Five Residues in the HtrI Transducer Membrane-proximal Domain Close the Cytoplasmic Proton-conducting Channel of Sensory Rhodopsin I*

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Transducer-free sensory rhodopsins carry out light-driven proton transport in Halobacterium salinarum membranes. Transducer binding converts the proton pumps to signal-relay devices in which the transport is inhibited. In sensory rhodopsin I (SRI) binding of its cognate transducer HtrI inhibits transport by closing a cytoplasmic proton-conducting channel necessary for proton uptake during the SRI photochemical reaction cycle. To investigate the channel closure, a series of HtrI mutants truncated in the membrane-proximal cytoplasmic portion of an SRI-HtrI fusion were constructed and expressed in H. salinarum membranes. We found that binding of the membrane-embedded portion of HtrI is insufficient for channel closure, whereas cytoplasmic extension of the second HtrI transmembrane helix by 13 residues blocks proton conduction through the channel as well as full-length HtrI. Specifically the closure activity is localized in this 13-residue membrane-proximal cytoplasmic domain to the 5 final residues, each of which incrementally contributes to reduction of proton conductivity. Moreover, these same residues in the dark incrementally and proportionally increase the pKₐ of the Asp-76 counterion to the protonated Schiff base chromophore in the membrane-embedded photoreactive site. We conclude that this critical region of HtrI alters the dark conformation of SRI as well as light-induced channel opening. The 5 residues in HtrI correspond in position to 5 residues demonstrated on the homologous NpHtrII to interact with the E-F loop of its cognate receptor NpSRII in the accompanying article (Yang, C.-S., Sineschekov, O., Spudich, E. N., and Spudich, J. L. (2004) J. Biol. Chem. 279, 42970–42976). These results strongly suggest that the membrane-proximal region of Htr proteins interact with their cognate sensory rhodopsin cytoplasmic domains as part of the signal-relay coupling between the proteins.

Halobacterium salinarum membranes contain 4 structurally similar seven-helix retinylidene proteins: bacteriorhodopsin (BR), halorhodopsin (HR), sensory rhodopsin I (SRI), and sensory rhodopsin II (SRII). SRI and SRII, together with their bound transducers HtrI and HtrII, respectively, mediate phototaxis responses (1–3). BR and HR are transport rhodopsins that carry out electrogenic outward proton and inward chloride translocation (4, 5). Without their transducers bound, the SRs also exhibit electrogenic proton pumping activity (6–9). This result and the similar atomic structures of BR (10, 11), HR (12), and NpSRII (13, 14) suggest a common mechanism for haloarchaeal rhodopsin transport and signaling (15). In particular, in BR, a light-induced outward tilting of helices (primarily helix F and to a smaller extent helix G) opens a cytoplasmic side channel important for proton uptake in its pumping cycle (16). Flash photolysis, proton flux measurements, and mutant data (15) and site-directed spin labeling (17) provide compelling evidence that a similar conformational change opens cytoplasmic proton-conducting channels in transducer-free SRs during their photocycles. Transducer binding inhibits the light-induced cytoplasmic side proton-conductivity increase and therefore either prevents the channel opening or blocks the channels (7, 18–20). Channel closure by the transducers appears to be a key part of the mechanism for converting SRs from proton pumps to sensory receptors (21) and understanding its structural basis is expected to provide insight into the SR-Htr signal-relay mechanism.

A 114-residue N-terminal Natronomonas pharaonis HtrII (NpHtrII) fragment containing the transmembrane domains (TM1 and TM2) and a 32-residue extension of TM2 into the cytoplasm (the “membrane-proximal domain”) closes the channel of NpSRII in a micellar system and in Xenopus oocytes (22). X-ray structures of NpSRII are available both for the free receptor (13, 14) and for receptor bound to this fragment (23). The atomic structures of NpSRII in the transducer-free form and in the NpSRII-NpHtrII(1–114) complex are essentially identical and therefore the mechanism of the channel closure is not evident from comparison of their dark structures. The structure of the interacting transmembrane domain of NpHtrII was refined at atomic resolution in the complex, and reveals that the membrane-embedded portions of helices F and G of NpSRII interact with TM1 and TM2, but the cytoplasmic portion of NpHtrII is unresolved in the structure of the complex. Therefore it is not clear from the crystallographic data whether the membrane-proximal domain of NpHtrII interacts with NpSRII and whether such a physical interaction might be responsible for inhibiting the channel activity.

