Structural Changes of Sensory Rhodopsin I and Its Transducer Protein Are Dependent on the Protonated State of Asp76[†]

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ABSTRACT: Sensory rhodopsin I (SRI) functions in both positive and negative phototaxis in complex with halobacterial transducer protein I (HtrI). Orange light activation of SRI results in deprotonation of the retinylidene chromophore of SRI to produce the S_{373} photocycle intermediate, the signaling state for positive phototaxis. In this study, we observed pH dependence on structural coupling between the two molecules upon the formation of the S_{373} intermediate by means of Fourier transform infrared spectroscopy. At alkaline pH, where Asp76 (one of the counterions of the protonated retinylidene Schiff base) is deprotonated, HtrI-dependent alteration of the light-induced difference spectra is limited to reduction of amide I bands at 1661 (+)/ 1647 (−) cm^{-1}, and perturbation of one of the protonated carboxylic acid bands occurs at 1734 (−) cm^{-1} (which appears to become ionized only when complexed with HtrI). However, at acidic pH, HtrI-complexed SRI exhibits not only light-induced reduction of the amide I changes but a wider range of spectral alterations including the appearance of several new amide I bands, perturbation of the chromophore-related vibrational modes, and other additional changes characteristic of tyrosine, glutamate, and aspartate residues. Since such pH dependence of structural changes was not observed in the complex of the D76N mutant of SRI, which behaves much like HtrI-complexed SRI in acidic conditions, we conclude that extensive orange light-induced conformational coupling between SRI and HtrI occurs only when Asp76 is neutralized.

Sensory rhodopsin I (SRI)[1] is a light sensor in haloarchaea that mediates attractant phototaxis toward orange light and repellent phototaxis toward near-UV light in an orange-light background (*1, 2*), while a related light sensor, sensory rhodopsin II (SRII, also called phoborhodopsin, pR) is specialized for mediating only negative phototaxis (*3–6*). SRI and SRII form 2:2 complexes with their transducer proteins, HtrI (*7*) and HtrII (*8*), respectively, and transmit light signals to a 2-component phosphosignaling cascade in the cytoplasm, which controls the switching of the flagella motor and is homologous to the chemotaxis pathway in bacterial chemotaxis (*9, 10*).

Light activation of the retinylidene chromophore in the light sensors triggers cyclic chemical reactions (photocycles) consisting of several sequential intermediate states (Figure 1), which are similar to those of bacteriorhodopsin (BR), a well-characterized light-driven proton pump in haloarchaea except that the sensors exhibit slower cycling rates (the recovery times are about 10 ms for BR and 1 s for SRI and SRII). The active state for the attractant responses mediated by SRI is attributable to the S_{373} state (λ_{max} = 373 nm) formed upon single-photon activation of the unphotolyzed state (SRI: λ_{max} = 587 nm), whereas the active state for the SRI-mediated repellent response is attributable to photoproducts of S_{373} with near-UV light, which include S^{b}_{520} (λ_{max} = 520 nm).

The absorption maximum of SRI has been shown to be affected by pH and ranged from 552 to 587 nm (*11*), which has been attributed to titration of Asp76 (*12*). The pK_a, which is 7.2 in the HtrI-free form, is elevated to 8.5 upon HtrI association (*13*), indicating that HtrI binding alters the conformation of SRI in such a way that the environment of Asp76 is altered. However, no vibrational modes due to the chromophore in the unphotolyzed and S_{373} states are affected upon association with HtrI as revealed by resonance Raman spectroscopy within the limits of the experimental accuracy (*14*). This observation suggests that HtrI binding does not directly alter the chromophore but the protein moiety of SRI near Asp76. The cytoplasmic channel is also blocked by HtrI binding so that the decay rate of S_{373} is insensitive to external pH between pH 5.0 and 8.0, whereas removal of HtrI renders the decay rate linearly dependent on pH in the same pH range, showing that the Schiff base reprotonates by taking up a proton from the cytoplasmic side (*11, 15*). Proton-

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[1] Abbreviations: BR, bacteriorhodopsin; SRI, sensory rhodopsin I (also called sensory rhodopsin, SR); SRII, sensory rhodopsin II (also called phoborhodopsin, pR); HtrI, halobacterial transducer I; HtrII, halobacterial transducer II; FT-IR, Fourier-transform infrared; DM, *n*-dodecyl-β-D-maltoside; PC, L-α-phosphatidylcholine; λ_{max}, the wavelength maximum of the visible absorption band.

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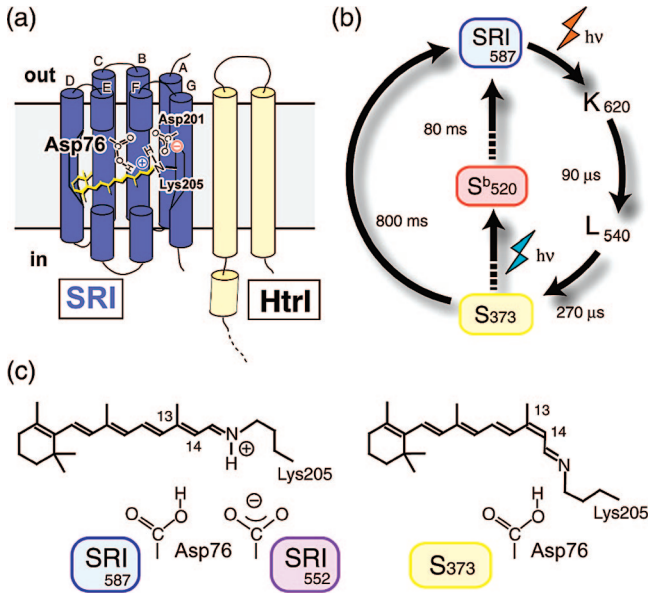

FIGURE 1: (a) Schematic structure of SRI and HtrI. Asp76 is protonated in the physiological condition when it forms a complex with HtrI. A positive charge on the Schiff base is considered to be stabilized by Asp201. (b) Photochemical reaction cycle of SRI. SRI absorbs orange light and forms the long-lived S_{373} intermediate, which forms the S^b_{520} intermediate upon the second photon absorption in the near-UV region. Approximate first order half-lives for the intermediates are taken from ref *9*. (c) Protonation states of Asp76 in the acidic form (SRI_{587}), alkaline form (SRI_{552}), and the S_{373} intermediate with their chromophore structures.

pumping by free SRI is suppressed by HtrI complexation, which is explained by the protonation of Asp76 and the blocking of the cytoplasmic channel of SRI by HtrI.

