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FTIR Analysis of the SII_{540} Intermediate of Sensory Rhodopsin II: Asp73 Is the Schiff Base Proton Acceptor†

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ABSTRACT: Sensory rhodopsin II (SRII), a repellent phototaxis receptor found in *Halobacterium salinarum*, has several homologous residues which have been found to be important for the proper functioning of bacteriorhodopsin (BR), a light-driven proton pump. These include Asp73, which in the case of bacteriorhodopsin (Asp85) functions as the Schiff base counterion and proton acceptor. We analyzed the photocycles of both wild-type SRII and the mutant D73E, both reconstituted in *Halobacterium salinarum* lipids, using FTIR difference spectroscopy under conditions that favor accumulation of the O-like, photocycle intermediate, SII_{540}. At both room temperature and −20 °C, the difference spectrum of SRII is similar to the BR→O_{640} difference spectrum of BR, especially in the configurationally sensitive retinal fingerprint region. This indicates that SII_{540} has an all-trans chromophore similar to the O_{640} intermediate in BR. A positive band at 1761 cm^{-1} downshifts 40 cm^{-1} in the mutant D73E, confirming that Asp73 undergoes a protonation reaction and functions in analogy to Asp85 in BR as a Schiff base proton acceptor. Several other bands in the C═O stretching regions are identified which reflect protonation or hydrogen bonding changes of additional Asp and/or Glu residues. Intense bands in the amide I region indicate that a protein conformational change occurs in the late SRII photocycle which may be similar to the conformational changes that occur in the late BR photocycle. However, unlike BR, this conformational change does not reverse during formation of the O-like intermediate, and the peptide groups giving rise to these bands are partially accessible for hydrogen/deuterium exchange. Implications of these findings for the mechanism of SRII signal transduction are discussed.

Sensory rhodopsin II (SRII,[1] also called phoborhodopsin) is a repellent phototaxis receptor found in the archeon *Halobacterium salinarum.* Along with sensory rhodopsin I (SRI), it modulates cell swimming behavior through light-activated signal transduction (*1*−*3*). It belongs to a larger family of homologous seven-helix proteins that form a protonated Schiff base with *all-trans*-retinal, including bacteriorhodopsin (BR), a light-driven proton pump, and halorhodopsin (HR), a light-driven chloride pump (*4*, *5*).

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[1] Abbreviations: SRII, sensory rhodopsin II; $SRII_{487}$, unphotolyzed form of sensory rhodopsin II; SRI, sensory rhodopsin I; BR, bacteriorhodopsin; pSRII, sensory rhodopsin II from *Natronobacterium pharaonis*; BR_{568}, light-adapted, unphotolyzed form of bacteriorhodopsin; LM, laurylmaltoside; FTIR, Fourier transform infrared spectroscopy.

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Because of similarities of these membrane proteins with the seven-helix rhodopsin retinal pigments and the wider family of seven-helix G-protein-coupled receptors, considerable interest has been focused on understanding the molecular mechanisms by which these retinal proteins function.

SRI, SRII, and BR all share a homologous Asp residue located in the C-helix. In the case of BR, this residue (Asp85) has been shown to serve as the counterion and proton acceptor for the retinylidene Schiff base during its light-activated photocycle and a critical link in the proton transport pathway (*6*, *7*). The disruption of the salt bridge between the Schiff base and Asp85 has also been implicated in switching BR from an extracellular-directed to an intracellular-directed proton transport pathway (*8*).

