Protonatable residues at the cytoplasmic end of transmembrane helix-2 in the signal transducer HtrI control photochemistry and function of sensory rhodopsin I

(phototaxis/bacterial chemotaxis/halobacteria/seven transmembrane helix receptor/retinal)

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ABSTRACT Neutral residue replacements were made of 21 acidic and basic residues within the N-terminal half of the *Halobacterium salinarum* signal transducer HtrI [the halobacterial transducer for sensory rhodopsin I (SRI)] by site-specific mutagenesis. The replacements are all within the region of HtrI that we previously concluded from deletion analysis to contain sites of interaction with the phototaxis receptor SRI. Immunoblotting shows plasmid expression of the *htrI-sopI* operon containing the mutations produces SRI and mutant HtrI in cells at near wild-type levels. Six of the HtrI mutations perturb photochemical kinetics of SRI and one reverses the phototaxis response. Substitution with neutral amino acids of Asp-86, Glu-87, and Glu-108 accelerate, and of Arg-70, Arg-84, and Arg-99 retard, the SRI photocycle. Opposite effects on photocycle rate cancel in double mutants containing one replaced acidic and one replaced basic residue. Laser flash spectroscopy shows the kinetic perturbations are due to alteration of the rate of reprotonation of the retinylidene Schiff base. All of these mutations permit normal attractant and repellent signaling. On the other hand, the substitution of Glu-56 with the isosteric glutamine converts the normally attractant effect of orange light to a repellent signal *in vivo* at neutral pH (inverted signaling). Low pH corrects the inversion due to Glu-56 → Gln and the apparent pK of the inversion is increased when arginine is substituted at position 56. The results indicate that the cytoplasmic end of transmembrane helix-2 and the initial part of the cytoplasmic domain contain interaction sites with SRI. To explain these and previous results, we propose a model in which (i) the HtrI region identified here forms part of an electrostatic bonding network that extends through the SRI protein and includes its photoactive site; (ii) alteration of this network by photoisomerization-induced Schiff base deprotonation and reprotonation shifts HtrI between attractant and repellent conformations; and (iii) HtrI mutations and extracellular pH alter the equilibrium ratios of these conformations.

Photon absorption by the seven-transmembrane helix phototaxis receptor sensory rhodopsin I (SRI; $\lambda_{\max}$ 587 nm) in *Halobacterium salinarum* membranes causes transient deprotonation of the retinylidene Schiff base and formation of the spectrally distinct species S₃₇₃ ($\lambda_{\max}$ 373 nm) (1). This process acts as an attractant signal for the cell, and absorption of a second photon by S₃₇₃ acts as a repellent signal (2, 3). SRI phototransformations are detected by an integral membrane protein, HtrI, a 57-kDa methyl-accepting transducer (4) that exhibits sequence similarity to eubacterial chemotaxis receptors (5) and is complexed with SRI (6, 7). The sequence-predicted structure of HtrI consists of 2 transmembrane helices within the N terminal $\approx$ 60 residues followed by an

$\approx$ 500-residue cytoplasmic domain. The C terminal 250 residues contain signaling regions that regulate a cytoplasmic pathway (8) which controls flagellar motor switching (for reviews, see refs. 9–11).

Genetic deletion of HtrI eliminates phototaxis and also alters SRI photochemical kinetics (6, 7, 12). In HtrI-free membranes the SRI Schiff base deprotonation causes transient proton release to the medium during the photocycle, and HtrI blocks this release but not deprotonation (12). As a consequence, in HtrI-free SRI the $t_{1/2}$ of S₃₇₃ decay, which requires reprotonation of the Schiff base (13) varies from $\approx$ 100 ms to $\approx$ 10 s as medium pH is varied from pH 4 to pH 7 (6). Interaction with HtrI results in an intermediate $t_{1/2}$ of $\approx$ 900 ms (18°C), which is pH-independent.

An important question is whether HtrI exerts its effects on SRI photochemistry by causing a global structural change in SRI or more directly through coupling of HtrI/SRI interaction sites to the SRI photoactive site. In the latter case, it is more likely one would find specific mutations in HtrI that do not disrupt the complex, but perturb the SRI photocycle. Therefore, we mutated the 21 sites in HtrI that meet the following criteria: (i) they are contained in the 247-residue N-terminal region of HtrI shown by an HtrI deletion construct to be sufficient to control SRI photochemistry (14); (ii) they are conserved (except for Glu-56, chosen for its apparent location in a transmembrane helix) in the two other known sensory rhodopsin (SRII) transducers, pHtrII and vHtrII, from the related halobacteria *Natranobacterium phaeononis* and *Haloferax vallismortis*; respectively (15); and (iii) they are acidic or basic and, therefore, might influence electrostatically the proton transfer reactions in the SRI photoactive site. The study revealed seven residues that effect SRI properties while maintaining functional coupling between the two proteins.