In alkaline conditions, the proton acceptor of the Schiff base in S_{373} formation was revealed to be Asp76 by FTIR spectroscopy (*12*). However, in acidic conditions where Asp76 is protonated in the unphotolyzed state, His166 was suggested as a candidate for the proton acceptor from the Schiff base upon S_{373} formation because it is located in the vicinity of the Schiff base on the cytoplasmic side, and mutations of His166 impair S_{373} formation (*16*). These modifications of the photochemistry and of the pK_a of Asp76 in SRI show that HtrI binding alters the conformation of SRI.

In contrast, the cognate transducer HtrII exerts little or no influence on the pK_a of the Schiff-base counterion Asp75 in the repellent light sensor SRII. In agreement with this, the influence of HtrII binding on the conformational changes of SRII elicited upon formation of the signaling state M were found to be confined to suppression of changes in the amide bands related to backbone conformational changes (*17, 18*) and in the appearance of bands that suggest disruption of the hydrogen-bonding between Tyr199 of SRII and Asn74 of HtrII (*19, 20*) in the interface of the two molecules according to FTIR spectroscopy. Otherwise, the light-induced changes of SRII are remarkably similar between the free and HtrII-complexed forms.

Here, we utilized FTIR spectroscopy to explore conformational changes exerted by HtrI in the SRI-to-S_{373} conversion. In contrast to the case of the SRII−HtrII complex, HtrI binding exerted numerous alterations in vibrational modes in the difference spectrum of S_{373} minus SRI at neutral and acidic pH where Asp76 is protonated or when Asp76 is mutated to Asn. These alterations are ascribable to conformational changes either in SRI caused by HtrI binding or in HtrI itself, related to the attractant-signaling conformational changes. However, at alkaline pH (9.5) where Asp76 is ionized, there were few HtrI-dependent alterations in the S_{373} minus SRI spectrum. We suggest that Asp76 protonation in SRI is prerequisite for functional coupling with HtrI to elicit attractant-signaling conformational changes.

MATERIALS AND METHODS

The genes of SRI or the SRI−HtrI fusion protein, in which the C-terminus of SRI is joined through a flexible linker peptide (ASASNGASA) to the N-terminus of HtrI truncated at position 147, were cloned into an expression vector pET-21d (Novagen) under the control of T7 promoter. Both free SRI and SRI−HtrI protein contain a His6 Tag at the C-terminus. The expression of the genes in BL21(DE3) was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside and 5 μM all-*trans*-retinal. The SRI and SRI−HtrI fusion proteins containing C-terminal histidine tags were expressed in *E. coli.*, solubilized with 1.0% *n*-dodecyl-β-D-maltoside (DM), and purified by Ni-NTA chromatography. Each sample, the SRI or the SRI−HtrI fusion protein, was then reconstituted into L-α-phosphatidylglycerol (PG) liposomes (SRI:PG = 1:50 molar ratio), where DM was removed with Bio-Beads (SM-2; Bio-Rad). The PG liposomes were washed three times with a buffer at pH 5.0 or 5.5 (2 mM citrate), or pH 9.5 (2 mM borate) containing 300 mM NaCl, and the sediment obtained after the third centrifugation was deposited and slowly dried on a BaF_2 window with a diameter of 18 mm at room temperature under high humidity. By using the sediment instead of the suspension of the sample, we were able to reduce the amount of salt precipitated on the dried films, which scatter measurement light in the mid-infrared region.

Low-temperature FTIR spectroscopy was performed as described previously (*18, 19*). After hydration with H_2O or D_2O, the sample was placed in a cell, which was mounted in an Oxford Optistat-DN cryostat placed into a FTS-7000 spectrometer (DIGILAB). Illumination with >480 nm light (VY-50, Toshiba) provided from a 1 kW halogen-tungsten lamp at 260 K for 120 s converted SRI to S_{373}. The spectra were constructed from 128 interferograms with spectral resolution of 2 cm^{-1}. The difference spectrum was calculated by subtracting the spectrum recorded before the illumination from the spectra recorded after the illumination. Three difference spectra obtained in this way were averaged for each S_{373} minus SRI spectrum. The S_{373} minus SRI spectrum at pH 5.0 without transducer contains a small contribution from photoreaction of the alkaline form of SRI. Therefore, the spectrum is subtracted to cancel the bands characteristic of the alkaline form, such as the bands at 1749 (+) and 1525 (−) cm^{-1} (the subtracting factor is 0.2). All spectra were normalized with respect to the C−C stretching vibration of the retinal chromophore at 1197 cm^{-1}.

RESULTS

Structural Changes of the Complex of Sensory Rhodopsin I and HtrI Are Dependent on the Environmental pH. HtrI-free SRI exhibits proton-pumping activity above pH 7.0 where Asp76 is deprotonated (the pK_a is ~7.2), whereas HtrI

binding elevates the pK_a to ~8.5 (*13*), rendering the aspartic acid protonated at the physiological pH in which SRI functions as a light sensor for positive phototaxis and transmits a signal to HtrI upon the formation of the attractant-signaling state S_{373} induced by orange/yellow-light illumination of the sample. To detect evidence of the conformational coupling between the two molecules, the S_{373} minus SRI difference FTIR spectra were compared between HtrI-free SRI and SRI with the C-terminus joined through a flexible linker peptide to the N-terminus of HtrI truncated at position 147. *H. salinarum* cells expressing the joined complex were shown to behave like those coexpressing separate SRI and HtrI, confirming a previous report that the linker peptide does not interfere with signaling (*7*), and the truncated HtrI at position 147 was shown to be sufficient to exhibit proper binding to SRI, which elevates the pK_a of Asp76 to pH 8.5 and makes the photocycle turnover rate insensitive to the pH changes in the outer milieu between pH 4 and 8 (*21, 22*). The pH of the film samples for the FTIR measurements were adjusted by washing the proteoliposome samples with either 2 mM citrate (pH 5.5) or 2 mM borate (pH 9.5), which would give pH values of the hydrated film sample similar to the buffer values and render Asp76 of the SRI samples either protonated or deprotonated, respectively, even with the HtrI-complexed samples. By use of UV–visible spectroscopy, we confirmed that only S_{373} states are formed and trapped at 260 K by illumination of the samples with yellow light (>480 nm) for both samples in acidic and alkaline conditions (data not shown). In Figure 2, the difference FTIR spectra of the S_{373} minus SRI states in the absence (red spectra) and presence (blue spectra) of HtrI are compared at acid (a) and alkaline pH (b).