C-terminal deletion analysis showed that the N-terminal 147
residues of HtrI (full-length HtrI contains 536 residues) are sufficient to close the channel of SRI in the native *H. salinarum* membrane (24). Shorter fragments either were not expressed well and/or folded improperly in the *H. salinarum* cell. Our main goal in the present study was to test more precisely which portions of HtrI are needed for channel closure and to determine whether specific residues are needed. We took advantage of expressing the SRI-HtrI fusion protein is fully functional in phototaxis and exhibits photochemical reactions indistinguishable from those of the wild-type SRI-HtrI complex (25), and that shortening the HtrI domain of the fusion proteins, even including complete removal of the membrane-proximal cytoplasmic portion, does not destabilize the fusion proteins, which could be expressed at high levels suitable for characterization in native cell membranes (results herein).

**RESULTS**

**Experimental Procedures**

**Strains and Plasmids**—Wild-type SRI-HtrI complex was expressed from its native promoter in plasmid pJK306 (26) and HtrI-free SRI and SRI-HtrI full-length fusion were expressed under the bop promoter in plasmids pTR2 and pXP6, respectively (18, 25). Plasmid pXP6 was constructed by recombination PCR using a 9-residue flexible linker (25, 27) to connect the C terminus of SRI and the N terminus of HtrI. Using pXP6 as template, the C-terminal truncated mutants of SRI-HtrI fusion, SRI-linker fusion, and SRI-HtrI-(54–66) were generated by PCR with specific primers containing AgeI and XbaI restriction enzyme sites. The PCR fragments replaced the AgeI and XbaI fragment of pXP6. Recombinant PCR was used to construct the SRI-HtrII fusion with the same method as that used for the SRI-HtrI fusion (25). SRI-HtrII-(1–302)-HtrI-(54–66) was constructed by PCR using SRI-HtrII fusion as template. All the single residue mutations were introduced by the two-step mega-primer PCR method with pfu polymerase (28). *H. salinarum* strain Pho81Wr- (BR HR SRI HtrI SRII HtrII and restriction-deficient) (29) was used as the recipient in plasmid transformations.

**Membrane Preparation**—Membranes were prepared by sonication of cells grown as described (30) to stationary phase in complex medium containing 1 μg/ml mevinolin. Membranes were pelleted for 1 h at 23,000 × g in a Beckman Optima™ L-100 XP ultracentrifuge and suspended in 4 M NaCl, 25 mM Tris-HCl (pH 6.8) at 2°C.

**Immunoblot Analysis**—5 μl of membrane suspension was added to 190 μl of SDS loading buffer and incubated at room temperature for 30 min. The mixture was separated by 12% SDS-PAGE. Proteins were electrophoretically transferred to polyvinylidene difluoride membrane at 4°C overnight and detected by anti-SRI antibody. Immunoblots were developed using the ECL Western blotting kit.

**Flash Photolysis**—Flash-induced absorption changes were measured by a laboratory constructed cross-beam spectrometer with a frequency doubled Nd:YAG laser (532 nm, 6-nm pulse, 40 mJ) providing the actinic flash. The flash duration was 0.08 Hz. A total of 12–36 transients were averaged for each trace at a constant temperature of 21°C.

**pH Titration**—The pH of the pigment suspension was measured with a Beckman 472 pH meter after sodium hydroxide solution was added in microliter quantities to the cuvette. Absorption difference spectra were recorded with a Cary 4000 UV-visible spectrophotometer (Varian Analytical Instruments, Walnut Creek, CA). The change in absorption of the suspension at 650 nm was used as a quantitative measure of the pH dependence of the blue-purple transition, from spectra adjusted for baseline drift by fixing the value at 750 nm, at which neither the blue nor purple SRI absorbs. Care was taken to accept only data sets in which the isosbestic point for the blue-purple transition was maintained throughout the titration. All samples were in 4 M NaCl. Fits to the titration data were performed in Origin 7.0 from Originlab (North Hampton, MA).