Evidence that Asp73 serves as the Schiff base counterion in SRII came from recent studies on the mutant D73N (*9*, *10*). D73N exhibited a red-shifted λ_{max} of 514 nm compared to 487 nm in wild-type SRII, consistent with the neutralization of the Schiff base counterion. D73N did not exhibit formation of the deprotonated M-like intermediate, SII_{360} (*9*). In addition, D73N causes constitutive activation of SRII in the dark as indicated by the 3-fold higher swimming reversal frequency and demethylation of HtrII, in analogy with effects of the same substitution for the Schiff base counterion in rhodopsin (Glu113). FTIR measurements of SRII from *Natronobacterium pharaonis* (pSRII) in detergent micelles (*10*) under conditions where an M-like intermediate accumulates revealed a positive band at 1764 cm^{-1} similar to a band first observed in the BR→M transition in BR (*11*). In the case of BR, this band was assigned using site-directed mutagenesis to the protonation of Asp85 (*6*). However, a similar assignment has not been made for SRII.

In this study, we report the first FTIR spectroscopy of SRII from *H. salinarum* and the SRII mutant D73E, both reconstituted into native halobacterial lipids, under conditions that favor the accumulation of the red-shifted intermediate, SII_{540} (sometimes referred to as the O intermediate of SRII). The structure and conformational changes which occur upon formation of SII_{540} are of importance since this intermediate is associated with signal transduction and the repellent response of SRII (*12*). On the basis of comparison with the BR→O_{640} difference spectrum of BR, it is concluded that SII_{540} has an *all-trans*-retinal chromophore similar to that of the O_{640} intermediate in bacteriorhodopsin. SII_{540} also exhibits a positive band at 1761 cm^{-1} in the carboxylic acid C═O stretch region. By comparison with the mutant D73E, this band is assigned to Asp73, thereby confirming that it serves as the Schiff base proton acceptor similar to Asp85 in bacteriorhodopsin. The D/H exchange-induced downshift of this band is identical to Asp85 in BR showing that it has a very similar environment as Asp85. The presence of intense bands in the amide I region in the late photocycle of BR and SRII also indicates that both proteins undergo similar conformational switching. However, unlike BR, where the conformational switch occurs between the M and N intermediates and decays by O_{640}, it has not yet reversed at the SII_{540} intermediate in SRII, consistent with the hypothesis that this conformational switch converts SRII to a signal-transducing form (*13*).

MATERIALS AND METHODS

Expression and Purification. (A) Materials. Synthetic DNA oligonucleotides were from Genosys (The Woodlands, TX), *Pfu* DNA polymerase from Stratagene (La Jolla, CA), Oxoid peptone from Unipath (Ogdensburg, NY), and laurylmaltoside (LM) from Anatrace (Maumee, OH). His•Bind resin (Novagen, Madison, WI) was prepared according to the manufacturer's instructions.

(B) Strain Construction. For high-level expression and purification, 660 bp fragments containing *sopII* or *sopII* mutated to encode SRII D73E were amplified by PCR to contain *Nco*I−*Nsi*I ends and placed under the control of the *bop* promoter in the *H. salinarum* expression vector pSO12 (*14*). The encoded proteins terminate with MHSESHHHHHH after Ala221 in wild-type SRII. The plasmids were used to transform Pho81Wr$^-$ to generate the strains SR217 (WT SRII) and SR235 (D73E SRII) (*15*).