The difference spectra of the free SRI in acidic and the alkaline conditions are in good agreement with earlier results (*12*), showing that S_{373} is formed under the conditions we employed. The state of the protonation of Asp76 in each of the pH conditions can be assessed by observing the C=O stretching mode of protonated carboxyl groups between 1800 and 1700 cm^{-1}, where the appearance of a positive band at 1749 cm^{-1} in alkaline conditions was attributed to the protonation of Asp76 upon S_{373} formation (Figure 2b), whereas in acidic conditions, the frequency shift of the protonated carboxyl mode from 1761 to 1750 cm^{-1} provided evidence of environmental changes of Asp76 without deprotonation (Figure 2a and Figure 4a) (*12*). The protonation state of Asp76, which affects the wavelength of the maximum absorption (λ_{max}) of the chromophore shifting form 552 to 587 nm, is also assessed from the frequencies of the ethylenic stretching modes of the chromophores (1518 and 1525 cm^{-1} for the SRI in acidic and alkaline conditions, respectively), which is inversely correlated with λ_{max} (Figure 2a and b). Although a sharp negative band at 1512 cm^{-1}, probably from an amide II band or a tyrosine residue as seen in the 1518-cm^{-1} band in the *p*SRII−*p*HtrII complex, overlaps the ethylenic mode at acidic pH, the overall band is clearly inversely correlated to the λ_{max} (Figure 3a). The assignments of the ethylenic stretches are consistent with the previous report (*12, 14, 23*). Incidentally, differences in amplitude between free SRI and the complex are explained by the reduction of amide II bands in the complex spectra overlapping with the ethylenic stretching modes.

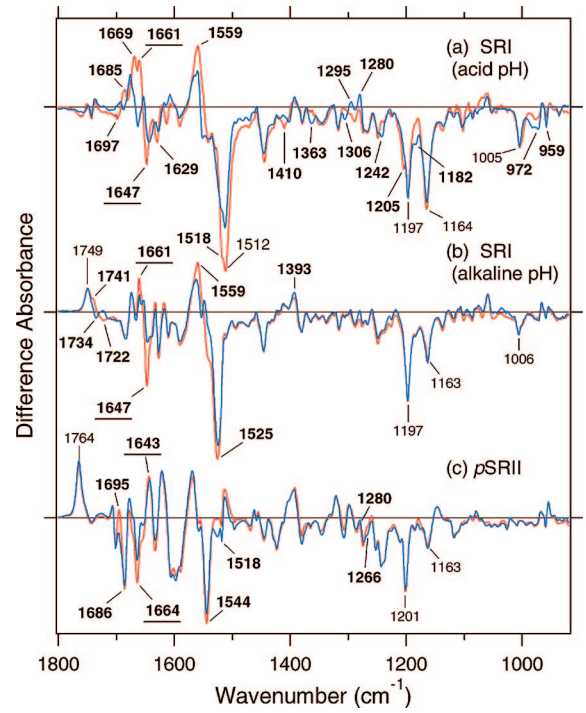

FIGURE 2: Infrared difference spectra of SRI (a and b) and *p*SRII (c) without (red curve) and with (blue curve) their transducer proteins (HtrI and HtrII, respectively). One division of the *y* axis corresponds to 0.009 absorbance units. (a) S_{373} minus SRI difference spectra at 260 K at acidic conditions (pH 5, receptor alone; pH 5.5, receptor with transducer). (b) S_{373} minus SRI difference spectra at 260 K at alkaline conditions (pH 9.5). (c) $pSRII_M$ minus *p*SRII difference spectra at 263 K at pH 7, which is reproduced from ref (*18*). The samples were hydrated with H_2O. The amplitudes of the spectra were normalized on the basis of the bands at 1197 cm^{-1} for SRI and at 1201 cm^{-1} for *p*SRII. Tagged frequencies in bold type represent the bands different between the receptor alone and the receptor−transducer complex. The amide I bands that reduce their intensities upon complex formation are underlined.

The SRI protein complexed with HtrI also exhibits identical behaviors of Asp76 and the shift of the ethylenic stretching vibrational mode with those of the free SRI, showing that Asp76 is protonated or deprotonated in acidic or alkaline conditions, respectively, and that Asp76 protonates upon S_{373} formation under alkaline conditions. However, clear contrast was seen in HtrI-complexed SRI between acidic and alkaline conditions in the spectral deviation between free and HtrI-complexed SRI in the other frequency domains. In acidic conditions, the S_{373} minus SRI difference spectrum was greatly affected by complex formation with HtrI over a wide spectral range (1800–900 cm^{-1}). These vibrational changes are attributable to both SRI and HtrI and are caused by interaction changes between SRI and HtrI upon complex formation and the formation of the S_{373} intermediate state (Figure 2a). Detailed explanations of these HtrI-induced vibrational changes are described below. In contrast, in alkaline conditions, the differences caused by the presence of HtrI on the spectrum are restricted to changes in the protonated carboxylic region (1760–1710 cm^{-1}) and the amide I region (1670–1630 cm^{-1}) (Figure 2b). In particular, the frequency domain in 1400–900 cm^{-1} is remarkably identical between the free and the HtrI-complexed SRI.

An interesting analogy is found in the *p*SRII−*p*HtrII complex (*18–20*) with a deprotonated counterion, Asp75, in physiological conditions (the pK_a value is 3.5) (*24, 25*) in

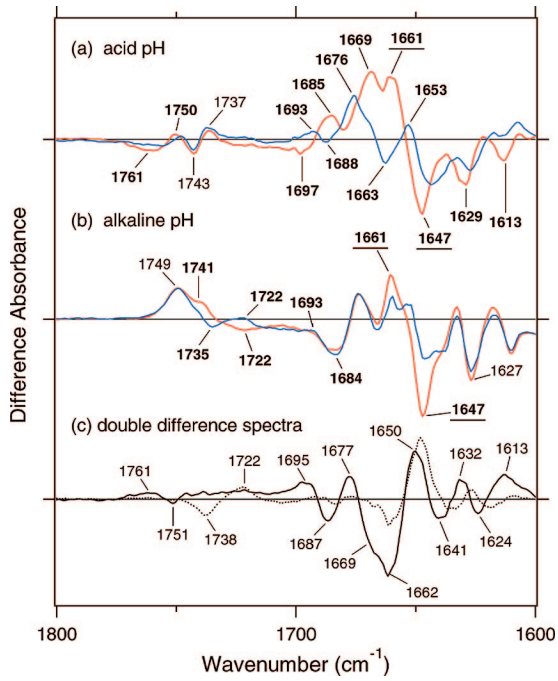

FIGURE 3: S_{373} minus SRI infrared difference spectra at pH 5.5 (blue curve) and pH 9.5 (red curves) compared between the wild type and D76N mutant of SRI in the presence of HtrI. The spectra were normalized according to the intensity of the negative band at 1197 cm^{-1}. The tags at 1749 and 1693 cm^{-1} indicate the C=O stretching vibrations of Asp76 and Asn76, respectively. One division of the *y* axis corresponds to 0.008 absorbance units.