MATERIALS AND METHODS

Restriction enzymes and T4 DNA ligase were from Promega, Sequenase was from United States Biochemical, and Pfu DNA polymerase was from Stratagene. Oligonucleotides (20–23 nucleotides) were purchased from Bioserve (Laurel, MD). Mevinolin was a gift from A. W. Alberts (Merck Sharp & Dohme).

Plasmids and Strains. Native or mutant forms of HtrI and native SRI were expressed from their native promoter by transformation of *Halobacterium salinarum* strain Pho81Wr[–]

Abbreviations: SRI, sensory rhodopsin I; HtrI, halobacterial transducer for SRI; SR₅₈₇, S₃₇₃, species in the SRI photochemical reaction cycle with maximum absorption at 587 nm and 373 nm, respectively; designations of mutated proteins make use of the standard one-letter abbreviations for amino acids—thus E56Q signifies the mutated protein in which the glutamyl residue at position 56 (designated Glu-56) is replaced with a glutaminyl residue.

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with plasmid pVJY1 and its mutated derivatives (14). Pho81W^r was isolated by screening for the absence of an endogenous restriction system (16) in single colony isolates of Pho81W [BR⁻, HR⁻, SRI⁻, SRII⁻, HtrI⁻, and carotenoid-deficient (BR, bacteriorhodopsin; HR, halorhodopsin)] (14). After selection for motility on swarm plates, *H. salinarium* cells were transformed with polyethylene glycol treatment (17). Halobacteria transformants expressing plasmid-encoded mevinolin-resistance were grown on spheroplast-regeneration plates at 37°C with 4 μ g of mevinolin per ml (18).

PCR Mutagenesis. The plasmid pKJ304 carrying the 758-bp *SpeI/SacI* fragment of *htrI* in pBluescript KS⁻ (Stratagene) was used as a template for mutagenesis following a two-step PCR method (19). T3 and T7 primers (Bioserve) and designed oligonucleotides containing specific mutations were used as primers for PCR. First, T7 and an oligonucleotide with the mutation were used to amplify a PCR fragment. The fragment was purified with QIAx II (Qiagen, Chatsworth, CA) and used as a second round primer. T3 primer and the double-stranded product of the first PCR were used to amplify a second round PCR product, which was purified and digested with *SpeI* and *SacI* restriction enzymes and the *SpeI/SacI* fragment ligated into pVJY1. The mutant plasmid was transformed into *Escherichia coli* DH5 α and the mutation was confirmed by sequencing. PCR was performed for 30 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min in a Programmable Thermal Controller-100 (MJ Research, Watertown, MA).

Each of the mutated *htrI* genes was inserted by replacing wild-type *htrI* in the native *htrI-sopI* operon on the *H. salinarium* expression plasmid pVJY1. Mutant HtrI and SRI were produced in *H. salinarium* transformant membranes at near wild-type concentration according to immunoblot analysis as described (6), except detection was by chemiluminescence using ECL Western blotting (Amersham) (data not shown).

Motion Analysis of Pho81W^r Transformants. Early stationary phase cultures were diluted 1:20 in fresh growth medium [conditioned medium (CM) (20)] and were incubated at 37°C for 1–2 hr with agitation. After the incubation period, phototaxis responses were measured with a computerized cell tracking system (Motion Analysis, Santa Rosa, CA) as described (20). Saturating intensities of photostimuli activating the SRI dark adapted form, SR₅₈₇, and its photoactive intermediate, S₃₇₃, at 600 nm in an infrared (≥ 700 nm) and 400 nm in a ≥ 580 nm background, respectively, were delivered to the cells from a Nikon 100 W Hg/Xe lamp. Data were collected and processed by a Sun SPARC-IPC workstation (Sun Microsystems, Mountain View, CA).

Preparation of Membrane Vesicles. The transformants were grown in 200 ml of CM with mevinolin (1 μ g/ml) at 37°C on a gyratory shaker at 240 rpm for 5 days. Membrane envelope vesicles were prepared by sonication as described (21). Membranes were pelleted for 1 hr at 48,000 rpm in a Beckman L3–50 ultracentrifuge and suspended with 4 M NaCl/25 mM Tris-HCl (pH 6.8).

Flash Photolysis. Flash-induced absorption changes were measured with a laboratory-constructed cross-beam flash spectrometer (6) with a frequency-doubled Nd-YAG laser (532 nm, 6 nsec pulse, 40 mJ) providing the actinic flash. The flashing frequency was 0.08 Hz. Eighteen to 20 transients were averaged for each trace at constant temperature (18°C). The amplitudes and $t_{1/2}$ values were calculated by fitting of single or double exponentials by using curve-fitting programs from SIGMAPLOT (Jandel, San Rafael, CA).

RESULTS

Photochemical Reaction Cycles of Mutants. Laser flash-induced absorption transients were monitored near the absorption maximum of SRI (590 nm) in vesicle suspensions to assess photocycle rates. The maximum absorbance change

amplitudes in all transformant membranes were similar to those of wild-type membranes, as shown for D86N and R99A in Fig. 1. Six HtrI mutations, R70A, R84A, R99A, D86N, E87Q, and E108Q, have significant effects on the photocycle $t_{1/2}$. The arginine replacements retard and the acidic residue replacements accelerate the photocycle (Fig. 2). All other mutations produce $t_{1/2}$ values within 1–2 SD of the set of four independent wild-type measurements, although D34N, E56Q, and D195N produce borderline low $t_{1/2}$ values.

Membranes containing each of the six HtrI mutations that alter the SRI photocycle rate exhibit flash-induced absorption difference spectra indistinguishable from wild-type, indicating photolytic formation of an S₃₇₃ intermediate (shown for R99A and D86N in Fig. 1). In each of the mutants, as in the wild-type, S₃₇₃ decay limits the rate of the photocycle, as shown by the kinetic agreement between absorbance decay at 400 nm and rise at 590 nm.

Mutations producing fast and slow photocycle rates were combined in the double mutants E87Q-R99A and R84A-D86A. Membranes from these double mutants exhibit photocycle $t_{1/2}$ values of 954 and 721 msec, respectively, showing that retardation and acceleration effects cancel.

All of the substitutions above were with neutral residues. For the six mutations that effect photocycle rate, there is a strict correlation of their effect with their likely charge in the wild-type protein; namely, positive-to-neutral replacements retard and negative-to-neutral replacements accelerate the photocycle. To test further this correlation with charge, a series

FIG. 1. Flash-induced absorption difference transients and spectra of SRI with mutant HtrI. Flash photolysis was at pH 6.8 and 18°C of Pho81W^r membrane suspensions transformed to contain wild-type HtrI (a and d), D86N (b and e), and R99A (c and f). Transients in a–c were recorded at 400 nm (upper trace) and 590 nm (lower trace). Maximum absorbance change amplitudes of the flash are plotted in d–f.

FIG. 2. Photocycle half-lives of SRI complexed with HtrI mutants. WT indicates pVJY1-transformed Pho81Wr⁻, which contains wild-type HtrI. The mean of four independent halftimes for decay of flash-induced absorption transients in wild-type membranes at 590 is plotted ± 1 and ± 2 SD indicated by the inner and outer dashed horizontal lines, respectively. Values from membranes containing single point mutations are plotted ± 1 SEM for three or four measurements. In addition to the seven mutations labeled, unlabeled points from left to right correspond to D34N, E77Q, E79Q, E96Q, R103A, R113A, E122Q, E124Q, E131Q, E160Q, E163Q, D169N, R173A, and D195N.

of residues were substituted in position 56 to provide a gradient of potential charges; namely, Glu-56 $\rightarrow$ Asp (more negative than Glu-56 on the basis of its lower pK_a), Glu-56 $\rightarrow$ Gln (neutral), and Glu-56 $\rightarrow$ Arg (positive). The resulting HtrI proteins, E56D, E56 (wild type), E56Q, and E56R, exhibit SRI photocycle rates that correlate with their charge and greater electronegativity producing a slower photocycle rate (Fig. 3).

Phototaxis Responses by Cells Containing Mutated HtrI. Cells containing the HtrI mutations were assayed for SRI signal transduction *in vivo* by using saturating photostimuli. All mutants except E56Q exhibit wild-type reversal responses to two saturating stimuli tested: a step-down in 600-nm light intensity and a step-up in 400-nm light intensity, which demonstrated the function of the SRI attractant and repellent signals, respectively. E56Q, however, exhibits inverted re-

FIG. 3. Photocycle half-lives for SRI in HtrI E56 mutants. Flash photolysis transients were monitored at 590 nm as in Fig. 2 for membranes with aspartyl (D), glutamyl (Q), and arginyl (R) residues substituted for the glutamyl (E) residue at HtrI position 56. Points are plotted as the mean ± 1 SEM of three values.

sponses to orange light revealing that this residue is important for attractant signal processing by HtrI. The wild-type responses to an abrupt increase and decrease in 600-nm light are suppression (Fig. 4 *Top Left*) and induction (*Middle Left*) of swimming reversals, respectively. E56Q exhibits the opposite reversal frequency changes (*Top* and *Middle Right*), and are repelled by normally attractant light. E56Q cells are repelled (*Bottom Right*), as is wild type (*Bottom Left*), to a pulse of violet light in an orange light background, a response attributable to photoactivity of the SRI photocycle intermediate, S₃₇₃ (2).

The Inverted Phenotype of E56 Mutants Depends on Extracellular pH. The response index, as defined in the legend to Fig. 5, is positive for an attractant response and negative for a repellent response. Wild-type cells exhibit attractant responses to 600-nm photostimuli when cells are suspended at pH values between 5 and 9 (Fig. 5a). The E56Q mutant exhibits inverted (i.e., repellent) responses at pH 5.5 and above; however, at pH_{ext} 5 the inversion is corrected and the normal attractant response occurs. Exchanging Glu-56 for the positively charged arginine shifts and broadens the apparent pK of the inversion, so that a repellent response is observed only at pH 8 and 9. The conservative residue replacement of Glu-56 with aspartic acid preserves the wild-type behavior.

Wild-type and E56Q cells respond to violet light (i.e., S₃₇₃ photoactivation) in an orange light background as a repellent at all pH_{ext} tested (Fig. 5b). S₃₇₃-mediated responses of E56D at pH5 and E56R at pH9, however, are negligible, while these mutants exhibit normal repellent responses at other pH_{ext} values.

DISCUSSION

The seven residues identified as altering SRI properties cluster in an ≈ 50 -residue region of HtrI which by hydrophathy analysis

FIG. 4. Phototaxis responses of E56Q and wild type: Pho81Wr⁻/pVJY1 and Pho81Wr⁻/E56Q. Two seconds after initiation of data acquisition, the cells were exposed to 4-sec addition (*Top*), 4-sec removal (*Middle*) of 600 $\pm$ 20 nm light, or a 15-msec pulse of 400 $\pm$ 5 nm light (*Bottom*). The ordinate shows fraction of cells undergoing reversals per 200 msec measured by computerized cell tracking.

FIG. 5. Phototaxis responses of HtrI mutants at residue position 56. Photostimuli were in (a) 4-sec removal of 600-nm light and in (b) a 15-msec 400-nm pulse in an orange light background (see *Materials and Methods*). The phototaxis index is calculated in sec^{-1} as the integral of the reversal frequency transient measured as in Fig. 4 between 0 and 2 sec minus the integral between 4 and 6 sec, where time 0 is the initiation of the light removal in a or the pulse in b. Attractant (reversal-suppressing) stimuli produce a positive index value, and repellent stimuli produce a negative index value. $\circ$, Wild type; $\bullet$, E56Q; $\blacktriangle$, E56R; $\blacksquare$, E56D.

is placed (4) at the cytoplasmic end of the second transmembrane helix (TM2) and in the adjacent portion of the hydrophilic domain (Fig. 6). The data shown here indicate this is a likely region for interaction of HtrI residues with those of SRI. Moreover, HtrI deletion analysis establishes that residues beyond position 147 are dispensable for receptor interaction (B. Perazzona, E. N. Spudich, and J.L.S., unpublished data). By hydropathy analysis alone we cannot assign whether Glu-56 is within the membrane domain or in the cytoplasm near the membrane surface. Functional interaction of the second HtrI transmembrane helix (TM2, Fig. 6) with the receptor is particularly interesting since disulfide-locking experiments (22) and mutagenesis (23) implicate the corresponding TM2 of the *E. coli* aspartate chemoreceptor as a mobile element responsible for signal transmission upon chemoeffector binding.