(C) Purification of His-Tagged SRII. Purification of His-tagged SRII was performed by a modification of the procedure previously described (*14*). Briefly, four 2 L flasks containing 1.8 L of Complex Medium (CM) + mevinolin (1.0 μg/μL) were inoculated with 5.5 mL of liquid culture of the transformant strains SR217 and SR235. Cultures were grown to stationary phase (OD_{600} = ~0.7) under illumination at 37 °C with 240 rpm shaking as described (*14*). Cells were harvested by centrifugation and resuspended in 240 mL of sonication buffer (4 M NaCl, 25 mM Tris-HCl, pH 6.8) in the presence of 40 mg of DNase (Sigma, St. Louis, MO) and dialyzed against 0.25 M KCl, 25 mM Tris-HCl, pH 6.8, at 4 °C for 18 h. The membranes were collected by centrifugation, and the pellets were resuspended in a total combined volume of 72 mL of MNI [50 mM 4-morpholineethanesulfonic acid (MES), 4 M NaCl, 5 mM imidazole, pH 6.0] + 1.0% w/v LM. His-tagged SRII was purified by Ni^{2+}-affinity chromatography by adding 1.8 mL of His•Bind resin to 72 mL of solubilized sample (150 μL/6 mL each) and shaken at 4 °C for 18 h. The bound resin was applied to a 50 cm glass chromatography column (Bio-Rad, Hercules, CA), and the flow-through was collected. The packed His-tagged SRII-bound resin was washed with 40 mL of MNI + 0.05% w/v LM, followed by the addition of 40 mL of elution buffer (4 M NaCl, 50 mM sodium acetate, 5 mM imidazole, 0.05% w/v LM, pH 4.0). The elutant was collected in 1.0 mL aliquots via a microfractionator (Gilson, Middleton, WI) in tubes containing 50 μL of 1.0 M 4-morpholinepropanesulfonic acid (MOPS), pH 7.5. The eluted protein was combined and concentrated in a stirred cell concentrator containing a 10 000 MW cutoff disk (YM10) membrane (Amicon, Beverly, MA) and stored at 4 °C.

(D) Halobacterial Polar Phospholipid Extraction. Extraction of halobacterial phospholipids was performed as described (*16*). Polar lipids were separated from nonpolar lipids by dissolving the lipid residue resulting from rotary evaporation in 20 mL of $CHCl_3$ and centrifuging the sample at 15500*g* for 20 min at 0 °C. The supernatant was combined with 200 mL of ice-cold acetone and centrifuged at 1400*g* for 10 min at 0 °C. The lipid pellet was dried under argon and redissolved in 20 mL of $CHCl_3$. After a second acetone treatment, the final dried lipid pellet was dissolved in $CHCl_3$ and stored under argon at −20 °C

(E) Proteoliposome Reconstitution. Purified His-tagged SRII was reconstituted in phospholipids by the dialysis procedure previously described (*14*). In the work reported here, His-tagged SRII (175 μg) was reconstituted in 875 μg of halobacterial phospholipids.

FTIR Difference Spectroscopy. Stock suspension of membranes (10 mg/mL) containing SRII or the mutant D73E in 2 M NaCl at pH 6.0 (50 mM MES buffer) were kept in the refrigerator prior to use. SRII films were prepared by depositing 5−10 μL of this suspension onto an AgCl window and then placing the sample in a drybox for approximately 1 h. Films were then rehydrated by placing 1−1.5 μL of H_2O near the edge of the window and then sealing the sample in a temperature-controlled IR cell (Model TFC, Harrick Scientific Corp., Ossining, NY) using a second AgCl window. For the purpose of deuterium/hydrogen (D/H) exchange, a drop of D_2O was first placed on a previously dried sample and allowed to re-dry in a drybox for approximately 1 h. Films were then rehydrated in the IR cell as described above with D_2O. Spectra were recorded as previously reported (*17*) with a Bio-Rad FTS-60A FTIR spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) using a liquid nitrogen cooled MCT detector. A Dolan-Jenner (Woburn, MA) model 180 illuminator (150 W, tungsten−halogen) and a fiber-optic light guide were used for sample illumination in combination with various optical filters (Corion Corp., Holliston, MA). Each light and dark spectrum consisted of multiple scans (see figure captions) recorded at 2 cm^{-1} resolution. At 20 °C, an averaged difference spectrum was obtained by recording successive spectra of the sample in the dark and light for 1−2 min using light normally with $\lambda > 475$ nm except where indicated (see figure captions), computing the difference for each cycle, and then averaging spectra for multiple cycles. At −20 °C, a "first push" difference spectrum was obtained by keeping the sample in the dark for at least 6 h and then recording the spectrum with the sample in the dark for 15 min immediately followed by a measurement of the sample while illuminated using a $\lambda > 475$ nm long-pass filter. After the first push, subsequent difference spectra were obtained by cycling the sample between dark and light using the same conditions (15 min and $\lambda > 475$ nm).