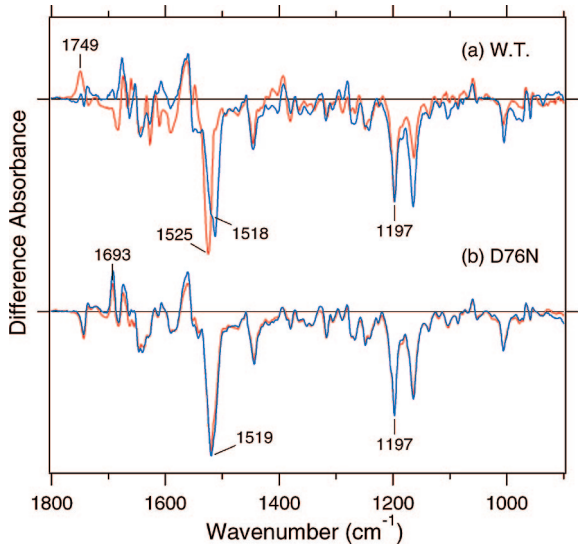

FIGURE 4: S_{373} minus SRI infrared difference spectra at acidic pH (a) and at alkaline pH (b) without (red curve) and with (blue curve) HtrI in the 1800–1600 cm^{-1} region. Spectra are reproduced from Figure 2. (c) The solid and dotted lines are double difference spectra calculated from the blue and red spectra in (a) and (b), respectively (blue minus red). One division of the *y* axis corresponds to 0.006 absorbance units. Tagged frequencies in bold type represent the bands different between the receptor alone and the receptor–transducer complex. The amide I bands that reduce their intensities upon complex formation are underlined.

which the effect of *p*HtrII binding on the FTIR difference spectrum in the transition from the dark state to the M state was restricted to the reduction of amide I bands at 1664 and 1643 cm^{-1} (*18–20*), and other minor changes at 1695 and 1686 cm^{-1}, and at 1518, 1280, and 1266 cm^{-1} ascribed to hydrogen-bonding interaction changes in the interface of the two molecules between Asn74 in *p*HtrII (*19, 20*) and Tyr199 in *p*SRII (*20*), respectively, upon conformational changes due to M formation. It is suggested that when the Schiff-base counterion Asp is unprotonated, the influence of the transducer on the photoreceptor might be similar between SRI and *p*SRII, and confined to suppression of backbone conformational changes of the photoreceptors. However, HtrI exerts more extensive perturbations on SRI-to-S_{373} conformational changes when Asp76 is protonated, suggesting that SRI couples to HtrI in a different manner compared to the Asp76-deprotonated form or to the *p*SRII–*p*HtrII complex. The difference in coupling might be associated with the differences between attractant- and repellent-signaling conformational coupling with each of the cognate transducers of these two systems.

The difference between SRI and SRII responsible for their opposite signals upon single photon excitation might reside in the inherent nature of the two molecules to invoke opposite structural changes consistent with the opposite direction of the shifts of the amide I bands between SRI and *p*SRII when the single photon products (S_{373} and M, respectively) are formed. In the case of SRI, the amide I band at 1647 cm^{-1} up-shifts to 1661 cm^{-1} upon the formation of S_{373}, while in *p*SRII, the amide I band at 1664 cm^{-1} down-shifts to 1643 cm^{-1} upon the formation of the M intermediate (Figure 2c). In the literature, an α_{II} helix, which has a distorted helical structure, exhibits a higher frequency (1667 cm^{-1}) than that of a typical α-helix (α_I-helix; 1655 cm^{-1}) (*26*). Therefore, the observed frequency shifts imply that the typical helical structure is distorted upon activation in SRI and vice versa in *p*SRII. These structural changes are suppressed by complexation both in SRI and *p*SRII.

pH-Dependent Changes of the SRI–HtrI Interaction Are Abolished in the D76N Mutant of SRI in the Presence of HtrI. The results above show that the pH of the sample has a drastic effect on the interaction between SRI and HtrI as well as on the protonation state of Asp76. This raises the question whether SRI–HtrI coupling upon light-induced protein structural changes depends on the protonation state of Asp76 in SRI, or alternatively whether high pH deprotonates residues other than Asp76, which reduce SRI–HtrI interaction, thereby lowering the pK_a of Asp76 to that of the transducer-free SRI with the consequence of Asp76 deprotonation. To distinguish these alternatives, we examined the D76N mutant of SRI in the presence of HtrI. This mutation was previously shown to permit function since D76N mediates phototaxis responses to attractant and repellent light stimuli (*27*). The spectral changes in the infrared difference spectra at alkaline and acidic pH are essentially identical in D76N SRI, and the spectra are very similar to that of the wild type in acidic conditions (Figure 3b), indicating that the mutant SRI couples HtrI in a manner similar to that of wild type. Our interpretation is that the HtrI-dependent structural changes eliminated in alkaline conditions in wild-type SRI are not caused by the deprotonation of other residues, but rather derive from the deprotonation of Asp76. Our first conclusion is that the light-induced HtrI-dependent structural changes of wild-type SRI and its transducer protein occur only when Asp76 is protonated in the unphotolyzed state. The spectral differences

in the acidic and alkaline conditions are discussed in the following section, step by step, according to the spectral window.

Transducer-Dependent Structural Changes of Sensory Rhodopsin I Observed in the 1800–1600-cm^{-1} Region. Figure 4 shows the S_{373} minus SRI difference spectra in the absence (red lines) and presence (blue lines) of HtrI in acidic (a) and alkaline conditions (b). The solid and dotted spectra in Figure 4c are double difference spectra between the HtrI-absent and HtrI-containing samples in acidic conditions (Figure 4a) and in alkaline conditions (Figure 4b), respectively. C═O stretching vibrational frequencies of the protonated carboxyl group range between 1800 and 1700 cm^{-1}. In acidic conditions, the C═O stretch modes of the protonated Asp76 were assigned to the 1761 and 1750 cm^{-1} bands for the initial state and S_{373}, respectively, because of the disappearance of these bands upon D76N mutations (*12*). In HtrI-complexed samples, those slightly down-shifted bands are assignable to the C═O stretch mode of the protonated Asp76 because they are also missing in D76N SRI with HtrI complexed (see Figure 3b). The slight shift of the vibrational modes of Asp76 indicates that HtrI alters SRI conformation in such a way that the strength of the hydrogen bond connected to Asp76 is slightly altered. Another protonated carboxyl group sensitive to the conformational changes in the transition from the initial state to S_{373} exhibiting bands at 1743 and 1737 cm^{-1} for the initial state and S_{373}, respectively, is likely to be located in the interior of the photoreceptor because the protonated state of the residue is stabilized both in the initial and S_{373} states. Since these bands are unaffected by HtrI binding (Figure 4a), this unidentified carboxyl group is suggested to be located distant from the HtrI binding domain (helix F or G). A conceivable candidate for the residue is Asp106 on helix D, corresponding to Asp115 of BR.

