Transducer-Binding and Transducer-Mutations Modulate Photoactive-Site-Deprotonation in Sensory Rhodopsin I†

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ABSTRACT: Sensory rhodopsin I (SRI) is a seven-transmembrane helix retinylidene protein that mediates color-sensitive phototaxis responses through its bound transducer HtrI in the archaeon *Halobacterium salinarum*. Deprotonation of the Schiff base attachment site of the chromophore accompanies formation of the SRI signaling state, S_{373}. We measured the rate of laser flash-induced S_{373} formation in the presence and absence of HtrI, and the effects of mutations in SRI or HtrI on the kinetics of this process. In the absence of HtrI, deprotonation occurs rapidly (halftime 10 μs) if the proton acceptor Asp76 is ionized (pK_a = ~7), and only very slowly (halftime > 10 ms) when Asp76 is protonated. Transducer-binding, although it increases the pK_a of Asp76 so that it is protonated throughout the range of pH studied, results in a first order, pH-independent rate of S_{373} formation of ~300 μs. Therefore, the complexation of HtrI facilitates the proton-transfer reaction, increasing the rate ~50-fold at pH6. Arrhenius analysis shows that HtrI-binding accelerates the reaction primarily by an entropic effect, suggesting HtrI constrains the SRI molecule in the complex. Function-perturbing mutations in SRI and HtrI also alter the rate of S_{373} formation and the λ_{max} of the parent state as assessed by laser flash-induced kinetic difference spectroscopy, and shifts to longer wavelength are correlated with slower deprotonation. The data indicate that HtrI affects electrostatic interactions of the protonated Schiff base and not only receives the signal from SRI but also optimizes the photochemical reaction process for SRI signaling.

Sensory rhodopsin I (λmax 587 nm) is a phototaxis receptor that mediates motility responses in the archaeon *Halobacterium salinarum* (*1*, *2*). Its seven helices form an internal pocket where the chromophore, all-trans retinal, is attached in a protonated Schiff base linkage to a lysine residue in Helix G. Photoisomerization of the retinal in the dark state, SR_{587}, triggers Schiff base deprotonation and forms the blue-shifted signaling conformation, S_{373} (λmax 373 nm). S_{373} returns thermally in seconds to SR_{587} completing a photocycle. During its lifetime, the S_{373} species transmits signals to the flagellar motors through a second membrane protein, the transducer HtrI, which is bound to SRI in a tight molecular complex.

HtrI has been demonstrated to influence the process of reprotonation of the Schiff base, measured by monitoring S_{373} decay to SR_{587}. In the absence of HtrI, the proton returns from the cytoplasm and the kinetics of S_{373} decay is sensitive to external pH, whereas in the presence of HtrI, the Schiff base is reprotonated from within the SRI−HtrI complex in a reaction independent of external pH (*3*, *4*). These effects have been interpreted in terms of HtrI blocking the opening of a proton channel from the cytoplasm to the photoactive center of SRI (*5*). Transducer-free SRI exhibits light-driven electrogenic pumping of protons across the membrane (*6*), which depends on the opening of such a channel in the latter half of the photocycle, and accordingly the binding of HtrI to SRI inhibits its pumping activity (*7*). Additional evidence that HtrI is coupled to SRI in a manner sensitive to the conformational change that has occurred in the later steps in the SRI photocycle has been provided by mutations in HtrI found to retard or accelerate S_{373} decay (*8*, *9*).

The above results demonstrated that its bound transducer affects SRI transformations late in the photocycle, i.e., after light-induced protein conformational changes in SRI have occurred. To characterize receptor/transducer interaction prior to these conformational changes, we asked whether HtrI influences the earlier proton transfer in the receptor, i.e., that occurring in light-induced deprotonation of the Schiff base as monitored by S_{373} formation. Microsecond-resolution flash photolysis reported here shows that the transducer indeed modulates deprotonation, demonstrating that even prior to light-induced protein conformational changes in SRI the bound transducer alters the structure at the photoactive site of the receptor.

