare, J. P., Gordeliy, V. I., Labahn, J., Buldt, G., Steinhoff, H. J., and Engelhard, M. (2004) *FEBS Lett* 564, 219-224

3. Spudich, J. L., and Jung, K. H. (2004) *Microbial rhodopsins: phylogenetic and functional diversity*. Handbook of Photosensory Receptors, in press. (Briggs, W., and Spudich, J. L., Eds.), Wiley-VCH, Weinheim

4. Zhang, X. N., Zhu, J., and Spudich, J. L. (1999) *Proc Natl Acad Sci U S A* 96, 857-862

5. Luecke, H., Schobert, B., Lanyi, J. K., Spudich, E. N., and Spudich, J. L. (2001) *Science* 293, 1499-1503

6. Royant, A., Nollert, P., Edman, K., Neutze, R., Landau, E. M., Pebay-Peyroula, E., and Navarro, J. (2001) *Proc Natl Acad Sci U S A* 98, 10131-10136

7. Gordeliy, V. I., Labahn, J., Moukhametzianov, R., Efremov, R., Granzin, J., Schlesinger, R., Buldt, G., Savopol, T., Scheidig, A. J., Klare, J. P., and Engelhard, M. (2002) *Nature* 419, 484-487

8. Hippler-Mreyen, S., Klare, J. P., Wegener, A. A., Seidel, R., Herrmann, C.,
Schmies, G., Nagel, G., Bamberg, E., and Engelhard, M. (2003) *J Mol Biol* 330, 1203-1213

9. Yang, C. S., and Spudich, J. L. (2001) *Biochemistry* 40, 14207-14214

10. Jung, K. H., and Spudich, J. L. (1998) *J Bacteriol* 180, 2033-2042

11. Zhang, X. N., and Spudich, J. L. (1998) *J Biol Chem* 273, 19722-19728

12. Spudich, E. N., and Spudich, J. L. (1993) *J Biol Chem* 268, 16095-16097

13. Subramaniam, S., and Henderson, R. (2000) *Nature* 406, 653-657

14. Wegener, A. A., Chizhov, I., Engelhard, M., and Steinhoff, H. J. (2000) *J Mol Biol* 301, 881-891

15. Wegener, A. A., Klare, J. P., Engelhard, M., and Steinhoff, H. J. (2001) *Embo J* 20, 5312-5319

16. Spudich, J. L. (1998) *Mol Microbiol* 28, 1051-1058

17. Chen, B., and Przybyla, A. E. (1994) *Biotechniques* 17, 657-659

18. Kunji, E. R., Spudich, E. N., Grisshammer, R., Henderson, R., and Spudich, J. L. (2001) *J Mol Biol* 308, 279-293

19. Koradi, R., Billeter, M., and Wuthrich, K. (1996) *J Mol Graph* 14, 51-55, 29-32

20. Sudo, Y., Iwamoto, M., Shimono, K., and Kamo, N. (2002) *J Photochem Photobiol B* 67, 171-176
21. Yang, C. S., Skiba, N. P., Mazzoni, M. R., and Hamm, H. E. (1999) *J Biol Chem* 274, 2379-2385

22. Skiba, N. P., Yang, C. S., Huang, T., Bae, H., and Hamm, H. E. (1999) *J Biol Chem* 274, 8770-8778

23. Kim, K. K., Yokota, H., and Kim, S. H. (1999) *Nature* 400, 787-792

24. Rink, T., Pfeiffer, M., Oesterhelt, D., Gerwert, K., and Steinhoff, H. J. (2000) *Biophys J* 78, 1519-1530

25. Xiao, W., Brown, L. S., Needleman, R., Lanyi, J. K., and Shin, Y. K. (2000) *J Mol Biol* 304, 715-721

26. Brown, L. S., Needleman, R., and Lanyi, J. K. (2002) *J Mol Biol* 317, 471-478

27. Thorgeirsson, T. E., Xiao, W., Brown, L. S., Needleman, R., Lanyi, J. K., and Shin, Y. K. (1997) *J Mol Biol* 273, 951-957

28. Alexiev, U., Rimke, I., and Pohlmann, T. (2003) *J Mol Biol* 328, 705-719

29. Franke, R. R., Sakmar, T. P., Graham, R. M., and Khorana, H. G. (1992) *J Biol Chem* 267, 14767-14774

30. Resek, J. F., Farahbakhsh, Z. T., Hubbell, W. L., and Khorana, H. G. (1993) *Biochemistry* 32, 12025-12032

31. Dunham, T. D., and Farrens, D. L. (1999) *J Biol Chem* 274, 1683-1690
The cytoplasmic membrane-proximal domain of the HtrII transducer interacts with the E-F loop of photoactivated natronomonas pharaonis sensory rhodopsin II
Chii-Shen Yang, Oleg Sineshchekov, Elena N. Spudich and John L. Spudich

J. Biol. Chem. published online July 15, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406504200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts