

Structural Changes of *Salinibacter* Sensory Rhodopsin I upon Formation of the K and M Photointermediates[†]

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ABSTRACT: Sensory rhodopsin I (SRI) is one of the most interesting photosensory receptors in nature because of its ability to mediate opposite signals depending on light color by photochromic one-photon and two-photon reactions. Recently, we characterized SRI from eubacterium *Salinibacter ruber* (SrSRI). This protein allows more detailed information about the structure and structural changes of SRI during its action to be obtained. In this paper, Fourier transform infrared (FTIR) spectroscopy is applied to SrSRI, and the spectral changes upon formation of the K and M intermediates are compared with those of other archaeal rhodopsins, SRI from *Halobacterium salinarum* (HsSRI), sensory rhodopsin II (SRII), bacteriorhodopsin (BR), and halorhodopsin (HR). Spectral comparison of the hydrogen out-of-plane (HOOP) vibrations of the retinal chromophore in the K intermediates shows that extended chromophore distortion takes place in SrSRI and HsSRI, as well as in SRII, whereas the distortion is localized in the Schiff base region in BR and HR. It appears that sensor and pump functions are distinguishable from the spectral feature of HOOP modes. The HOOP band at 864 cm⁻¹ in SRII, important for negative phototaxis, is absent in SrSRI, suggesting differences in signal transfer mechanism between SRI and SRII. The strongly hydrogen-bound water molecule, important for proton pumps, is observed at 2172 cm⁻¹ in SrSRI, as well as in BR and SRII. The formation of the M intermediate accompanies the appearance of peaks at 1753 (+) and 1743 (–) cm⁻¹, which can be interpreted as the protonation signal of the counterion (Asp72) and the proton release signal from an unidentified carboxylic acid, respectively. The structure and structural changes of SrSRI are discussed on the basis of the present infrared spectral comparisons with other rhodopsins.

Microorganisms respond and adapt to various environmental stimuli. They show attractant and repellent responses (taxis) to survive in various environments where they are living. Taxis to chemicals including to some amino acids, temperature, pH, and light is known as chemotaxis, thermotaxis, pH-taxis, and phototaxis, respectively (1). The archaeon *Halobacterium salinarum* has two photoreceptors regulating negative and positive phototaxis. One of them, sensory rhodopsin I (SRI)¹ works as a dual receptor both for negative and positive phototaxis (2). Sensory rhodopsin II (SRII, also known as phoborhodopsin, pR) is a negative phototaxis receptor in the cells (3). SRI and SRII form 2:2 complexes with their transducer proteins, halobacterial

transducer protein I for SRI (HtrI) and halobacterial transducer protein II for SRII (HtrII) (4, 5), and transmit light signals to a two-component signal transduction cascade, which controls the flagella motor rotation (6).

SRI is one of the most interesting photosensory receptors in nature because of its novel ability to mediate opposite signals depending on light color by photochromic one- and two-photon reactions (2, 7). However, the molecular mechanism of SRI is not understood in atomic terms. On the other hand, the signal relay mechanism of SRII is well characterized by using various methods because SRII from *Natronomonas pharaonis* (NpSRII) has high stability in dilute salt solutions and is much more resistant against detergents (8, 9), whereas HsSRI is unstable in those conditions. Recently, we characterized an extremely stable SRI-like protein from a eubacterium *Salinibacter ruber*, which was named SrSRI (10). This protein is the first eubacterial SRI as a functional protein. SrSRI has an all-*trans* retinal as a chromophore with an absorption maximum at 558 nm and shows a slow photocycle. These properties are close to those of HsSRI. SrSRI is highly stable within the membrane and in detergent micelles even in the absence of NaCl. In addition, a functional expression system utilizing *Escherichia coli* cells can provide a large amount of the proteins (10).

Besides SRI and SRII, *H. salinarum* has two other retinal proteins, bacteriorhodopsin (BR) and halorhodopsin (HR),

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¹ Abbreviations: SrSRI, sensory rhodopsin I from *Salinibacter ruber*; FTIR, Fourier transform infrared; SrSRI_K, K-intermediate of SrSRI; SrSRI_M, M-intermediate of SrSRI; HsSRI, sensory rhodopsin I from *Halobacterium salinarum*; BR, light-adapted bacteriorhodopsin from *Halobacterium salinarum*; HR, halorhodopsin from *Natronomonas pharaonis*; SRII, sensory rhodopsin II from *Natronomonas pharaonis*; BR-T, P200T/V210Y/A215T triple mutant of BR; HOOP, hydrogen out-of-plane.

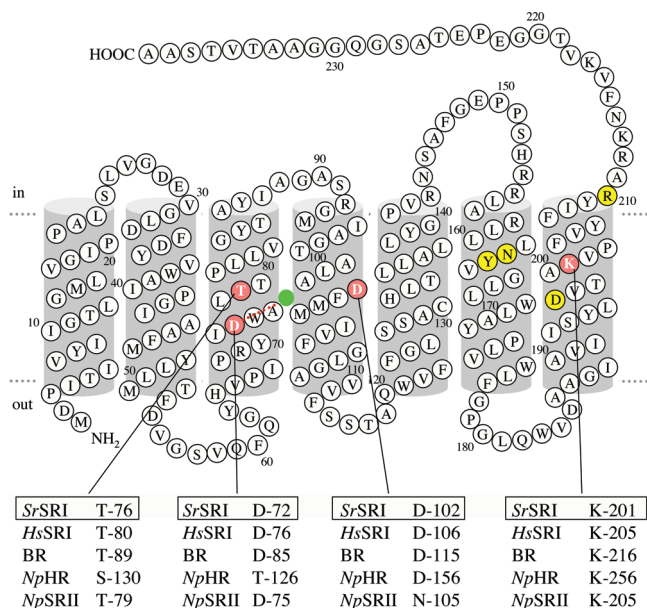


FIGURE 1: Predicted secondary structure of *Salinibacter ruber* sensory rhodopsin I (*SrSRI*). Names of the other rhodopsins are *HsSRI*, sensory rhodopsin I from *Halobacterium salinarum*; BR, bacteriorhodopsin from *Halobacterium salinarum*; *NpHR*, halorhodopsin from *Natronomonas pharaonis*; and *NpSRII*, sensory rhodopsin II from *Natronomonas pharaonis*. Amino acid residues colored with yellow are necessary for function in *HsSRI*. The N165F, H166Y, and D201N mutations convert the normally attractant signal to a repellent signal (7, 60, 61). The R215W is a suppressor mutation (62). We focused on the residues colored pink. A strongly hydrogen-bonded water molecule is shown as a filled green circle.

which work as light-driven ion pumps, whose functions are distinctly different from those of SRI and SRII. Namely, BR and HR function as a light-driven outward proton pump and a light-driven inward chloride pump, respectively. The original state of SRI ($\lambda_{\max} = 587$ nm) and its long-lived photointermediate (the M-intermediate; $\lambda_{\max} = 373$ nm) are important for positive and negative phototaxis, respectively (6). SRII is a negative phototaxis receptor in haloarchaea, including *H. salinarum* and *N. pharaonis*. Those are called *HsSRII* and *NpSRII*, respectively, and their absorption maxima are at about 500 nm (8). Thus, haloarchaea are attracted to light with wavelengths longer than 520 nm, and they avoid light with wavelengths shorter than 520 nm due to the functions of SRI and SRII (6). Light at >520 nm can activate the ion pumping rhodopsins (BR and HR) to obtain light-energy, and cells avoid light of shorter wavelengths, which contains harmful near-UV. Interestingly it was reported that SRI and SRII are able to function as an outward proton pump like BR only in the absence of the cognate transducer proteins (11, 12), suggesting that these retinal proteins mentioned above are presumably evolved from the same protein and have similar structures (Figure 1). In fact, the rhodopsins have seven transmembrane helices and a retinal chromophore bound to a specific lysine residue of helix G via a protonated Schiff base linkage (13). To stabilize the positive charge of a protonated Schiff base inside the protein, highly conserved charged groups are present in the Schiff base region: arginine and aspartate of helix C and aspartate of helix G. Water molecules also stabilize the charged group in the region (13). In particular, three water molecules are involved in the pentagonal cluster structure

(14, 15). A strongly hydrogen-bonded water molecule located between the Schiff base and the counterion exists in the light-driven proton pumps, BR (16), transducer-free SRII (17), HR with azide (18), and D212N BR mutant with NaCl (19). Thr204 in SRII located around the retinal chromophore (The distance from the C14 atom to the hydroxyl oxygen of Thr204 is 4.4 Å.) is quite an important residue for negative phototaxis (15, 20). Thus, structural changes of the Schiff base region and internal water molecules are directly linked to functions of retinal proteins.

Light absorption of these retinal proteins triggers a cyclic reaction that is comprised of a series of intermediates, designated alphabetically (21). The *trans*–*cis* photoisomerization of the retinal chromophore leads to the formation of the K intermediate. In proton pumps, the primary proton transfer takes place in the L to M transition. A proton is transferred from the protonated Schiff base (Lys205 for *HsSRI*; Lys216 for BR; Lys205 for SRII) to an aspartate of helix C (Asp76 for *HsSRI*; Asp85 for BR; Asp75 for SRII). In BR, a proton is released simultaneously from a protonated water cluster to the extracellular side (22, 23). Consequently the proton was transferred from the cytoplasmic side to the extracellular side. Thus, the molecular properties of ion-pumping rhodopsins, especially BR, have been extensively studied.

How about photosensory rhodopsins? SRII has been well-characterized over the past several years using various methods because of its high protein stability (24). The crystallographic structure of SRII and the SRII/HtrII complex had been achieved (5, 15), and light-induced structural changes were also identified by various methods, including UV–vis, electron paramagnetic resonance (EPR) spectroscopy, etc. Interestingly, the structure and structural changes are almost the same as those of BR, implying that the functional differentiation was caused by small structural differences in some amino acid side chain(s) or main chain(s). In fact, Sudo and Spudich recently reported that substitution of only three residues, P200T/V210Y/A215T, converted BR into a sensor for negative phototaxis like SRII (BR-T), indicating similar structural changes between BR and SRII (25). On the other hand, little is known about the molecular mechanism(s) of interactions between SRI and HtrI, about structural changes, or about the signal relay mechanism of positive phototaxis.

Here, we utilized Fourier transform infrared (FTIR) spectroscopy to explore structural changes of *SrSRI*. FTIR spectroscopy is a powerful tool to investigate structure–function relationship in rhodopsins. In the case of SRII, a transducer-dependent specific interaction of Thr204 was first found (26), followed by phototaxis analysis revealing the important role of Thr204 in function (20). Then, the origin of such a specific interaction related to Thr204 was revealed from a positive correlation between the increment of a retinal vibration in the K intermediate and the physiological phototaxis response (27, 28). In *HsSRI*, earlier studies identified characteristic vibrational bands such as Asn53 (29) and Asp76 (30). It was also shown that *HsSRI*_M contains 13-*cis* retinal (31). Hydrated film samples of *SrSRI* were stable in the absence of salt, in contrast to *HsSRI* (32). This enabled us to obtain accurate light-induced difference spectra upon formation of the K and M intermediates in the entire mid-infrared region. In this study, we report light-induced

difference FTIR spectra at neutral pH, where the Schiff base counterion (Asp72) is deprotonated. The obtained spectra for *SrSRI* are compared with those of other archaeal rhodopsins such as BR, HR, *HsSRI*, and *SRII*.

MATERIALS AND METHODS

Preparation of *SrSRI* and *HsSRI* Samples. *SrSRI* and *HsSRI* were prepared as described previously (10, 32, 33). Briefly, the *SrSRI* and *HsSRI* proteins with a six-histidine tag at the C-terminus were expressed in *E. coli* BL21(DE3) cells, solubilized with 1.0% *n*-dodecyl β -D-maltoside (DDM), and purified with a Ni²⁺-NTA column (QIAGEN, Valencia, CA, USA) as described previously (34). Purified samples were then reconstituted into L- α -phosphatidylglycerol (PG) liposomes (*SRI*/PG = 1:50 molar ratio) by removing the detergent with Bio-Beads (SM2, Bio-Rad, Hercules, CA).

FTIR Spectroscopy. Low-temperature FTIR spectroscopy was performed with 2-cm⁻¹ resolution as described previously (17). The *SrSRI* samples in PG liposomes were washed with a 2 mM phosphate buffer (pH 7.0). Then a 90 μ L aliquot of the sample (0.1–1 mg) was dried on a BaF₂ window with a diameter of 18 mm. The *HsSRI* samples in PG liposomes were washed with a 15 mM borate buffer (pH 8.3) containing 300 mM NaCl. Then a 40 μ L aliquot of the sample (0.1–1 mg) was dried on a BaF₂ window with a diameter of 18 mm, and deposition of excess salt was washed away with a 15 mM borate buffer (pH 8.3) containing 15 mM NaCl. After hydration with H₂O, D₂O, or D₂¹⁸O, the sample was placed in a cell, which was mounted in an Oxford DN-1704 cryostat equipped in the Bio-Rad FTS-40 spectrometer.

The *SrSRI*_K minus *SrSRI* difference spectra were measured at 77 K as follows. Illumination of the *SrSRI* film with a 450 nm light for 2 min at pH 7.0 and 77 K converted *SrSRI* to *SrSRI*_K, and subsequent illumination with >640 nm light forced *SrSRI*_K to revert to *SrSRI*. The *SrSRI*_M minus *SrSRI* difference spectra were measured at 260 K and pH 7.0 as follows. To convert *SrSRI* to *SrSRI*_M, the sample was irradiated with >480 nm light for 2 min, after which subsequent 1 min illumination with UV light changed *SrSRI*_M back to *SrSRI*. The difference spectrum was calculated from the spectra constructed with 128 interferograms before and after the illumination. Twenty-four or sixteen spectra obtained in this way were averaged for the *SrSRI*_K minus *SrSRI* or *SrSRI*_M minus *SrSRI* spectra. The *HsSRI*_K minus *HsSRI* difference spectra were measured at 77 K. Illumination of the *HsSRI* film with 500 nm light for 2 min at pH 7.0 and 77 K converted *SrSRI* to *SrSRI*_K, and subsequent 1 min illumination with >680 nm light forced *HsSRI*_K to revert to *HsSRI*.

BR_K minus BR, HR_K minus HR, *SRII*_K minus *SRII*, BR_T minus BR-T spectra, BR_M minus BR, *HsSRI*_M minus *HsSRI*, and *SRII*_M minus *SRII* spectra were taken from Shibata et al. (35), Shibata et al. (36), Kandori et al. (34), Sudo et al. (37), Tanimoto et al. (38), Furutani et al. (32), and Furutani et al. (39), respectively. All spectra were normalized with respect to the C–C or C=C stretching vibration of the retinal chromophore.

RESULTS

Infrared Spectral Changes of *SrSRI* and *HsSRI* upon Formation of K Photointermediate. Figure 2a,b shows the K minus initial state difference FTIR spectra of *SrSRI* and

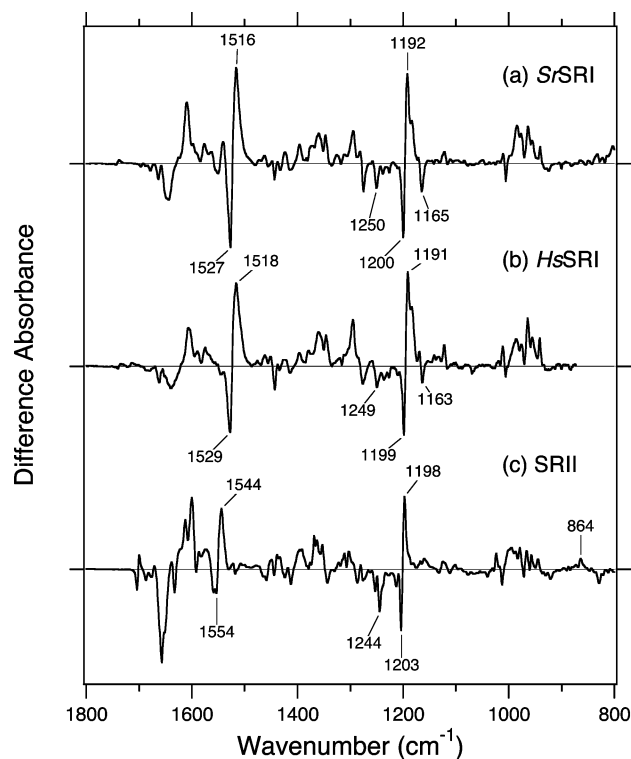


FIGURE 2: *SrSRI*_K minus *SrSRI* (a) and *HsSRI*_K minus *HsSRI* (b) difference infrared spectra measured at 77 K at pH 7.0 and 8.5, respectively, in the 1800–800 cm⁻¹ region. The spectrum of *HsSRI* is deleted at <872 cm⁻¹. The *HsSRI*_K minus *HsSRI* spectrum (b) is multiplied by 4.2 for sake of comparison. The samples were hydrated with H₂O. The *SRII*_K minus *SRII* difference FTIR spectrum (c) is reproduced from ref 34 for comparison. One division of y axis corresponds to 0.018 absorbance units.

HsSRI, respectively. The *SRII*_K minus *SRII* (Figure 2c) is reproduced from ref 34 for comparison. The spectrum of *HsSRI* was too noisy at <872 cm⁻¹ because of scattering, so that the region was removed. The bands at 1527 (–)/1516 (+) cm⁻¹ for *SrSRI*, 1529 (–)/1518 (+) cm⁻¹ for *HsSRI*, and 1554 (–)/1544 (+) cm⁻¹ for *SRII* correspond to the ethylenic C=C stretching vibrations of the retinal chromophore. The lower frequency shift corresponds to the spectral red-shift upon formation of the K intermediate. The bands at 1250 (–), 1200 (–), 1192 (+), and 1165 (–) cm⁻¹ for *SrSRI*, 1249 (–), 1199 (–), 1191 (+), and 1163 (–) cm⁻¹ for *HsSRI*, and 1244 (–), 1203 (–), and 1198 (+) cm⁻¹ for *SRII* correspond to the C–C stretching vibrations of the retinal chromophore, and the frequency shift represents retinal isomerization from the all-*trans* to 13-*cis* form. Thus, these results indicate the formation of the K intermediate both in *SrSRI* and *HsSRI* as well as in *SRII*. Negative bands were observed at 1165 cm⁻¹ for *SrSRI* and 1163 cm⁻¹ for *HsSRI*, but not for *SRII*. It is likely that the bands originate from C10–C11 stretching vibration, because the corresponding band of BR appears at 1170 cm⁻¹ and is assigned to C10–C11 stretching vibration (40).

Figure 3 shows the spectral changes in the 1780–1560 cm⁻¹ region, where most signals originate from vibrations of protein. One exception is the C=N stretching vibration of the retinal Schiff base. Among various D₂O sensitive bands of *SRII* (c), the C=N stretch has been assigned at 1650 cm⁻¹ in H₂O and 1633 cm⁻¹ in D₂O (34). The 1646 and 1622 cm⁻¹ bands for *SrSRI* are tentatively assigned to the C=N stretches of the retinal Schiff base in H₂O and D₂O,

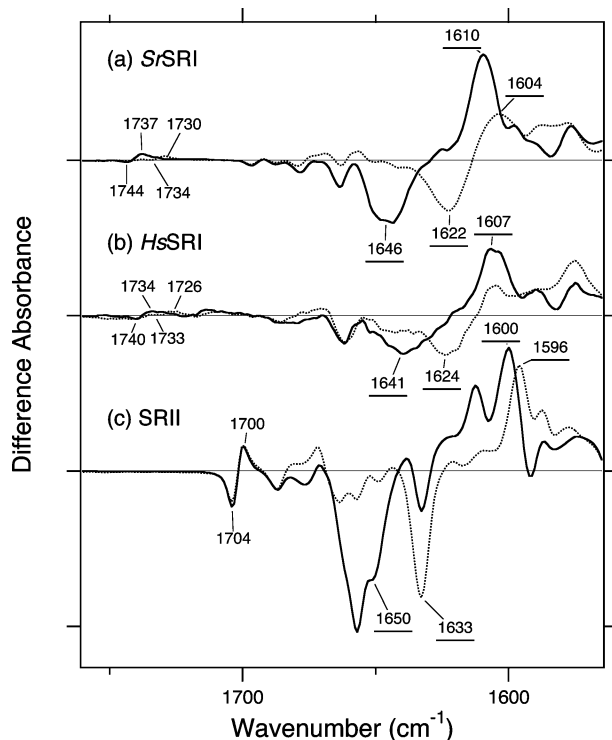


FIGURE 3: *SrSRI_K* minus *SrSRI* (a) and *HsSRI_K* minus *HsSRI* (b) difference infrared spectra measured at pH 7.0 and 8.5, respectively, in the 1780–1565 cm^{-1} region. The *HsSRI_K* minus *HsSRI* spectra are multiplied by 4.2 for sake of comparison. The samples were hydrated with H_2O (—) or D_2O (···). Underlined values correspond to C=N stretching vibration. The *SRII_K* minus *SRII* difference FTIR spectra (c) are reproduced from ref 34 for comparison. One division of y axis corresponds to 0.008 absorbance units.

respectively. Previous resonance Raman of *HsSRI* reported those frequencies at 1634 and 1616 cm^{-1} (31). Therefore, the 1641 and 1624 cm^{-1} bands in Figure 3b probably originate from the Schiff base C=N stretches of *HsSRI* in H_2O and D_2O , respectively. The spectral upshift in H_2O is caused by the coupling of the N–H bending vibration of the Schiff base, and the difference in frequency between H_2O and D_2O has been regarded as the marker of hydrogen-bonding strength of the Schiff base nitrogen. Frequency differences in *SrSRI* ($1646 - 1622 = 24 \text{ cm}^{-1}$) and *HsSRI* ($1641 - 1624 = 17 \text{ cm}^{-1}$) are similar to the value in *SRII* ($1650 - 1633 = 17 \text{ cm}^{-1}$), indicating that the hydrogen-bonding strengths of the Schiff base in SRIs are similar to that of *SRII*. In the K state, the C=N stretching vibrations appear at 1610–1600 cm^{-1} , though the band in D_2O is unclear only for *HsSRI* (Figure 3b).

In *SRII*, the 1704 (–)/1700 (+) cm^{-1} bands were assigned as the C=O stretching vibration of Asn105, and the strong peaks are characteristic for *SRII* (41). Corresponding amino acids are Asp102 in *SrSRI* and Asp106 in *HsSRI*. In *SrSRI* and *HsSRI*, the 1744 (–)/1737 (+) and 1740 (–)/1734 (+) cm^{-1} bands in H_2O shifted to 1734 (–)/1730 (+) and 1733 (–)/1726 (+) cm^{-1} in D_2O (Figure 2a,b), respectively. They were ascribable to the C=O stretching vibrations of Asp102 for *SrSRI* and Asp106 in *HsSRI*, respectively. The corresponding bands appear at 1742 (–)/1733 (+) cm^{-1} in BR and 1744 (–)/1737 (+) cm^{-1} in HR (41–43), and their intensities are similar to those of *SrSRI* and *HsSRI*. It should be noted that the carboxylic C=O stretch of the N105D mutant *SRII* exhibits intense bands at 1744(–)/1739(+) cm^{-1}

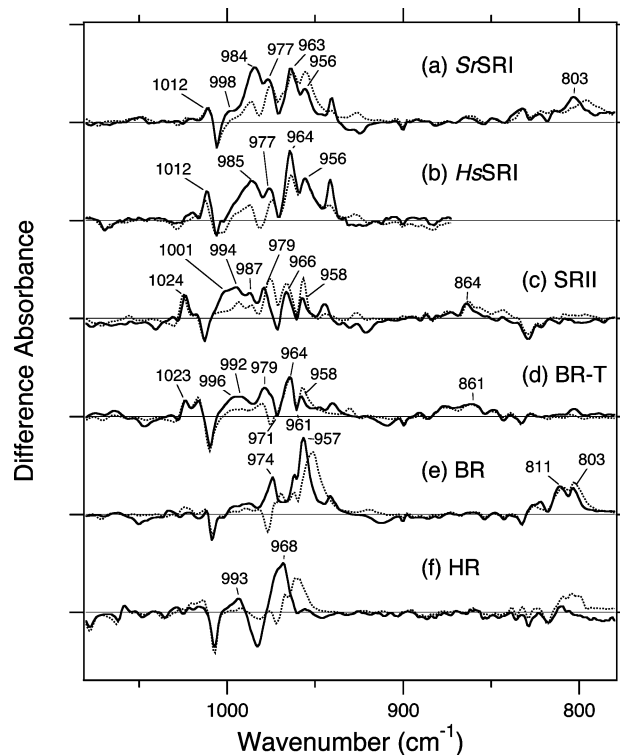


FIGURE 4: *SrSRI_K* minus *SrSRI* (a) and *HsSRI_K* minus *HsSRI* (b), difference infrared spectra measured at pH 7.0 and 8.5, respectively, in the 1080–780 cm^{-1} region. The spectrum of *HsSRI* was deleted at $<872 \text{ cm}^{-1}$. The *HsSRI_K* minus *HsSRI* spectra are multiplied by 4.2 for sake of comparison. The samples were hydrated with H_2O (—) or D_2O (···). The *SRII_K* minus *SRII* (c), *BR-T_K* minus *BR-T* (d), *BR_K* minus *BR* (e), and *HR_K* minus *HR* (f) difference FTIR spectra are reproduced from refs 34, 37, 35, and 36, respectively, for comparison. One division of y axis corresponds to 0.006 absorbance units.

in H_2O and at 1734(–)/1728(+) cm^{-1} in D_2O (41). Thus, the structure and structural changes at position 102 in *SrSRI* (106 in *HsSRI*) are similar to those of ion-pumping rhodopsins.

Figure 4 shows spectral changes in the 1080–780 cm^{-1} region, where hydrogen out-of-plane (HOOP) vibrations of the retinal molecule are mainly observed. Difference FTIR spectra for *SRII* (c), *BR-T* (d), *BR* (e), and *HR* (f) are reproduced from refs 34, 37, 35, and 36, respectively, for comparison. The *SrSRI_K* minus *SrSRI* and the *HsSRI_K* minus *HsSRI* spectra exhibit intense positive peaks at 998, 984, 977, 963, and 956 cm^{-1} for *SrSRI*, and at 985, 977, 964, and 956 cm^{-1} for *HsSRI*, which would be tentatively assigned to HOOP vibrations of the retinal chromophore. These results suggest that the structural changes spread to the middle part of the retinal chromophore in *SRI*. By using all seven monodeuterated retinal analogues, Furutani et al. assigned the 966 (+)/971 (–) and 958 (+)/961 (–) cm^{-1} bands in *SRII* as the C7=C8 and C11=C12 Au (irreducible representation of diene vibrations) HOOP modes, respectively, and the positive bands at 1001, 994, 987, and 979 cm^{-1} as the C15-HOOP vibrations (40). From the similarity in frequency, the bands at 998, 984, and 977 cm^{-1} in *SrSRI* are likely to originate from C15-HOOP (Figure 4a). The bands at 963 and 956 cm^{-1} are likely to originate from C7=C8 and C11=C12 Au HOOP vibration, respectively. These bands were also observed in a BR mutant engineered to transmit photosensory signals like *SRII* (*BR-T*) at 964 (+)/971 (–), 958 (+)/961 (–), 996 (+), 992 (+), and 979

(+) cm^{-1} (Figure 4d), implying the importance of the extended chromophore distortion for phototaxis signaling. In addition, a positive band at 1012 cm^{-1} appears both in SrSRI_K and HsSRI_K . A similar band is also present at 1024 cm^{-1} in SRII_K and at 1023 cm^{-1} in BR-T_K , which was assigned to symmetric rocking of methyl groups connected to the C9 and C13 atoms (37, 40).

On the other hand, only two intense positive peaks were observed for ion-pumping rhodopsins, at 974 and 957 cm^{-1} for BR (Figure 4e) and at 993 and 968 cm^{-1} for HR (f). For the BR spectrum (e), the D_2O -sensitive intense bands at 974 (+) and 957 (+) cm^{-1} were assigned as HOOP vibrations of C15–H and N–H groups (44), indicating that the structural changes of BR upon formation of the K intermediate are localized to the Schiff base region unlike photosensory rhodopsins. Thus, the HOOP modes were highly different between photosensors (Figure 4a–d) and ion-pumps (e, f).

The bands at 864 and 861 cm^{-1} were assigned as C14–HOOP in SRII (Figure 4c) and BR-T (d), respectively, the presence of which is highly correlated with negative phototaxis (28). It appears that SrSRI does not possess such a band (Figure 4a). Instead, SrSRI possesses a peak at 803 cm^{-1} , which was absent for SRII and BR-T but present for BR. In the BR spectra (Figure 4e), two intense positive bands at 811 and 803 cm^{-1} are likely to originate from wagging vibrations of the retinal chromophore, because resonance Raman study reported that such vibrations appeared in the 900 – 800 cm^{-1} region (45). Therefore the same band in SrSRI would originate from the vibration, although the band at 803 cm^{-1} is more largely shifted upon D_2O hydration than those in BR.

Figure 5 shows the spectral changes in D_2O in the 2560 – 1900 cm^{-1} region, which contains O–D and N–D stretching vibrations. The measurements for SRII , BR, and HR are reproduced from the published spectra (34–36). The bands at 2515 (–)/ 2474 (+) cm^{-1} of SRII (b) and 2506 (–)/ 2466 (+) cm^{-1} of BR (c) were previously assigned as the O–D stretch of Thr79 and Thr89, respectively (46, 47). Therefore, similar bands at 2526 (–)/ 2471 (+) cm^{-1} of SrSRI (a) that show no isotope shift of D_2^{18}O possibly originate from the O–D stretch of a conserved threonine residue (Thr76) (Figure 1). A low frequency (2526 cm^{-1}) as threonine O–D stretch is an indication of a strong hydrogen bond of Thr76 in SrSRI , presumably with a counterion, Asp72. A lower frequency shift from 2526 to 2471 cm^{-1} implies a further strengthened hydrogen bond of Thr76 upon retinal photoisomerization.

Some peaks in this frequency region exhibit spectral downshift by $\sim 10\text{ cm}^{-1}$ in D_2^{18}O (blue spectra in Figure 5), which originate from O–D stretching vibrations of water molecules. Proton-pumping rhodopsins possess strongly hydrogen-bonded water molecule(s), whose O–D stretch is located at $<2400\text{ cm}^{-1}$ (48). The water stretches of SRII at 2307 and 2215 cm^{-1} (Figure 5b) and those of BR at 2323 , 2292 , and 2171 cm^{-1} (Figure 5c) correspond to strongly hydrogen-bonded water molecules (16, 17). HR has only moderately hydrogen-bonded water molecules at 2535 , 2501 , and 2445 cm^{-1} in the unphotolyzed state, though retinal isomerization strengthens the water hydrogen bond as shown at 2384 and 2263 cm^{-1} (Figure 5d) (36). In the case of SrSRI , a strongly hydrogen-bonded water molecule was observed at 2172 cm^{-1} (Figure 5a). In addition, water bands were

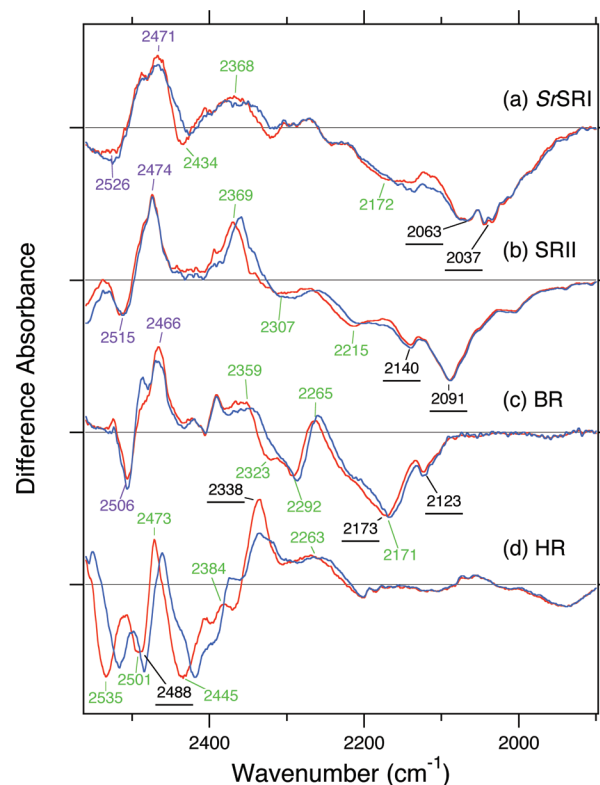


FIGURE 5: SrSRI_K minus SrSRI (a) difference infrared spectra measured at pH 7.0 in the 2560 – 1900 cm^{-1} region. The samples were hydrated with D_2O (red line) and with D_2^{18}O (blue line). Underline, green label, and purple label correspond to C=N stretching vibration, vibration of water molecules, and vibration of threonine residue, respectively. The SRII_K minus SRII (b), BR_K minus BR (c), and HR_K minus HR (d) difference FTIR spectra are reproduced from refs 34, 35, and 36, respectively, for comparison. One division of y axis corresponds to 0.0012 absorbance units.

observed at 2434 (–)/ 2368 (+) cm^{-1} (Figure 5a), as well as 2651 and 2611 cm^{-1} (data not shown).

The peaks at 2063 and 2037 cm^{-1} in SrSRI (Figure 5a) do not originate from the water O–D stretch. The peaks at 2140 and 2091 cm^{-1} in SRII (b) and those at 2173 and 2123 cm^{-1} in BR (c) were previously assigned to the N–D stretches of the Schiff base, using ^{15}N -Lys containing proteins (47, 49). Therefore, the 2063 and 2037 cm^{-1} bands in SrSRI are good candidates for the Schiff base vibration. Lower frequencies of these bands indicate that hydrogen-bonding strength of the N–D group in SrSRI is stronger than those of BR and SRII , which is consistent with the analysis of C=N stretching vibrations (Figure 3) (34, 35).

Infrared Spectral Changes of SrSRI , HsSRI , and SRII upon Formation of M Photointermediate. Figure 6a shows the M minus initial state difference FTIR spectra of SrSRI (a). Difference FTIR spectra for HsSRI (b) and SRII (c) are reproduced from refs 32 and 39, respectively, for comparison. The bands at 1525 (–)/ 1562 (+) cm^{-1} for SrSRI , 1525 (–)/ 1559 (+) cm^{-1} for HsSRI , and 1545 (–)/ 1568 (+) cm^{-1} for SRII correspond to the ethylenic C=C stretching vibrations of the retinal chromophore, but amide-II vibrations also possibly appear. In fact, resonance Raman data reported the C=C stretches of the M state of HsSRI at 1568 cm^{-1} (31), not at 1559 cm^{-1} . Negative bands at 1249 , 1199 , and 1164 cm^{-1} for SrSRI are attributable to the C–C stretching vibration of the retinal chromophore, where intensity of the corresponding positive bands is much reduced because of

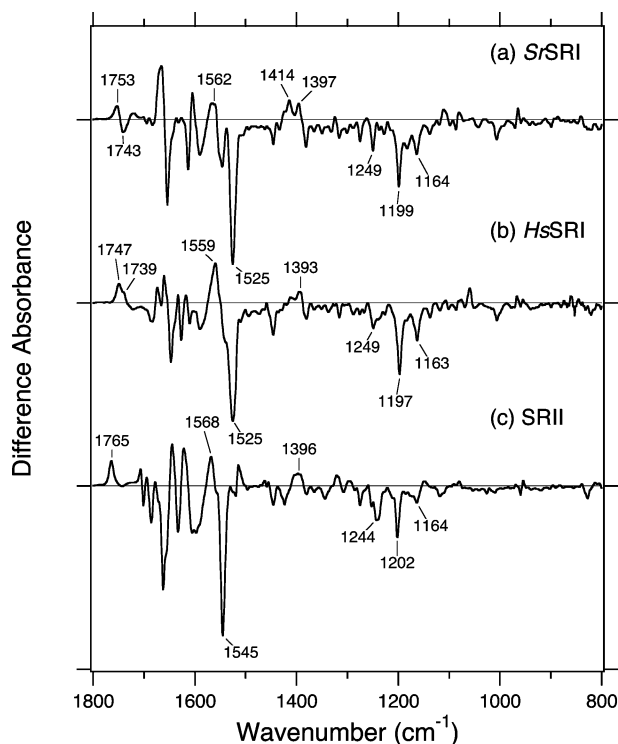


FIGURE 6: $SrSRI_M$ minus $SrSRI$ (a) difference infrared spectra measured at 260 K at pH 7.0 in the 1800–800 cm^{-1} region. The samples were hydrated with H_2O . The $HsSRI_M$ minus $HsSRI$ (b) and $SRII_M$ minus $SRII$ (c) difference FTIR spectra are reproduced from refs 32 and 39, respectively, for comparison. One division of y axis corresponds to 0.01 absorbance units.

deprotonation of the Schiff base. All three spectra show a positive peak at 1397–1393 cm^{-1} . In addition, the $SrSRI_M$ minus $SrSRI$ spectrum shows an additional peak at 1414 cm^{-1} (Figure 6a). Since COO^- stretching vibrations of carboxylate appear in this frequency region, the positive band may correspond to deprotonation of a protonated carboxylic acid at 1743 cm^{-1} .

Figure 7 shows the spectral changes in the 1780–1600 cm^{-1} region. When the Schiff base counterion (Asp72 for $SrSRI$; Asp76 for $HsSRI$; Asp75 for $SRII$) is deprotonated, the M formation accompanies a proton transfer from the Schiff base to the counterion. The experimental conditions are indeed the cause, because the pH is higher than the pK_a of the counterion. In $SRII$, the positive band at 1765 cm^{-1} in H_2O and at 1753 cm^{-1} in D_2O represents the protonation signal of Asp75 (Figure 7c) (39). In $HsSRI$, the positive band at 1749 cm^{-1} band in H_2O and at 1739 cm^{-1} in D_2O were assigned as the $C=O$ stretching vibration of Asp76 (Figure 7b) (30, 32). In the case of $SrSRI$, we observed a positive band at 1753 cm^{-1} in H_2O , which shifts to 1742 cm^{-1} in D_2O (Figure 7a). Therefore, it is reasonable to assign the band as the $C=O$ stretching vibration of Asp72.

Interestingly, the $SrSRI_M$ minus $SrSRI$ spectrum exhibits an additional negative band at 1743 cm^{-1} in H_2O , shifting to 1734 cm^{-1} in D_2O . At present, we cannot exclude the possibility that the bands at 1753 (+)/1743 (–) cm^{-1} originate from a carboxylic acid (hydrogen-bonding alteration of a protonated carboxylic acid), but it is unlikely because the M formation probably accompanies a proton transfer to Asp72. Thus, a protonated carboxylic acid undergoes deprotonation upon formation of the M intermediate. A positive band at 1414 cm^{-1} (Figure 6a) possibly corresponds to the

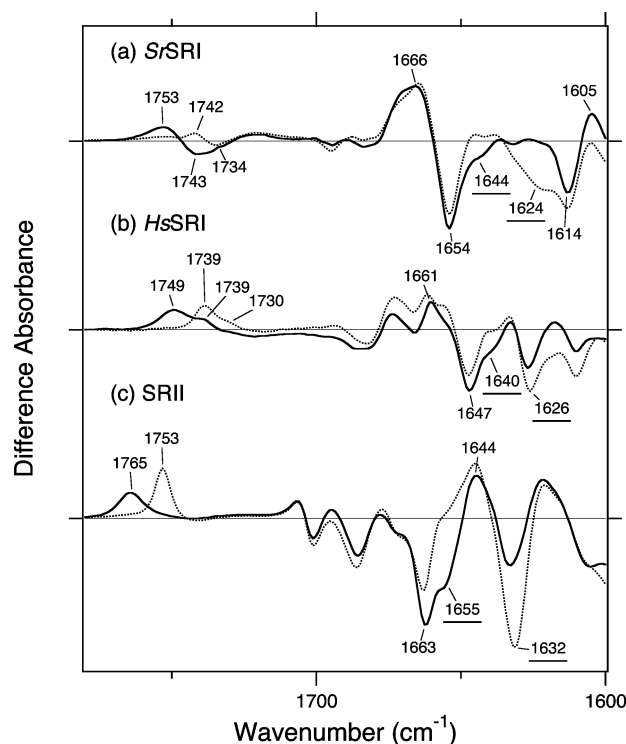


FIGURE 7: $SrSRI_M$ minus $SrSRI$ (a) difference infrared spectra measured at 260 K at pH 7.0 in the 1780–1600 cm^{-1} region. The samples were hydrated with H_2O (—) or D_2O (····). Underlined values corresponds to $C=N$ stretching vibration. The $HsSRI_M$ minus $HsSRI$ (b) and $SRII_M$ minus $SRII$ (c) difference FTIR spectra are reproduced from refs 32 and 39, respectively, for comparison. One division of y axis corresponds to 0.01 absorbance units.

signal of the carboxylate deprotonation. In BR, the M formation accompanies proton release from a protonated water cluster, not from a carboxylic acid (22, 23). The present observation may indicate proton release from a carboxylic acid at the extracellular side of $SrSRI$, which is also the case in some BR mutants (50).

Figure 7 shows the $C=N$ stretching vibrations in H_2O and D_2O : at 1644 and 1624 cm^{-1} in $SrSRI$ (a), at 1640 and 1626 cm^{-1} in $HsSRI$ (b), and at 1655 and 1632 cm^{-1} in $SRII$ (c), respectively. On the other hand, H/D-insensitive bands can be identified as an amide-I vibration. Figure 7 shows such bands at 1666 (+)/1654 (–) cm^{-1} for $SrSRI$ (a), at 1661 (+)/1647 (–) cm^{-1} for $HsSRI$ (b), and at 1663 (–)/1644 (+) cm^{-1} for $SRII$ (c), which correspond to an amide-I vibration of the α -helix. Interestingly, frequency shifts were opposite between SRI and $SRII$, where a downshift and an upshift were observed, respectively. This indicates that the M formation accompanies a weakened hydrogen bond of the α -helix in SRI but a strengthened hydrogen bond of the α -helix in $SRII$. In other words, activation of SRI or $SRII$ may involve breakage or formation of the helical structure.

Figure 8 shows the spectral changes in D_2O in the 2740–1900 cm^{-1} region. Difference FTIR spectra for $SRII$ (b) and BR (c) are reproduced from refs 39 and 38, respectively, for comparison. The bands at 2704 (+), 2690 (–), and 2640 (+) cm^{-1} in the $SrSRI_M$ minus $SrSRI$ spectrum (Figure 8a) can be identified as water O–D stretching vibrations, whereas no isotope effect of water molecules was observed at <2500 cm^{-1} . Absence of the negative 2172 cm^{-1} band (Figure 5a) in Figure 8a indicates that the strongly

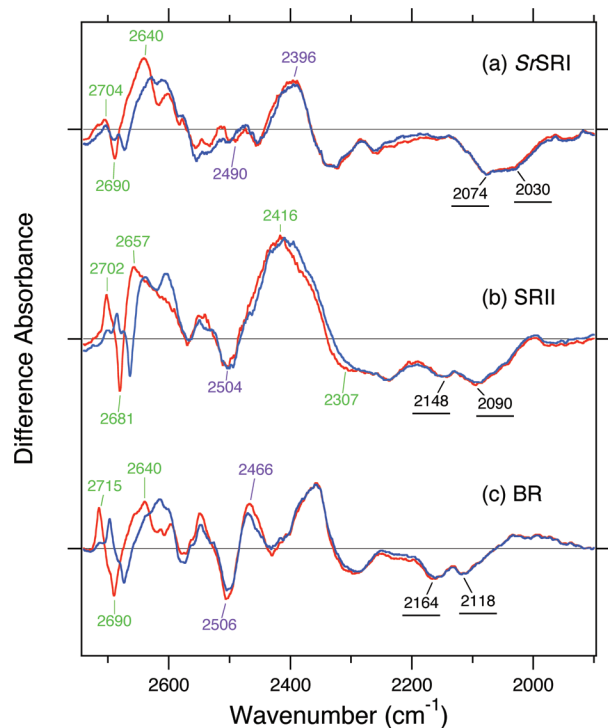


FIGURE 8: $SrSRI_M$ minus $SrSRI$ (a) difference infrared spectra measured at 260 K at pH 7.0 in the 2760–1900 cm^{-1} region. The samples were hydrated with D_2O (red line) and with $D_2^{18}O$ (blue line). Underline, green label, and purple label correspond to C=N stretching vibration, vibration of water molecules, and vibration of threonine residue, respectively. The $SRII_M$ minus $SRII$ (b) and BR_M minus BR (c) difference FTIR spectra are reproduced from refs 39 and 38, respectively, for comparison. One division of y axis corresponds to 0.0012 absorbance units.

hydrogen-bonded water molecule in $SrSRI$ restores its hydrogen-bonding strength upon formation of the M intermediate. This is also the case for strongly hydrogen-bonded water molecules in BR (2323, 2292, and 2171 cm^{-1} bands in Figure 5c) and $SRII$ (2215 cm^{-1} band in Figure 5b), but only the negative water band at 2307 cm^{-1} in $SRII$ changes its hydrogen bond in $SRII_M$, presumably shifting to 2416 cm^{-1} (Figure 8b).

The negative bands at 2148 and 2090 cm^{-1} in $SRII$ (Figure 8b) and at 2164 and 2118 cm^{-1} in BR (c) were assigned to the N–D stretch of the Schiff base (38, 51). Similar bands were observed at 2074 and 2030 cm^{-1} in $SrSRI$ (Figure 8a), whose frequencies are similar to those in the $SrSRI_K$ minus $SrSRI$ spectrum (Figure 5a). Therefore it is likely that the bands originate from the N–D stretch of the Schiff base. There are also bands at 2504 (–) cm^{-1} in $SRII$ (Figure 8b) and at 2506 (–) and 2466 (+) cm^{-1} in BR (c), which were assigned as the O–D stretch of Thr79 and Thr89, respectively (46, 47). Similarly, the bands at 2490 (–) and 2396 (+) cm^{-1} in $SrSRI$ could originate from the O–D stretch of Thr76.

Figure 9 shows the infrared spectra of the $SrSRI_K$ minus $SrSRI$ (a) and $SrSRI_M$ minus $SrSRI$ (b) in D_2O in the 3600–3000 cm^{-1} region, where H/D unexchangeable O–H and N–H stretching vibrations appear. The candidates are N–H stretching vibrations of the peptide backbone (amide-A) and O–H or N–H stretching vibrations of threonine, tyrosine, and tryptophan. In the case of $SRII$, transducer-dependent bands were observed at 3479 (–)/3369 (+) cm^{-1} , and a mutation study revealed the bands to have originated

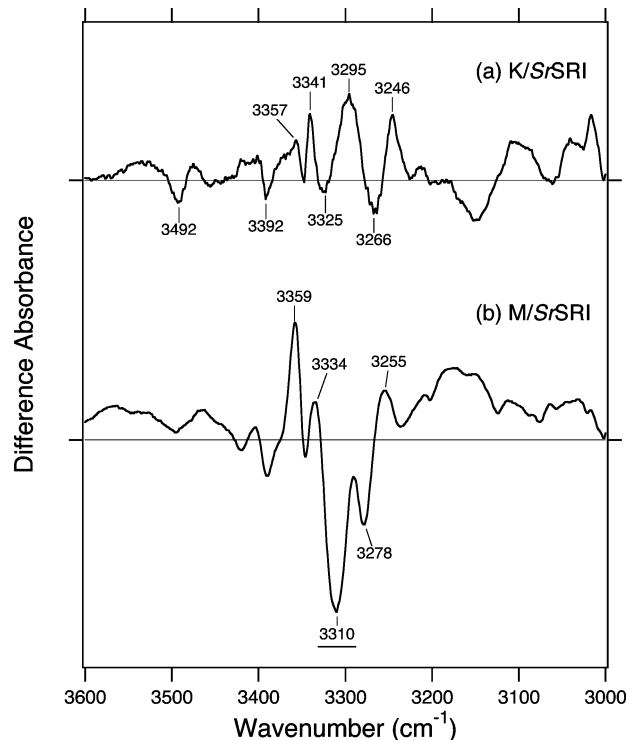


FIGURE 9: $SrSRI_K$ minus $SrSRI$ (a) and $SrSRI_M$ minus $SrSRI$ (b) difference infrared spectra in the 3600–3000 cm^{-1} region, which are multiplied by 1 and 0.66 from Figure 2a and Figure 6a, respectively, to exhibit the same negative intensities at 1250 and 1200 cm^{-1} . Underlined value corresponds to amide-A vibration. One division of y axis corresponds to 0.0015 absorbance units.

from an O–H stretch of Thr204 (26). Interestingly, importance of Thr204 for negative phototaxis was revealed after the FTIR observation (20). Thus, the high-frequency region contains rich structural information related to function. In the $SrSRI_K$ minus $SrSRI$ spectrum, the bands at 3492 (–), 3392 (–), and 3357 (+) cm^{-1} would originate from O–H stretching vibrations of threonine, tyrosine, or tryptophan residue(s).

The N–H stretching vibrations of the peptide backbone (amide-A) also appear in this frequency region. Since N–H and C=O groups form hydrogen bonds in the α -helix, we expect a similar spectral feature for the amide-A and amide-I regions, respectively, and this is indeed the case for the $SrSRI_M$ minus $SrSRI$ spectrum. Figure 9b shows peaks at 3359 (+), 3334 (+), 3310 (–), 3278 (–), and 3255 (+) cm^{-1} in the amide-A region, while Figure 7a shows peaks at 1666 (+), 1654 (–), 1614 (–), and 1605 (+) cm^{-1} in the amide-I region. Thus, negative bands at 3310 and 1654 cm^{-1} correspond with each other in the α -helix, and the M formation accompanies distortion of the α -helix as shown by the upshift to 3359 or 3334 cm^{-1} and 1666 cm^{-1} , respectively. On the other hand, negative bands at 3278 and 1614 cm^{-1} also correspond with each other, and the M formation leads to the spectral downshift. Unlike the former case, the secondary structure is unclear from the frequencies, where $SrSRI$ may form a β -sheet, or some unusual structure.

DISCUSSION

Many organisms utilize light as a signal. SRI is one of the most interesting photosensory receptors because of its ability to mediate opposite signals depending on the color

of light by use of photochromic reactions. However, the molecular mechanism for its function is not well understood at the atomic level. Recently we successfully prepared SRI from a eubacteria source (*Salinibacter ruber*) for the first time (to date found only in the haloarchaea). Most importantly, the *SrSRI* protein is stable even in the absence of NaCl and amenable to study, unlike SRI from other sources (10). Here, we utilized FTIR spectroscopy to explore structural changes of *SrSRI* and focused on similarities and dissimilarities between *SrSRI* and other rhodopsins to determine what are important structural changes for function of SRI.

The archaeon *Halobacterium salinarum*, a halophilic prokaryote, contains four retinal proteins, BR, HR, SRI, and SRII. The high-resolution crystal structures of BR, HR, and SRII and the SRII-HtrII complex were reported from 1997 through 2006 (5, 15, 52, 53), whereas the structure of SRI has not been determined yet. While high stability of *SrSRI* (10) could lead to crystallization in the future, we will discuss similarities and dissimilarities between *SrSRI* and other rhodopsins based on the present FTIR observations.

Similarities and Dissimilarities in the Initial State. Protein functions are entirely different among BR (proton pump), HR (chloride pump), and SRII (negative phototaxis sensor), but their X-ray crystallographic structures are very similar among them (5, 15, 52, 53). Thus, it is reasonable to infer that SRI has a similar protein architecture to other archaeal-type rhodopsins. The present FTIR study indeed provides this aspect, though FTIR spectroscopy cannot determine the tertiary structure. Similar retinal bands such as C=C and C-C stretching vibrations in *SrSRI* and *HsSRI* (Figure 2) to other rhodopsins imply a similar chromophore structure in SRI. Negative amide-I bands at 1654 and 1647 cm^{-1} for *SrSRI* and *HsSRI* (Figure 7), respectively, are characteristic of the α -helix, and absolute IR spectrum of *SrSRI* has a peak at 1657 cm^{-1} (data not shown). Thus, SRI probably possesses an all-*trans* retinal chromophore in seven transmembrane α -helices as do BR, HR, and SRII.