In alkaline conditions, the C═O stretching band at 1749 cm^{-1} showing protonation of Asp76 upon S_{373} formation was assigned previously from the fact that the corresponding band shifted toward a lower frequency side in the D76E mutant in a manner consistent with the expected frequency downshift of the C═O stretch mode of Glu compared to that of Asp (*12*). The band was unaffected by HtrI binding (Figure 4b), in clear contrast with the case of the sample in acidic conditions, suggesting that the interaction of SRI and HtrI is different in alkaline conditions. However, another protonated carboxylic group sensitive to the light-induced conformational changes and showing bands at 1722 and 1741 cm^{-1} in the initial state and S_{373}, respectively, appears to be perturbed in the presence of HtrI (shifted to 1735 and 1722 cm^{-1}, respectively). Assignment of these bands needs the study of mutations, but presumably they originate from a carboxyl group in SRI near the SRI−HtrI interface.

Mironova et al. reported that the C═O stretching vibrations of Asn53 in HtrI were observed at 1692 cm^{-1} for the S_{373} state and 1686 cm^{-1} for the unphotolyzed state. The corresponding bands seem to appear at 1695 (+)/1687 (−) cm^{-1} in our measurement conditions (Figure 4c). The slight difference in frequency is probably due to the difference in measurement conditions (lipid composition, ATR or transmission, temperature, and resolution of spectra). While these bands are not prominent in the double difference spectra recorded in alkaline conditions (Figure 4c; dotted line), the small bands at 1693 (+)/ 1684 (−) cm^{-1} are substantially

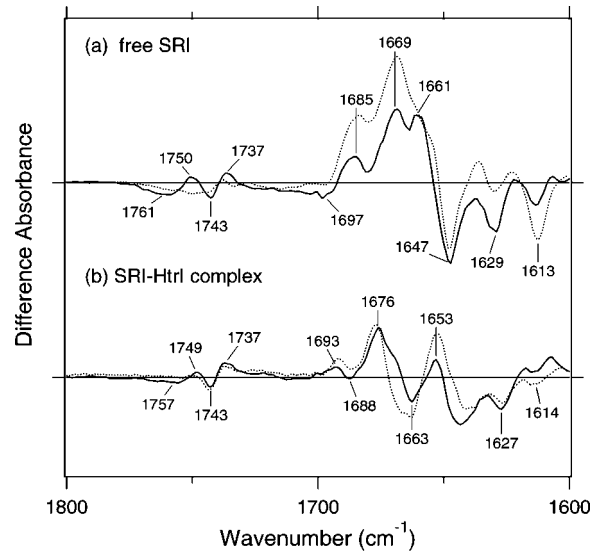

FIGURE 5: Effect of hydration with D_2O on the S_{373} minus SRI infrared difference spectra at acidic pH without (a) and with (b) HtrI in the 1800–1600 cm^{-1} region. The samples were hydrated with H_2O (solid line) and D_2O (dotted line), respectively. One division of the *y* axis corresponds to 0.006 absorbance units.

observed in the difference spectrum of the SRI−HtrI complex (Figure 4b, blue line). Disappearance of the bands at 1697 (−)/1685 (+) cm^{-1}, which is observed in the free SRI at acidic pH (Figure 4a), may apparently increase the intensities of the Asn53 bands in the double difference spectra (Figure 4c; solid line) or smaller numbers of interaction changes in alkaline conditions may diminish the environmental changes of Asn53. Mironova et al. suggested that Asn53 in HtrI regulates the pK_a of Asp76 in SRI directly or indirectly, which could explain the small intensity of the Asn53 bands in alkaline conditions. Mutation studies in alkaline conditions are needed for distinguishing these possibilities.

One of the hydrogen-bonding partners of Asp76, the protonated Schiff base, exhibits a C═N stretching vibrational mode between 1650 and 1600 cm^{-1}. Ref *23* reported that the C═NH and C═ND stretching vibrations were observed at 1634 and 1616 cm^{-1} in free SRI and 1635 and 1618 cm^{-1} in the SRI−HtrI complex, while ref *14* showed that they were 1628 and 1620 cm^{-1} in free SRI, respectively. Although there are some differences, it is reasonable to suppose that the C═N stretching vibrations of SRI appear in the range of 1635–1628 cm^{-1} (C═NH) and 1620–1616 cm^{-1} (C═ND).

The mode can be identified as the bands that show downshifts in frequency upon deuteration of the Schiff base when the sample is hydrated with D_2O, and the extent of the deuterium shift is known to correlate with the strength of hydrogen bonding. On the basis of this criterion, the negative bands at 1629 cm^{-1} (in H_2O) and 1613 cm^{-1} (in D_2O) observed in free SRI in acidic conditions are attributable to the change of the C═N stretching mode of the Schiff base (Figure 5a). The corresponding band in the HtrI-complexed SRI in acidic conditions is not clear, but likely to be shifted to 1627 cm^{-1} (in H_2O) and 1614 cm^{-1} (in D_2O) (Figure 5b). The lower effect of D_2O hydration on reduction and the increase of the C═NH and C═ND band intensities may be explained by less accessibility of water or disruption of the hydrogen bonding network around the Schiff base in the complex, which reduces the H−D exchange rate of the C═NH group.

Other bands in this frequency domain with no deuterium shifts (Figure 5a and b) are identifiable as amide I bands, which reflect conformational changes of the backbone peptide. As noted above, HtrI binding shifted most of the bands or reduced their intensities compared to the HtrI-free SRI sample. These features were confirmed both in H_2O and D_2O conditions, while some bands in D_2O are larger than those observed in H_2O because of relatively reduced linearity in this frequency region due to large infrared absorption of water. The relatively large amount of salts in the samples makes it difficult to control the amount of water. Reduction of the intensity of the amide I bands (1669 (+) and 1661 (+) cm^{-1}, and 1647 (−) cm^{-1} in acidic conditions, and bands at 1661 (+) and 1647 (−) cm^{-1} in alkaline conditions) in the presence of HtrI (Figure 4a and b) suggests that HtrI binding inhibits helix displacements of SRI upon the formation of S_{373} regardless of the protonation state of Asp76. However, the amide I bands appearing only in the presence of HtrI (positive bands at 1676 and 1653 cm^{-1}, and the negative band at 1663 cm^{-1} (Figure 5b, a dotted line)) suggest structural changes of either SRI or HtrI induced by the complexed partner. Such HtrI complex-specific bands were not observed in the samples in alkaline conditions, suggesting that SRI with deprotonated Asp76 is largely uncoupled from HtrI or that at least the manner of complexation is markedly different from that of SRI with protonated Asp76.