MATERIALS AND METHODS

Plasmids and Recipient Strain. Wild-type or mutant HtrI/SRI complexes were expressed from their native promotor in plasmid pKJ306 derivatives (*9*) and free SRI was expressed under the *bop* promoter in plasmid pSO7 derivatives (*10*). The mutations were introduced by the two-step mega-primer PCR method with Pfu polymerase (*11*). Pho81Wr$^-$ (BR$^-$, HR$^-$, SRI$^-$, SRII$^-$, HtrI$^-$, HtrII$^-$, carotenoid deficient, and restriction negative (*12*)) was used for the *H. salinarum* recipient in plasmid transformations.

Sample Preparation. Halobacterial cells were transformed using a modification of the poly(ethylene glycol)-mediated

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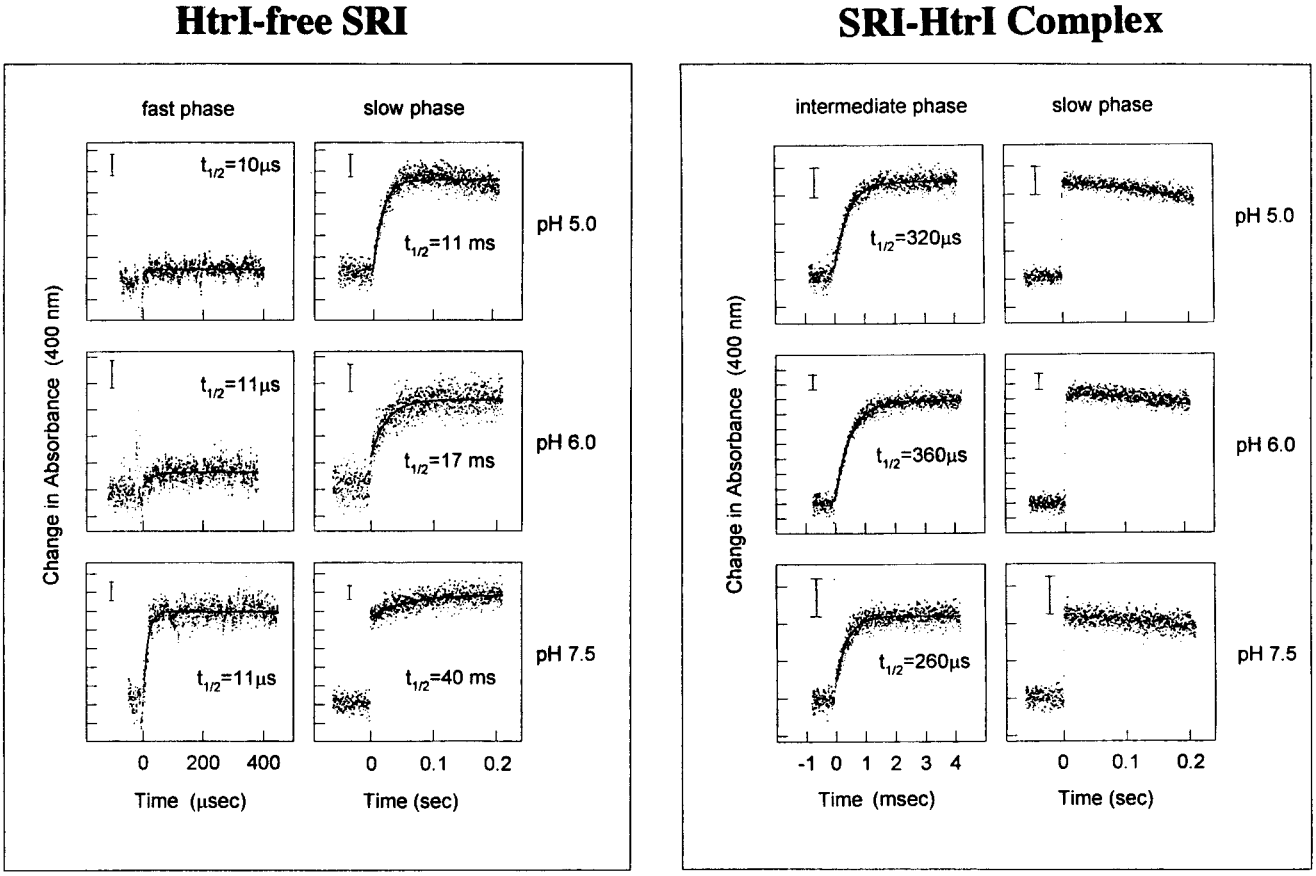