**Motion Analysis**—Motility responses to SRI photoactivation were assayed by computer-assisted cell tracking and motion analysis as described (26). Swimming cells were subjected to a 4-s step-down in light in 600–20 nm light to test attractant signaling, and a 100-ms step-up in 380–600 nm light to test repellent signaling.

**The Membrane-proximal Domain of HtrI Is Required to Close the Cytoplasmic Channel of SRI**—HtrI-free SRI has a similar proton pumping activity as BR, releasing a proton to the extracellular side from the protonated Schiff base in the photoactive site and in the second half of the photocycle taking up a proton through the cytoplasmic channel of the molecule (6). The photocycle kinetics of free SRI is rate-limited by the return of the proton to the SRI photoactive site, and therefore is strongly pH-dependent for the open channel SRI (19). The proton return is readily monitored as the decay of the unprotonated Schiff base photocycle intermediate (called "M" or S373, λmaj 373 nm) to the far red-shifted Schiff base-protonated state with absorption maximum at either 587 or 552 nm (depending on whether Asp-76 near the Schiff base is neutral or ionized, respectively). The pH dependence of the decay of M, which kinetically matches the return of the unphotolyzed state (25) in HtrI-free SRI under the conditions in this study was found to exhibit a slope of 1.0 in the range of pH between 5.0 and 7.5 (Fig. 1); i.e. the rate of Schiff base reprotoonation is directly proportional to the proton concentration of the medium.

In the HtrI-complexed SRI the proton is not exchanged with the medium (18) and therefore the reprotoonation of the Schiff base is relatively insensitive to external proton concentration (19). The decay of M exhibits a slight acceleration with higher pH and the slope of the log of the M decay half-time versus proton pH is slightly negative (about −0.07) when the channel is closed (Fig. 1). The pH dependence of the photocycle rate therefore provides a robust quantitative measure of the extent of channel closure.

To test whether the membrane-proximal cytoplasmic domain of HtrI closes the channel, a series of truncation mutants of SRI-HtrI fusion were constructed (Fig. 2) and expressed in *H. salinarum* membranes. Membrane suspensions of these mutants immunoblotted with anti-SRI antibody showed that all of the fusions are intact in the membrane, with no detectable proteolytic bands (Fig. 3). All the fusions were expressed under the control of the bop promoter and have higher yields than that of SRI expressed under the native promoter in the htrI sopI operon. The SRI-HtrI fragment fusions all have similar expression yields (Fig. 3).

The photocycles of SRI-HtrI-(1–53) and SRI-HtrI-(1–61) exhibit pH dependence similar to that of free SRI but with the slopes reproducibly decreased by −10% (slopes 0.88 and 0.86, respectively; Fig. 4). SRI-HtrI-(1–72), SRI-HtrI-(1–85), and SRI-HtrI-(1–147) exhibit a small negative pH dependence iden-
tical to that of full-length SRI-HtrI complex (Fig. 4). We conclude that the N-terminal 61 residues of HtrI are not sufficient to close the channel, whereas the N-terminal 72 residues of HtrI are sufficient to close the channel just as effectively as longer HtrI fragments.

SRI-HtrI-(1–53) is predicted to contain the two transmembrane segments of the transducer but no cytoplasmic extension (Fig. 2) based on aligning HtrI with NpHtrII for which the membrane-embedded portion is known from the crystal structure (23). The small decrease in the slope in SRI-HtrI-(1–53) argues that the transmembrane portion alone of HtrI binds to SRI but does not significantly close the channel. Also the pH dependence shifts 1 unit to higher pH (compare Figs. 1 and 4). To exclude that the 9-residue linker, which connects the C terminus of SRI and N terminus of HtrI, is responsible for this small decrease and pH shift, we constructed an SRI-linker fusion without an HtrI fragment, which showed a pH dependence identical to that of free SRI. To further confirm that the photocycle change of SRI-HtrI-(1–53) derives from the specific interaction between the SRI receptor and the transmembrane portion of HtrI, an SRI-HtrII fusion was constructed. HtrII, the cognate transducer for sensory rhodopsin II (SRII), does not function with SRI (31). This fusion also exhibited the same pH dependence as free SRI.