Transducer-Dependent Structural Changes of Sensory Rhodopsin I Observed in the 1450–1070-cm^{-1} Region. Many of the bands in this frequency domain are affected by HtrI complex formation only at acidic pH, as is clearly seen in the double difference spectra between the free and HtrI-complexed SRI shown in Figure 6c (a solid line at acid pH and a dotted line at alkaline pH). This frequency region is contributed to by vibrational modes of symmetric stretching vibrations of deprotonated carboxylate groups (CO_2^-) (1440–1335 cm^{-1}), C−O stretching vibrations of a phenol group of a tyrosine residue (1270–1235 cm^{-1}), and C−H bending and C−C stretching vibrations of the retinal chromophore (1400–1100 cm^{-1}). Identification of each of the bands to a specific mode of a residue requires the use of mutant proteins or specifically isotope-labeled protein, although as described below, it is possible to make rough assignments on the basis of currently accumulated knowledge on the vibrational modes of the side chains of the residues and chromophore.

Bands insensitive to D_2O hydration and appearing in the 1440–1335-cm^{-1} region are attributable to symmetric stretching vibrations of deprotonated carboxylates (CO_2^-) of aspartate or glutamate groups (*28*) that undergo environmental changes upon the formation of S_{373}. The bands that meet these criteria are those at 1410 (−)/1395 (+) cm^{-1} and at 1393 (+) /1363 (−) cm^{-1} observed in the free SRI and SRI−HtrI complex, respectively (Figure 7a and b) in acidic conditions. Since large HtrI-dependent shifts are observed in the negative bands but small shifts in S_{373}, the interaction between the carboxylic group and HtrI is likely to be present in the initial state, but weaker in the S_{373} state. Assignment of the bands to carboxylates of specific residues requires the use of mutation or specifically isotope-labeling of carboxyl groups. However, these HtrI-dependent bands are most likely due to a carboxylate located near the SRI−HtrI interface,

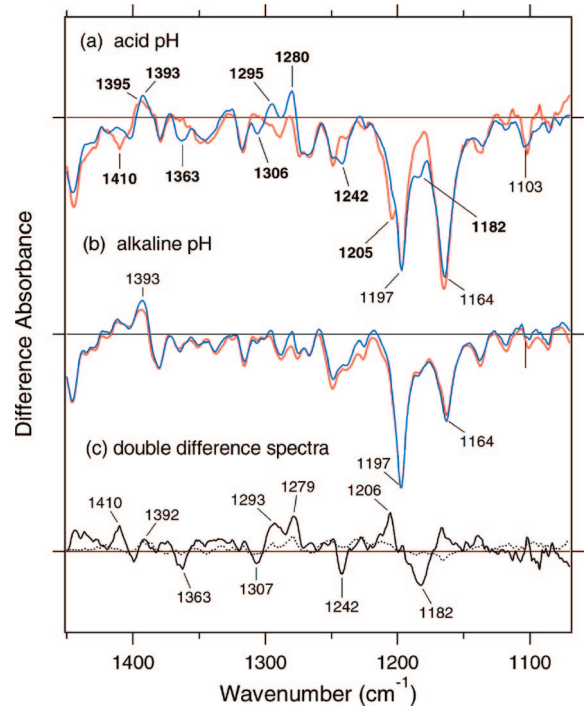

FIGURE 6: S_{373} minus SRI infrared difference spectra at acidic pH (a) and at alkaline pH (b) without (red curve) and with (blue curve) HtrI in the 1450–1070 cm^{-1} region. Spectra are reproduced from Figure 2. (c) The solid line is a double difference spectrum calculated from the blue and red spectra in (a). The double difference spectrum at alkaline pH is shown as a dotted line for comparison. One division of the *y* axis corresponds to 0.0055 absorbance units. Tagged frequensied in bold type represent the bands different between SRI alone and the SRI−HtrI complex.

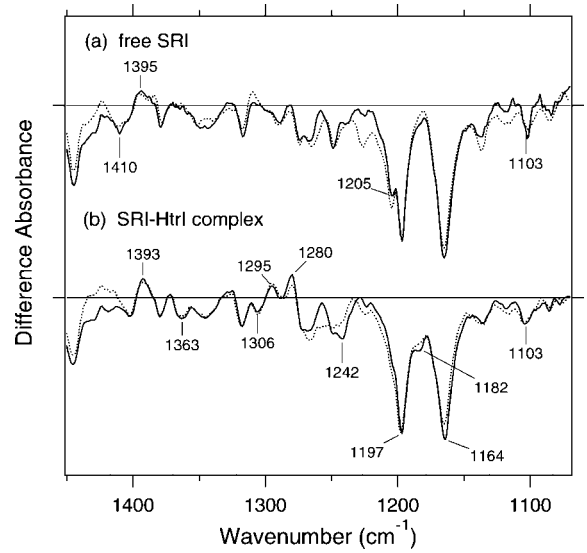

FIGURE 7: Effect of hydration with D_2O on the S_{373} minus SRI infrared difference spectra at acidic pH without (a) and with (b) HtrI in the 1450–1070 cm^{-1} region. The samples were hydrated with H_2O (solid line) and D_2O (dotted line), respectively. One division of the *y* axis corresponds to 0.0055 absorbance units.

although a possibility that other carboxylates outside the interface such as Asp201, which is part of a complex counterion to the protonated Schiff base could be perturbed upon HtrI association cannot be excluded since the binding alters the SRI conformation. Thus, candidates for the band are any of the three glutamate residues (Glu155, Glu187, and Glu223) in helices F and G of SRI.

Deuteration-sensitive bands are attributable to the vibrational mode of the residues with a protonatable group. Of them, the C−O stretching mode of the phenol group of a tyrosine was previously shown in the case of the *p*SRII−*p*HtrII complex to appear at 1280 (+) and 1266 (−) cm^{-1}, which was assigned by the use of the Y199F mutant of the M state and the initial state in *p*SRII, respectively (*20*) (Figure 2c). The deuteraration-sensitive bands in similar frequencies at 1280 (+)/ 1242 (−) cm^{-1} (Figure 7b) observed in the S_{373} minus SRI difference spectra at acidic pH (Figure 6a) are therefore likely to derive from a C−O stretch mode of a tyrosine either in SRI or in HtrI, which undergoes changes upon the formation of the S_{373} intermediate. The candidates are Tyr197, 208, 210, and 213 in helix G of SRI, and Tyr10 and 18 in TM1 in HtrI, which are predicted to reside near the interaction surface.