FIGURE 1: Analysis of the rate of S_{373} formation at different pH in the absence (HtrI-free SRI) and presence (HtrI−SRI complex) of HtrI. Formation of S_{373} in *H. salinarum* membrane pellets (0.5 mm path length) was measured at 400 nm, 18 °C, in various time windows. Thirty flash photolysis transients were averaged for each determination. In each panel the 532 nm laser flash was at $t = 0$. The length of the bar in each panel is 10^{-3} absorbance units.

spheroplast transformation method (*13*). The cells containing mutant protein were grown to late stationary phase in CM (complex medium, pH 6.0) with mevinolin (1 μg/mL) at 37 °C for 7 days. Membranes were prepared by sonication as described (*8*). Prior to measurements, wet membrane pellets were prepared by centrifugation at 75 000 rpm for 30 min in a Beckman TL-100 tabletop ultracentrifuge and placed in 0.5 mm path length demountable rectangular cuvettes (glass, 334−2500 nm, Starna Cells, Atascadero, CA) for reducing light scattering. The membranes were pelleted, resuspended in 4 M NaCl/25 mM Tris or MES buffer at pH values indicated in the figures, and recentrifuged to produce the pellet.

Flash Photolysis. Flash-induced absorption changes were recorded by a cross-beam spectrophotometer with a 532 nm, 40 mJ/6 ns pulse Nd:YAG laser (Surelite I, Continuum, Santa Clara, CA) and RSM-1000 spectrophotometer (On-line Instrument System, Inc. Bogart, GA). The absorbance transients due to S_{373} formation were monitored at 400 nm. Thirty transients were averaged for each trace at 18 °C, 25 °C, 35 °C, and 45 °C, and fifty for each 230 nm scan. The $t_{1/2}$ of S_{373} deprotonation was calculated by a single-exponential curve-fit using SigmaPlot (Jandel, San Rafael, CA).

RESULTS AND DISCUSSION

HtrI Modulates the Kinetics of S_{373} Formation in SRI. The rise of S_{373} exhibits two kinetic components in free SRI, each fit well by a single exponential (Figure 1). A fast ~10 μs component is detected with small amplitude at pH 5.0 and increases in amplitude with increasing pH to dominate the process at pH 7.5 (leftmost panels, Figure 1). A far slower deprotonation rate (11−40 ms halftime) has small amplitude at pH 7.5 and replaces the fast phase to dominate the transient at pH 5.0. These data can be understood in terms of the titration of Asp76, which has been demonstrated to serve as a primary counterion to, and proton acceptor from, the Schiff base in HtrI-free SRI (*14*). In free SRI, Asp76 has a pK_a near 7 (*5*, *15*). Therefore, at pH 7.5 it is mostly ionized and serves as a proton acceptor from the Schiff base, producing the 10−11 μs rate of S_{373} formation. At pH 5, Asp76 is mostly protonated and, therefore, proton transfer from the Schiff base occurs to an alternative unknown acceptor at a far slower rate (>10 ms).

In the HtrI−SRI complex, the pK_a of Asp76 is 8.5 (*16*) and, therefore, at all pH values tested in Figure 1 Asp76 is unavailable as a proton acceptor in the HtrI−SRI complex. If HtrI did not effect the availability of a proton acceptor from the Schiff base, then we would expect a very slow rate of S_{373} formation of >10 ms as in the free receptor. In contrast, a first-order rate of ~300 μs is observed at all pH values (rightmost panels, Figure 1), as was observed for wild-type membranes in a comprehensive analysis of the SRI photocycle (*17*). Therefore, we conclude that the complexation of HtrI to SRI facilitates the deprotonation of the Schiff base, increasing the rate ~50-fold at pH 6.

Similar to our results, a rapid deprotonation rate (2−5 μs) of HtrI-free SRI has also been observed in envelope vesicles at 23 °C (*18*). However, in contrast, an identical rate of deprotonation of 380 μs in both the complexed SRI and free SRI has been reported (*19*). The time-resolution in the latter study was cited as 130 μs, which would be sufficient to detect the difference. However, no process faster than 380 μs appears under any conditions in the data in ref *19*. Therefore, a possible explanation of the disagreement is that the time resolution may have been larger than that reported.

The results reported here show that the presence of HtrI modulates the rate of formation of the S_{373} intermediate as well as its rate of decay as was demonstrated previously (*3*). Similarly, HtrII presence in the membrane modulates both formation and decay of the deprotonated intermediate in the SRII photocycle (*7*).

How does the HtrI interaction with SRI accelerate Schiff base deprotonation? One possibility is that the unassigned proton acceptor residue resides on HtrI and is positioned near the Schiff base in the SRI−HtrI complex. Alternatively, HtrI-binding alters the structure of the active site rendering an acceptor within SRI more accessible to the Schiff base. Neither of those possibilities can be definitively excluded by the available data, but mutagenesis studies argue against HtrI itself, accepting the Schiff base proton. In a deletion study, the 147 N-terminal residues of the 536-residue HtrI protein were found to be sufficient to modulate the photocycle rate of SRI (*12*). We made neutral residue replacements in the 147-residue region of each of the 21 potentially charged residues that are conserved with other transducers and found no significant reduction in flash-induced yield of S_{373}, demonstrating that none of these residues is an obligate proton acceptor from the Schiff base (*8*). Transducer chimeras show that the N-terminal 60 residues of HtrI, comprising its two transmembrane helices, fused to the cytoplasmic portion of HtrII exhibit functional interaction and control of the SRI photocycle (*20*), suggesting that HtrI binding alters SRI photochemistry via an effect on SRI protein conformation mediated by helix−helix contacts within the membrane.

Correlated Effects of Mutations in HtrI and SRI on S_{373} Formation and Absorption Maximum of the Pigment. Residue substitutions of Glu56 in HtrI, a function-perturbing residue in or near the second transmembrane helix (TM2) of HtrI, modulate the S_{373} decay rate to an extent that depends on the electronegativity of the residue introduced (*8*). Several suppressor mutations near the end of TM2 also accelerate or retard the S_{373} lifetime (*9*). These effects suggest that this region of HtrI is important for the coupling of HtrI to SRI. S_{373} formation is also influenced by the electronegativity of the residue at position 56 in HtrI, but in the opposite way as S_{373} decay: the more positive residues lengthen the time of S_{373} formation, whereas, as previously observed, the more positive residues accelerate S_{373} decay (Figure 2). These inversely correlated effects indicate that electronegativity at position 56 of HtrI destabilizes the protonated Schiff base of SRI, and further substantiates that electrostatic interactions of Glu56 with SRI or with other HtrI residues are involved in the coupling of HtrI to the SRI photoactive site.

One would predict that destabilizing the protonated Schiff base in the dark state, thereby raising the energy level of the ground state of the pigment, would shift the pigment absorption to longer wavelength (*21*). Such a trend is indeed evident in the correlation between the rate of S_{373} rise and the absorption maximum of the pigment deduced from flash-induced absorption difference spectra (Figure 3). A possible mechanism for this effect is that the mutations alter the strength of the Schiff base proton and counterion interaction, and that the counterion is the proton acceptor. A weakened protonated Schiff base/counterion association, either by increasing interionic distance or solvation of the counterion, is expected to shift the absorption maximum to longer wavelengths (*22*), and the diminished interaction with the Schiff base may also retard the proton transfer from the

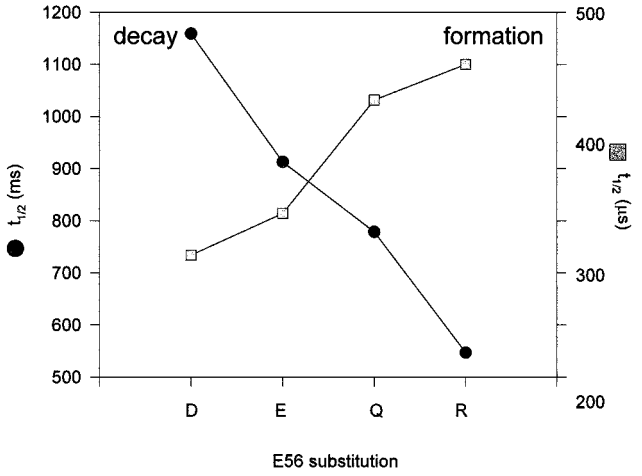

FIGURE 2: The rate of formation and decay of S_{373} in membrane containing E56 substitutions in HtrI of decreasing electronegativity. Circles show the half time of S_{373} decay (left axis scale). Rectangles show the half time for S_{373} formation (right axis scale).

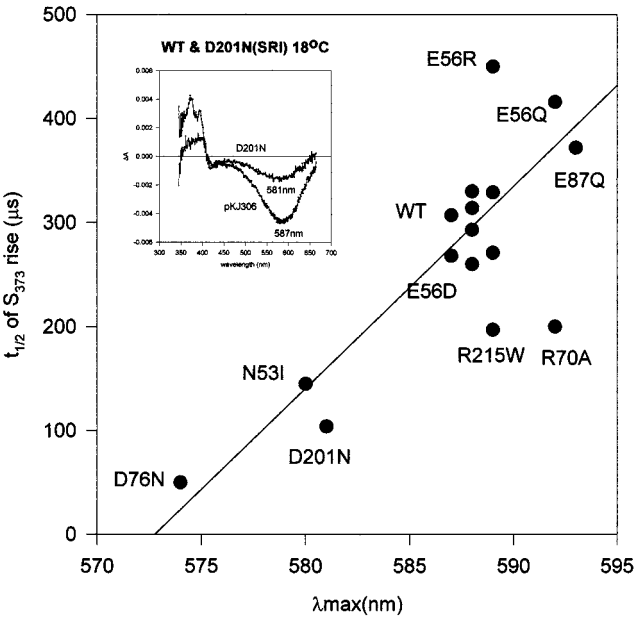

FIGURE 3: The mutations of HtrI and SRI observed to alter S_{373} lifetime and λ_{max} and seven determinations of wild-type (WT) are plotted. Inset: Flash-induced absorption difference spectra of wild-type (pKJ306) and the D201N mutant of SRI in HtrI/SRI complex membranes at pH 6.8 to illustrate the type of data from which λ_{max} values were estimated. The spectra were measured between 340 and 660 nm. Fifty flash-induced difference spectra were averaged after 16 ms following the laser flash at 18 °C. The scanning resolution was 1000 scans/sec and 1.1 nm/point for the 230 nm range by the RSM-1000. The λmax of the spectrum was calculated by a multifunctional curve-fit using SigmaPlot.

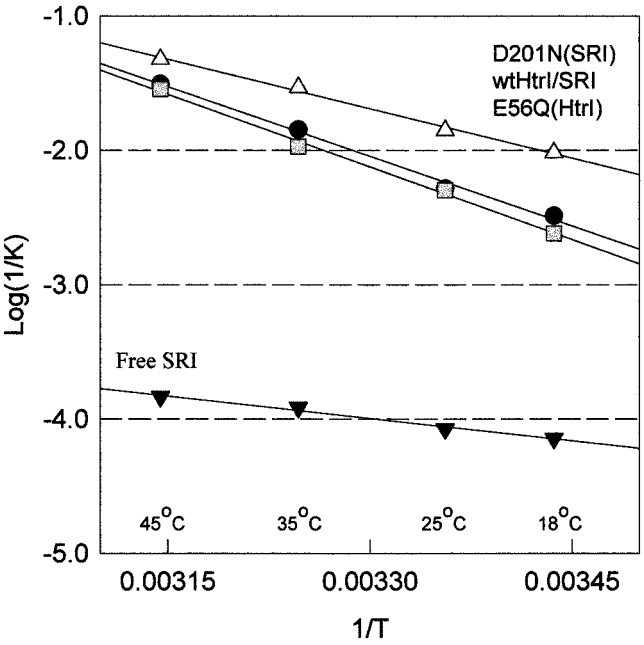

FIGURE 4: Temperature dependence of the rate constants of S_{373} formation in wild-type free and complexed SRI, SRI mutant D201N, and SRI complexed with HtrI mutant E56Q. The rate constants were obtained by exponential fitting of the traces at 400 nm measured at 18, 25, 35, and 45 °C, and the logarithms of the rate were plotted against 1/*T*. The membrane pellets in 0.5 mm demountable cuvettes were incubated at least 20 min to reach the equilibrium temperature.

Schiff base to the counterion. Asp76, the primary counterion and proton acceptor in the proton-translocating form of SRI (SR_{552}) (*14*) is not ionized in the SR_{587} form in the SRI−HtrI complex and, therefore, an unknown proton acceptor-(s) fulfills these functions.

Effect of HtrI and Mutations on Thermodynamic Parameters of Schiff Base Deprotonation. The rate constants for S_{373} rise were measured at 18 °C, 25 °C, 35 °C, and 45 °C in the free and complexed SRI, and the logarithm of the rates were plotted against reciprocal of temperature (Figure 4). Each plot fit well to a straight line. The effect of the mutations in the HtrI/SRI complex on ΔS and ΔH are relatively small but E56Q in HtrI changes ΔS and D201N in SRI changes both ΔS and ΔH.

The effect on binding of HtrI to SRI on ΔS is relatively large. Since the acceleration of the S_{373} formation by HtrI binding is an entropic effect (Figure 4), HtrI-binding may constrain the SRI molecule. Cryoelectron crystallography of BR has revealed a conformational change inducing movements of helices F and G that appear to open a cytoplasmic channel in the M or N photointermediates (*23*, *24*). Assuming the analogous change occurs in SRI, the entropic effect implies that the conformational change is destabilized by the constraint from HtrI binding. Also, the proton pumping inhibition by HtrI could be explained by its blocking the cytoplasmic proton channel of SRI. Modulation of the kinetics of S_{373} decay by HtrI complexation indicated that HtrI-binding changes the hydrogen bonding network around the Schiff base to facilitate its protonation (*3*). Also, there is evidence that the binding of HtrII influences the electrostatic interactions or hydrogen-bonding network in the SRII photoactive site, accelerating the Schiff base proton transfer (*25*). Thus, in both sensory rhodopsins complexation with their transducers modulates Schiff base deprotonation, the earliest chemical event observed following photoisomerization. We interpret this effect as indicating conformational coupling of the transducers to their cognate receptors' photoactive sites.

Such protein coupling may involve similar long-range electrostatic interactions as have been reported for the G-protein transducin's effect on mammalian rhodopsin. Rhodopsin is photoconverted via a series of intermediate species to a signaling state, MetaII ($\lambda_{max} = 380$ nm), in which the retinylidene Schiff base proton has been transferred to a glutamate residue on the protein (*26*). Complexation with transducin stabilizes the MetaII species resulting in an enhancement of MetaII formation following flash photolysis (*27*, *28*). The stabilization is attributed to transducin binding to loops on the cytoplasmic surface of the receptor, indicating an allosteric effect on the proton-transfer reaction analogous to HtrI modulation of proton transfer in the SRI photoactive site.

Electrostatic coupling of distant charges to the photoactive site in retinylidene proteins may be responsible for several additional observations: (1) cytoplasmic phosphorylation shifts the MetaI/MetaII equilibrium toward MetaII in rhodopsin, thus it stabilizes the active conformation MetaII (*29*); (2) surface-bound cations effect the photoactive site of bacteriorhodopsin (*30*); (3) global conformational changes are detected in response to residue substitutions as well as photocycle transitions in bacteriorhodopsin (*31*).

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