Fine Mapping of the HtrI Channel Closure Activity in the Membrane-proximal Domain

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Extending the cytoplasmic domain by 8 residues (SRI-HtrI-(1–61)) did not increase channel closure, whereas extending it by 19 (SRI-HtrI-(1–72)) is sufficient for the full channel closure effect. To examine this region in finer detail, we made truncated fusion mutants at each residue between positions 61 and 72 of HtrI. Partial closure of the channel is observed for lengths of the transducer up to SRI-HtrI-(1–66), which is the shortest that exhibits a pH dependence slope indistinguishable from that of full-length HtrI (Fig. 6). Therefore the N-terminal 66 residues of HtrI, containing TM1, TM2, and the first 13 cytoplasmic amino acids, are sufficient to close the channel. The 5 residues from 62 through 66 in HtrI contribute most to the channel closure of SRI.

HtrI binding increases the single-flash yield of M in the SRI photocycle because the transducer suppresses thermal branching reactions from S610 (K) and S560 (L) states to the unphotolyzed state (32). The flash yield of SRI-HtrI-(1–53) is intermediate between that of free and HtrI-complexed SRI (Fig. 5), confirming again that the transmembrane domain of HtrI alone exhibits some interaction with the SRI receptor.

Fine Mapping of the HtrI Channel Closure Activity in the Membrane-proximal Domain—Extending the cytoplasmic domain by 8 residues (SRI-HtrI-(1–61)) did not increase channel closure, whereas extending it by 19 (SRI-HtrI-(1–72)) is sufficient for the full channel closure effect. To examine this region in finer detail, we made truncated fusion mutants at each residue between positions 61 and 72 of HtrI. Partial closure of the channel is observed for lengths of the transducer up to SRI-HtrI-(1–66), which is the shortest that exhibits a pH dependence slope indistinguishable from that of full-length HtrI (Fig. 6). Therefore the N-terminal 66 residues of HtrI, containing TM1, TM2, and the first 13 cytoplasmic amino acids, are sufficient to close the channel. The 5 residues from 62 through 66 in HtrI contribute most to the channel closure of SRI.

Channel Closure by the Membrane-proximal Domain Requires the Membrane-embedded Domain of HtrI—To test whether the cytoplasmic 13-residue stretch itself can close the channel of SRI without the specific interaction between the transmembrane domains of HtrI and the receptor, two fusions
were constructed: first, SRI was fused to this 13-residue stretch with the same linker (SRI-HtrI-(54–66)), and second, the 13 residues were substituted into the cytoplasmic portion of HtrII in the SRI-HtrII fusion (SRI-HtrII-(1–302)-HtrI-(54–66)). The photocycle kinetics of both fusions exhibit the same pH-dependent photocycles as HtrI-free SRI. Our interpretation is that the presence of the 13-residue stretch itself is not sufficient to block the channel, but requires specific interactions between the SRI receptor and HtrI transducer in the membrane domain.

The \( pK_a \) of Asp-76 in the SRI Incrementally Increases in a Manner Correlated with the Closure of the Cytoplasmic Channel—SRI exhibits a blue-purple transition at alkaline pH, caused by the deprotonation of Asp-76 of SRI. In the blue species (\( \lambda_{\text{max}} = 587 \) nm) Asp-76 is protonated, and in the purple species (\( \lambda_{\text{max}} = 552 \)) Asp-76 is unprotonated. In HtrI-free SRI, the \( pK_a \) of Asp-76 is 7.2–7.4 (6, 8). Binding of full-length HtrI, in addition to blocking the SRI cytoplasmic channel, increases this \( pK_a \) of Asp-76 to 8.6–8.9 (25). The different absorption maxima among the truncation mutants of SRI-HtrI fusion at pH 7.5 show an incremental increase of the \( pK_a \) correlated with the incremental closure of the channel. The pH titration of the purple-blue transition of SRI-HtrI-(1–53) yields a \( pK_a \) of 7.8 (with free SRI showing a \( pK_a \) of 7.3), further confirming that the membrane portion of HtrI itself, which only slightly closes the channel, interacts with the SRI receptor. Extension of the cytoplasmic portion of HtrI in the fusion mutants further incrementally increases the \( pK_a \) of Asp-76 to 8.6 at position 66 at HtrI, closely correlating with the complete blocking of the channel (Fig. 7).