It should be noticed that the positive bands at 1393 and 1280 cm^{-1} are also observed in the D76N mutant of SRI complexed with HtrI (Figure 3b), but the amplitudes are substantially smaller than those of the wild type. It may suggest that the protein–protein interaction with HtrI slightly modified in the D76N mutant, while it preserves phototaxis responses to attractant and repellent light stimuli.

Other HtrI-dependent bands at 1306 (−), 1295 (+), 1205 (−), and 1182 (−) cm^{-1} (Figure 6a and c) are deuteration-insensitive (Figure 7a and b), implying perturbation of a nonprotonatable residue or the chromophore. The 1306 (−) and 1295 (+) cm^{-1} bands appear to originate from a single vibrational mode of a residue in either SRI or HtrI undergoing change upon S_{373} formation in the SRI−HtrI complex, while the 1205 (−) and 1182 (−) cm^{-1} bands are likely to belong to a single vibrational mode of a residue or the chromophore in the initial state of SRI undergoing change upon complex formation with HtrI. The intensity of the C−C stretching vibrational modes of the chromophore with unprotonated Schiff base becomes considerably smaller compared to the protonated Schiff base showing only negative bands (1197 and 1164 cm^{-1}) due to the C−C stretch modes (C14–C15 and C10–C11 stretching modes, respectively) of the Schiff-base protonated chromophore in the difference S_{373}-minus-SRI spectrum (Figure 6a and b). Therefore, the latter bands appearing only in the negative side might be attributed to a C−C stretching mode of the chromophore perturbed upon HtrI complexation.

Transducer-Dependent Structural Changes of Sensory Rhodopsin I Observed in the 1030–935-cm^{-1} Region. This frequency region is contributed to by the symmetric methyl rocking vibration (1010–1000 cm^{-1}), the hydrogen-out-of-plane (HOOP) vibrations (990–920 cm^{-1}) of the chromophore, and the C−H and N−H wagging modes of amino acid residues. When the Schiff base is deuterated, the N−D in-plane vibrations (970–940 cm^{-1}) of the Schiff base also appear in this frequency range (Figure 8). HtrI binding has significant effects also on these vibrations in acidic pH as shown in Figure 8a and c by the disappearance of the 999 (−) cm^{-1} band and intensification of the 983 (−), 972 (−), 965 (+), 959 (−), and 944 (+) cm^{-1} bands. The 983 and 972 cm^{-1} bands can be assigned to the N−H HOOP mode or C−H HOOP modes coupled to N−H HOOP of the retinal chromophore in the initial state since it disappears upon D_2O hydration (Figure 9b). The bands at 965 (+) and 959 (−) cm^{-1}, which are deuteration insensitive, are attributable either to C−H HOOP modes of the chromophore uncoupled with HOOP near the Schiff base or wagging modes of C−H or N−H of an amino acid residue. Intensification of these bands indicates alteration in the binding pocket of the chromophore in SRI so that the chromophore is more twisted when HtrI is bound.

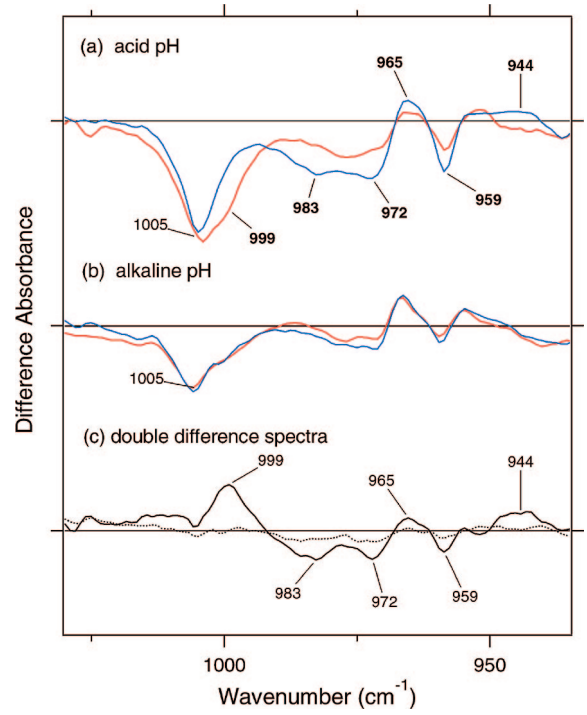

FIGURE 8: S_{373} minus SRI infrared difference spectra at acidic pH (a) and at alkaline pH (b) without (red curve) and with (blue curve) HtrI in the 1030–935 cm^{-1} region. Spectra are reproduced from Figure 2. (c) The solid line is a double difference spectrum calculated from the red and blue spectra in (a). The double difference spectrum at alkaline pH is also shown as a dotted line for comparison. One division of the *y* axis corresponds to 0.0025 absorbance units. Tagged frequensies in bold type represent the bands different between SRI alone and the SRI−HtrI complex.

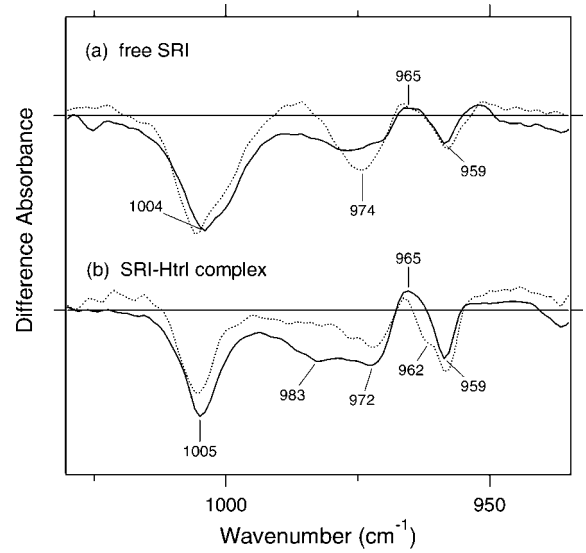

FIGURE 9: Effect of hydration with D_2O on the S_{373} minus SRI infrared difference spectra at acidic pH without (a) and with (b) HtrI in the 1030–935 cm^{-1} region. The samples were hydrated with H_2O (solid line) and D_2O (dotted line), respectively. One division of the *y* axis corresponds to 0.003 absorbance units.

The bands that appear in the frequency range in the deuterated samples (Figure 9a and b) are attributable to the N−D in-plane wagging (bending) vibrations of the chromophore (*23*), which appear at 974 (−) or 962 (−) cm^{-1} for the free or HtrI-complexed SRI, respectively. It is possible that the negative band at 962 cm^{-1} originates from the C15-HOOP downshifted by uncoupling with N−HOOP. Isotope labeling of the retinal is needed for conclusive assignment.