The data demonstrate that neutral substitution of three anionic residues, Asp-86, Glu-87, and Glu-108, accelerate reprotonation of the Schiff base, as assessed by S_{373} decay, while neutral substitution of three cationic residues, Arg-70, Arg-84, and Arg-99, retard this process. This result is a significant addition to the body of evidence that SRI and HtrI physically interact. The previous evidence for interaction (6, 7, 12) is all based on the fact that *total* deletion of HtrI from the membrane affects SRI photochemistry. These data did not exclude an indirect effect mediated by HtrI influencing other

FIG. 6. Regions of HtrI modulating SRI photocycle kinetics and signaling function. Open and closed circles represent HtrI sites mutated in this study. $\bullet$, Residues whose substitution effects S_{373} reprotonation rate in the SRI photocycle. E56 in HtrI is labeled to emphasize its critical role in attractant signaling (see text), as was previously observed also for D201 in SRI (24). Reading from C terminal to E56, the remaining closed circles represent R70, R84, D86, E87, R99, and E108. $\circ$, Residues whose substitution had no effect in this study (listed in legend to Fig. 2). The dotted line indicates the fusion of residues 147–501, the minimal fragment of HtrI found by deletion analysis to produce a wild-type pH-insensitive SRI photocycle (B. Perazzona, E. N. Spudich, J.L.S., unpublished data). The C-terminal fragment beyond residue 501 is also dispensable according to a separate construct. The arrangement of transmembrane helices of SRI and HtrI is not known and was chosen arbitrarily for illustration.

components which in turn interact with SRI. The effects of more subtle modification of HtrI on SRI shown here favor direct interaction over an indirect interaction via an additional component between SRI and HtrI. Furthermore, recently HtrI has been shown to copurify with SRI through an SRI-affinity column, arguing for a direct interaction (E. N. Spudich, P. Dag, and J.L.S., unpublished data).

Several results indicate that electrostatic interactions are responsible for the effects of the HtrI mutations on SRI photocycling and signaling: (i) the correlation of anion neutralization with acceleration of Schiff base reprotonation, and cation neutralization with retardation; (ii) the cancellation of photocycle perturbations in double mutants containing acidic and basic residue replacements with neutral residues; (iii) the correlation of electronegativity of residues at position 56 and slower photocycle rate; and (iv) the low pH_{ext} correction of E56Q and E56R mutants, suggesting that protonation of some other residue cancels their effect. These results also indicate that the seven residues are shielded from charge screening by the $\approx 4 \text{ M K}^+$ and Cl^- concentrations in the cytoplasm.

These effects, as well as the previously reported change in pK_a of Asp-76 in SRI caused by HtrI interaction (24, 25), suggest an electrostatic bonding network connects the SRI/HtrI interaction surface to the SRI photoactive site. A chain of ionic and hydrogen bonds consisting of protein residues and bound water is believed to mediate long-range interactions between the Schiff base and the cytoplasmically accessible residues participating in proton pumping by bacteriorhodopsin

(26–28). Because HtrI-free SRI translocates protons (25), it is reasonable to suggest this aspect of bacteriorhodopsin structure is conserved.

Two classes of models are suggested by the inverted responses reported here and that of the SRI mutant D201N (24). The first assumes two conformations of the SRI-HtrI complex in a metastable equilibrium, one conformation suppressing and the other inducing swimming reversals. Photostimuli shift the complex between the two conformations causing reversal suppression or induction responses. The effect of mutations that invert the attractant response would be to stabilize the inappropriate conformation in the dark or during the photocycle. Such an effect may be analogous to mutations in the visual pigment rhodopsin that produce constitutively active receptors (29). Low pH would correct the inverted response phenotype by stabilizing the conformation favored in the wild-type receptor.

A second type of explanation is based on the adaptation process [via methylation changes on HtrI (30–32) that occurs in response to photostimulation. In the wild type, the adaptation system produces the opposite signal to nullify the excitation signal produced by photostimuli. If cells contain a mutation in the complex that blocks the excitation signal but still allows the adaptation system to be triggered, a behavioral response opposite to that of wild-type cells might occur. However, the rapidity of the inverted response favors an explanation based on shifted conformations rather than on the slow adaptation system.

Distinguishing and refining these models will require further understanding of structural and functional effects of the HtrI Glu-56 and SRI Asp-201 mutations. In any case it is clear these two residues are important in the attractant signaling mechanism.

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