**FIG. 3.** Immunoblot analysis of membranes containing wild-type SRI, truncated SRI-HtrI fusion, SRI-linker, SRI-HtrI fusion, and SRI-HtrII fusion. Membrane suspensions were subjected to 12% SDS-PAGE and probed with anti-SRI antibody.

**FIG. 4.** Effect of pH on the photocycle rates of SRI-HtrI truncation fusion mutants, SRI-HtrI-(1–53), SRI-HtrI-(1–61), and HtrI-free SRI exhibit similar pH-sensitive photocycles. The slopes of log M half-life versus pH are: SRI-HtrI-(1–53), 0.88; SRI-HtrI-(1–61), 0.86; SRI-HtrI-(1–72), SRI-HtrI-(1–85), and SRI-HtrI-(1–147) slopes are in the range -0.05 to -0.10, indistinguishable within the reproducibility of the method from that of the full-length SRI-HtrI complex.

**FIG. 5.** Flash yield difference between HtrI-free SRI, SRI-HtrI-(1–53), and SRI-HtrI (full-length) at different pH values. The relative flash yields were calculated as \( \Delta A_{400}/A_{587} \), where \( \Delta A_{400} \) is the maximum amplitude of absorption change at 400 nm following the flash and \( A_{587} \) is the SRI absorbance at 587 nm, measured by difference spectroscopy using retinal regeneration of the NH\(_2\)OH-bleached membrane.

**FIG. 6.** The pH dependence of photocycle rates for different SRI-HtrI fusion with different extents of truncation. The slopes of pH dependence (log M half-life versus pH) of photocycle rate are plotted against the HtrI position at which SRI-HtrI truncation mutants terminate. 0 indicates HtrI-free SRI.
HtrI Membrane-proximal Domain Modulation of SRI

The Effects of Mutations in the 5-Residue Region on the Phototaxis Response—To test whether the 5 residues most effective in channel closure (62–66 in HtrI) are also functionally important for phototaxis, Ala residues were introduced into each of the first 3 positions (K62A, E63A, and I64A) and Ser residues into each of the final 2 positions (A65S and A66S) in the HtrI portion of the SRI-HtrI full-length fusion. Photocycle kinetics of these mutants exhibited a small negative pH dependence indistinguishable from that of the wild type SRI-HtrI complex, indicating that the channel closure effect depends on the presence of residues at these positions but does not depend on their specific type.

We assessed swimming behavior responses to 1-photon stimulation with orange light (600 ± 20 nm), which induce attractant responses in cells expressing the wild-type SRI-HtrI full-length fusion, and to 2-photon stimulation to white light (380–600 nm), which induce repellent responses. Both responses were completely eliminated by the mutation I64A in the HtrI portion of the SRI-HtrI full-length fusion (data not shown). The other 4 position mutants exhibited wild-type attractant and repellent responses to saturating stimuli. Residue Ile-64, in the middle of the 5 channel closing positions, is therefore singularly critical for signaling.

**DISCUSSION**

The results above show that (i) 13 residues extending into the cytoplasm from the second transmembrane helix (TM2) of HtrI are sufficient for closure of the cytoplasmic channel opened in transducer-free SRI during its photocycle. This is a much shorter region than the 94 cytoplasmic residues found sufficient in a previous study (24), the key advantage here being the use of SRI-HtrI fusions that allow much shorter HtrI fragments to be stabilized. (ii) The final 5 residues (62–66) are nearly completely responsible for the closure effect, and (iii) channel closure by this membrane-proximal domain requires the membrane-embedded domain of HtrI, which alone interacts with SRI altering to a small extent its properties, but without significantly closing the channel. (iv) The same membrane-proximal HtrI residues raise the pKₐ of Asp-76, a buried residue far from the cytoplasmic loops of the SRI protein predicted to be near the HtrI membrane-proximal domain. It is therefore clear that the SRI dark conformation is altered by the presence of the position 62–66 residues in HtrI.