DISCUSSION

Previous studies revealed that HtrI association with SRI increases the flash yield of S_{373}, elevates the pK_a of Asp76 from ∼7.2 to ∼8.5, and makes the photocycle's turnover rate independent of pH in the outer milieu (*13, 15, 22*). All these properties are conferred by an HtrI fragment consisting of the N-terminal 66 amino acid residues (*22*), which is composed of the two-TM domain (1–53) and part of the HAMP domain. Recently, it was reported that Asn53 mutants of HtrI (N53A, N53D, and N53Q) make the photcycle's turnover rate dependent on pH and have an effect on the S_{373} minus SRI infrared difference spectra (*29*). The flash-yield increase and the Asp76's pK_a elevation indicated that HtrI binding alters the conformation of SRI. Here, we have found definitive evidence by analyzing the S_{373}-minus-SRI FTIR spectra: the truncated HtrI at position 147 joined to the receptor indeed induce extensive alterations of SRI in terms of the hydrogen-bonding network connecting the protonated Schiff base and Asp76 of the backbone conformation, of the chromophore twist, and of the perturbation of the side chains of several residues likely to be situated in the interface of SRI and HtrI.

These structural perturbations by HtrI were seen only when Asp76 is protonated but largely lost when it is unprotonated, except that the backbone conformational changes were suppressed, and a carboxylate group was perturbed. Changing pH to alkaline conditions for D76N SRI in complex with HtrI did not result in the loss of perturbations. Therefore, the loss of many of these perturbations is not a consequence of the dissociation of SRI and HtrI in alkaline conditions, which, had it occurred, would result in the deprotonation of Asp76, but rather are the consequence of conformational changes of SRI caused by deprotonation of Asp76.

Even in SRI with deprotonated Asp76, the bound HtrI suppressed light-induced conformational changes as revealed by attenuation of the changes in the amide I and II bands in transition to the initial state to S_{373}. This suppression is a point of similarity with the photophobic receptor, *p*SRII, interaction with its cognate transducer, *p*HtrII. The *p*SRII−*p*HtrII complex differs from SRI−HtrI in that the Asp counterion is unprotonated in the dark and protonated upon M formation.

However, many of the additional conformational couplings of SRI and HtrI as revealed in the present FTIR data are seen only in the state of SRI with protonated Asp76 or with D76N mutation, indicating a coupling between the Asp76-protonation-induced conformational change and SRI−HtrI association. In contrast, in *p*SRII, the D75N mutation does not affect the interaction with *p*HtrII as suggested from the FTIR results in which *p*HtrII-induced perturbation of the difference FTIR spectrum between the initial state and the signaling state of D75N is similar to that of wild-type *p*SRII (*20*), in line with the fact that the D75N mutant of *p*SRII (Schiff base counterion neutralized) exhibits a wild type phototaxis phenotype with *p*HtrII (*30–32*). The notion might be also related to the fact that pK_a elevation of Asp76 requires the interaction of the cytoplasmic portion of HtrI with SRI (*22*), suggesting a coupling between the Asp76-related conformational change of SRI and the interaction of the cytoplasmic portion of HtrI, whereas little or no influence of *p*HtrII binding on the pK_a of Asp75 was found for *p*SRII (our unpublished results).

The minimum length of the HtrI required to prevent proton pumping by SRI and to elevate the pK_a of Asp76 to the same degree as that of the full length HtrI was shown to be 66 (*22*), demonstrating that HtrI(1–66) contains the specific interaction site(s) responsible. Recent FTIR studies confirmed this conclusion by comparing the S_{373}-minus-SRI spectra for SRI bound to HtrI truncated at different positions (*29*). The SRI−HtrI(1–71) complex gave light-induced difference spectra identical to that of HtrI(1–147) (and also to the spectrum in this study) but not HtrI(1–52), showing that residues 53–71 are needed to exert perturbations on the S_{373}-minus-SRI spectrum. The minimum length that provides transducer specificity for SRI binding might be trimmed to HtrI(1–60) on the basis of an earlier publication, in which a chimera transducer with the N-terminal 60 residues of HtrI with the rest of the molecule swapped with the corresponding portion of HsHtrII exhibited a wild-type SRI−HtrI phototaxis phenotype (*30*). The result indicates that residues 1–66 are necessary for blocking the proton pumping of SRI and elevation of the pK_a of Asp76, but the slightly shorter 1–60 are sufficient for the specificity of HtrI binding to SRI. One of the key residues was reported to be Asn53 by the previous FTIR result (*29*). Mutations at the Asn53 position were found to impair the complexation effect on the photocycle's turnover rate and the S_{373}-minus-SRI difference spectra, suggesting that the residue is important for binding to SRI. Consistent with this observation, the Asn53 mutant N53I has been shown to have greatly reduced phototaxis responses (*33*). However, N53I exhibited strong phototaxis responses in the presence of other mutations (inverting mutations SRI D201N or HtrI E56Q) ruling out a strict dependence on Asn53 for functional SRI−HtrI coupling (*33*).

In *H. salinarum* SRII, Asn substitution of Asp73 (corresponding to Asp76 in SRI) makes the complex partially constitutively active in signaling (*31, 32*), showing that the disruption of the salt bridge between the Schiff base and Asp73 triggers partial activation of the receptor. Therefore, SRI with protonated Asp76 in the dark can be regarded as a partially repellent-signaling state, which has been described as an equilibrium between the attractant-signaling state (A conformer) and the repellent-signaling state (R conformer) (*33, 34*). In fact, cells expressing the D76N SRI−HtrI complex have a significantly higher dark reversal rate (*35, 36*) compared to those expressing the wild-type SRII−HtrII complex (*32*). However, the partially constitutively active SRII mutants with position 73 neutralized exhibit further repellent signals upon photoexcitation when the degree of constitutive activity was below a saturating level, while SRI−HtrI delivers attractant signals (suppresses reversals) upon single photon excitation (orange-light excitation). The functional difference must reside in the specific coupling of HtrI to SRI, which shows clear differences compared to that

of the *p*SRII−*p*HtrII complex as revealed in this FTIR study. This notion is also consistent with the fact that most of the mutation sites in SRI that cause inversion of signaling by SRI−HtrI are in the F and G helices of SRI (*33, 34*), which constitute the binding site of HtrI. FTIR studies of the inverted mutants will help elucidate SRI−HtrI association, which is key to understanding the ability of the SRI−HtrI complex to switch between attractant and repellent signaling.

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