A correlation between proton pumping activity and existence of strongly hydrogen-bonded water molecule(s) ($<2400 \text{ cm}^{-1}$ as water O-D stretch) was discovered by Kandori's group by using various rhodopsins and their mutants (48). Figure 5b,c shows that at $<2400 \text{ cm}^{-1}$, there are two water bands for SRII (2307 and 2215 cm^{-1}) and three water bands for BR (2323, 2292, and 2171 cm^{-1}), both of which pump protons. In contrast, strongly hydrogen-bonded water molecules were not observed for HR (Figure 5d), *Neurospora* rhodopsin, and bovine rhodopsin (17, 36, 54, 55), which do not pump protons. Figure 5a shows the presence of a water O-D stretch at 2172 cm^{-1} for *SrSRI*, suggesting proton-pump activity for *SrSRI*. In fact, light-induced pH changes of the *SrSRI*-containing vesicles revealed that *SrSRI* could pump protons as well as BR and SRII (unpublished data). A similar spectrum of *SrSRI* in the X-D stretching vibrational region to those of BR and SRII (Figure 5), namely, strongly hydrogen-bonded water O-D and Schiff base N-D stretches, also suggest that a water molecule bridges between the Schiff base and Asp72 in *SrSRI*.

Similarities and Dissimilarities in the K Intermediate. Then, we focused on similarities and dissimilarities in the K intermediate. The *SrSRI*_K minus *SrSRI* spectrum is almost identical to that of *HsSRI* (Figures 2–4), implying a similar

structure and structural changes upon formation of the K intermediate between them. It appears that HOOP vibrations are different between photosensors and ion pumps (Figure 4), where HOOP vibrations of SRI and SRII are extended widely along the retinal chromophore. In contrast, the HOOP modes are more localized at the Schiff base region for BR and HR. Spectral analysis of water bands shows that hydrogen-bonding destabilization is important for energy storage in proton pumps, but energy storage for photosensory receptors probably requires a more steric effect, such as a steric constraint between the C14-D group of retinal and Thr204 in SRII (28).

In 2008, Ito et al. demonstrated a positive correlation between vibrational amplitudes of the C14-H HOOP vibration at 864 cm^{-1} at 77 K (Figure 4c) and the physiological phototaxis response (28). The band was also observable at 861 cm^{-1} for BR-T (Figure 4d), but not for *SrSRI* (Figure 4a). This suggests that the changes of C14-H HOOP are a specific feature among sensors for negative phototaxis. It is reasonable because the counterpart of the C14 atom, Thr204 of SRII, is not conserved in other rhodopsins, including SRIs. The distortion extended along the polyene chain of the retinal in SRIs may be due to the photoisomerization-induced steric trigger involving the retinylidene C13 methyl group previously demonstrated in *HsSRI* (56), even though the trigger is a different one than in SRII.

The *SrSRI*_K minus *SrSRI* spectrum at 3600–3000 cm^{-1} in D₂O (Figure 9a) provides undeuterated O-H or N-H vibrations. Since 2003, Kandori's group have demonstrated that the 110 cm^{-1} upshift of the O-H stretching vibration of Thr204 in SRII and its alteration of hydrogen bond with Tyr174 are required and essential for light-signal transduction to HtrII (20, 26). In the case of BR, O-H stretching vibrations of threonine (57) and N-H stretching vibrations of tryptophan (58) were also studied in detail. Thus, it will be important to identify the bands at 3492, 3392, and 3357 cm^{-1} in Figure 9a, which is one of our future focuses.

Similarities and Dissimilarities in the M Intermediate. Because the active (signaling) intermediate of SRII-HtrII and SRI-HtrI complexes is referred to as the M intermediate (2), it is important to analyze the structure and structural changes upon formation of the M intermediate. In the M intermediate, appearance of a protonation signal of the counterion is a common feature among rhodopsins (SRIs, SRII, BR) except for HR, which has no M intermediate (59). In *SrSRI*, the 1753 cm^{-1} band in H₂O (1742 cm^{-1} in D₂O) is ascribable to a C=O stretching vibration of carboxylate (Figure 7a), and interpreted in terms of protonation of Asp72 by the proton transfer from the protonated Schiff base (Lys201) (Figure 10).

In addition, *SrSRI* has a negative band at 1743 cm^{-1} in H₂O (1734 cm^{-1} in D₂O) (Figure 7a). A corresponding positive peak is observed at 1414 (or 1397) cm^{-1} as a band for deprotonated carboxylate (Figure 6a). Therefore, we infer that in *SrSRI*, a proton is released from the carboxylate to the extracellular side, although a proton was released from a protonated water cluster in BR (22, 23).

The most different aspect between SRI and SRII is the structural changes of the peptide backbone upon formation of the M intermediate (Figures 7 and 9). As described above, the analysis of the M intermediate is important not only for photosensors but also for ion-pumping rhodopsins, because

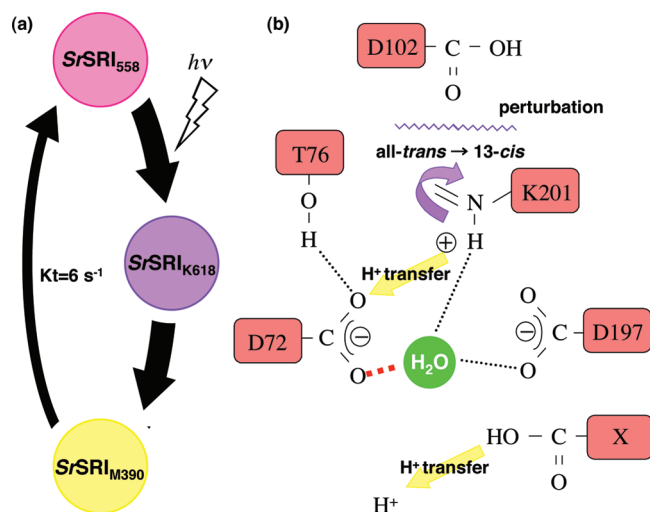


FIGURE 10: (a) Schematic of the photochemical reaction cycle of SrSRI identified in a previous study (10). K_t is the decay rate constant of SrSRI_M. (b) Structural changes upon formation of K (purple) and M intermediate (yellow). Our results suggest that a strongly hydrogen-bonded water molecule exists between Asp72 and Lys201 in SrSRI. Upon formation of the K intermediate, retinal isomerizes from the all-trans to the 13-cis form, and Asp102 is affected by the structural changes. The primary proton transfer takes place upon formation of the M intermediate. Then a proton is transferred from the protonated Schiff base (Lys201) to Asp72, and a proton may be released from a carboxylic residue.

of its role on the function. Frequency changes of amide-I vibration of the α -helix were observed at 1666 (+)/1654 (−) cm^{-1} in SrSRI, at 1661 (+)/1647 (−) cm^{-1} in HsSRI, and at 1663 (−)/1644 (+) cm^{-1} in SRII (Figure 7). Namely, these bands in SRIs and in SRII showed a higher and lower frequency shift, respectively. The amide-A vibrations at 3359 (+) and 3334 (+)/3310 (−) cm^{-1} are likely to correspond to the amide-I vibrations at 1666 (+)/1654 (−) cm^{-1} , which probe structural changes in the α -helix. The weakened hydrogen bond of the peptide backbone in SRI is in contrast to those in SRII. The different modes in protein structural changes between SRI and SRII may be correlated with their function, dual receptors both for positive and negative phototaxis versus a receptor for negative phototaxis.

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