The correlation of the effects of HtrI on the Asp-76 pKₐ and channel conductivity during the photocycle suggests a model based on two SRI dark conformations in equilibrium: one with a low Asp-76 pKₐ able to open the cytoplasmic channel during the photocycle and the other with a high Asp-76 pKₐ in which this opening (or more accurately the associated proton-conductivity increase) is suppressed. In terms of this two-conformation description, the 5-residue region stabilizes the second conformation, with incremental increases in the equilibrium shift occurring with incremental lengthening of the HtrI fragment from 61 to 66 residues.

What is the mechanism by which residues 62–66 alter the SRI conformation? One mechanism would be direct interaction of these residues with the cytoplasmic region of SRI, in particular with the E-F loop of SRI. According to modeling the SRI-HtrI complex on the structure of the related NpSRII-NpHtrII complex, the SRI E-F loop would be at approximately the same distance from the membrane as the 5-residue region in the cytoplasmic extension of HtrI. A direct interaction model is favored by the demonstration of close proximity between the NpSRII E-F loop and the membrane-proximal domain of its transducer NpHtrII as described in the accompanying article (33) by sulphydryl cross-linking, probe accessibility measurements, and FRET. In that study, 5 residues in NpHtrII were found to be within energy-transferring distances from a fluorescent probe positioned on S154C on the NpSRII E-F loop. Most strikingly, in alignments of HtrI and NpHtrII, these 5 residues (91–95) in NpHtrII correspond to residues 62–66 in HtrI.

Residues 62, 65, and 66 are almost entirely responsible for the channel closure, consistent with a helical turn in which they contact SRI, and in which residues 63 and 64 would be facing away from the interaction region. Residue 64 is shown here to be critical for phototaxis signaling, perhaps because of its involvement in HtrI homodimer interaction in this region (34).

We cannot exclude the distinctly different mechanism that the structure in the membrane-embedded portion of the HtrI-SRI interface could be altered by the cytoplasmic extension, and hence a conformational equilibrium of SRI between partially interacting (open channel) and fully interacting (closed channel) forms is shifted incrementally by extending the cytoplasmic domain. Such a mechanism would require assuming that the relatively small differences in helical length between SRI-HtrI(1–61) through SRI-HtrI(1–66) would cause a significant alteration in the transmembrane structure of HtrI. This interpretation would be consistent with the observation that shortening of NpHtrII from 114 to 101 residues eliminates binding of NpHtrII truncation mutants to NpSRII in detergent micelles (22), although this phenomenon may be because of non-native folding by the shorter truncated NpHtrII. Considering all of these points we favor the direct interaction model as explaining the results most simply.

The membrane-proximal domains of HtrI and HtrII are predicted to be HAMP domains, which consist of two helical amphipathic sequences (AS-1 and AS-2) connected by a connector of undefined structure, which is observed in most methyl-accepting chemotaxis proteins and in sensor kinases in bacteria (35, 36). The membrane-proximal region of HtrI required for
channel closure of SRI identified in this study belong to the AS-1 region of the HAMP domain. Mutations in HAMP domains of Tsr, Tar, NarX, EnvZ, and HtrI result in biased signal transduction causing constitutive or inverted signaling (26, 37–41). Most interestingly, all suppressor mutations to SRI inverted signaling mutants found in HtrI are located in this region (26).

The small but readily detected effects of the HtrI(1–53) fragment, which does not contain a cytoplasmic extension, on SRI properties demonstrates that the membrane-embedded portion of HtrI binds to SRI, as would be expected from the crystallographically demonstrated interaction of NpSRII and NpHtrII (23). Our results indicate that this membrane interaction is necessary for the cytoplasmic membrane-proximal domain to close the channel in SRI.

In BR, the main conformational change induced by light is the tilting of helices, especially helix F, which opens its cytoplasmic proton-conducting channel. Cryoelectron crystallography of BR reveals that the greatest movement of BR induced by light is in the cytoplasmic portion of helix F and the E-F loop (16). Hence the interaction of SRI and HtrI within the membrane may function to hold the membrane-proximal cytoplasmic domain in the proper orientation to detect the similar SRI motion in the cytoplasmic portion of helix F and the E-F loop. In this view the signal transfer from SRI to HtrI would occur in the membrane-proximal cytoplasmic domain of the complex. Hence transmembrane channel, such as occurs in chemotaxis receptors (42), would be bypassed and the HAMP domain would function as a signal-input rather than signal-relaying domain to close the channel in SRI.

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