RESULTS

SII_{540} Has an all-trans-Retinal Chromophore Similar to the O_{640} Intermediate in BR. FTIR difference spectra were obtained for SRII devoid of HtrII at 20 °C for samples hydrated in H_2O (Figure 1) as described (Materials and Methods). The SRII photocycle includes at least two long-lived intermediates: a blue-shifted intermediate (SII_{360}) and a longer lived red-shifted intermediate (SII_{540}). Based on recent optical flash spectroscopic measurements of SRII devoid of HtrII in *H. salinarum* membranes (*18*), the M contributions at 20 °C should be low, since the ratio between the O decay and M→O decay half-times is approximately 50/1. SRII should therefore accumulate predominantly SII_{540} when illuminated with light of wavelengths $\lambda > 470$ nm, and the difference spectrum should reflect primarily the SRII→SII_{540} transition. We cannot, however, eliminate the possibility that other photointermediates such as SII_{360} contribute to the difference spectra with the conditions used.

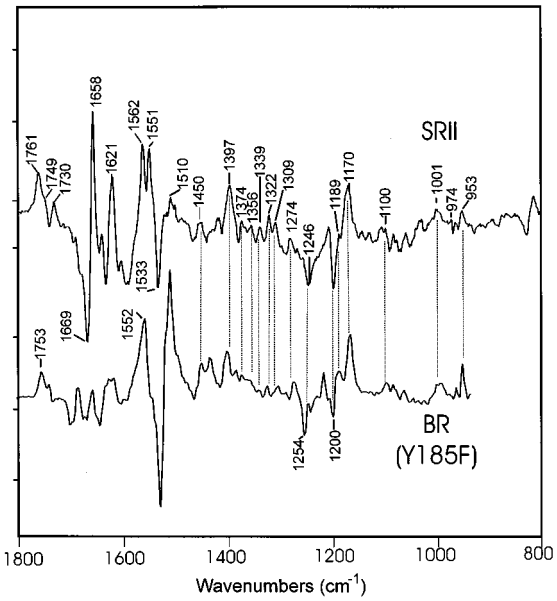

FIGURE 1: Comparison of difference spectra recorded near room temperature for SRII and the bacteriorhodopsin mutant Y185F. (Top) FTIR difference spectrum of native SRII (WT) recorded at 20 °C and 2 cm^{-1} resolution. Each difference spectrum is the subtraction of a spectrum in the dark (85 scans, 2 min) and a spectrum in the light (475 nm long-pass filter, 85 scans, 2 min); and consists of the average of 466 such differences. Spacing of *Y*-axis (absorbance) markers corresponds to 4×10^{-5} OD. (Bottom) Time-resolved room-temperature BR→O FTIR difference spectrum of the mutant Y185. Data were obtained only for the region between 800 and 1800 cm^{-1} (for further details, see ref *20*).

Due to its red-shifted absorption and appearance late in the SRII photocycle, it has been predicted that SII_{540} is similar to the O intermediate in the bacteriorhodopsin photocycle (*18, 19*). This prediction is supported by similarities between the difference spectrum of SRII and the time-resolved BR→O_{640} difference spectrum of BR. The latter was obtained from the BR mutant Y185F, which exhibits a slow O_{640} decay at high pH (*20*−*23*). For this reason, the O_{640} intermediate accumulates to a greater extent in the photocycle of Y185F compared to native BR and is therefore easier to observe in a pure form in the late photocycle by time-resolved FTIR-difference spectroscopy. As seen in Figure 1, common bands in both spectra appear in the retinal fingerprint region (1150−1400 cm^{-1}), which is highly sensitive to the retinylidene chromophore configuration, near 1170 (+), 1189 (shoulder, +), 1200 (−), 1246 (−), 1274 (+), 1309 (+), 1322 (+), 1339 (+), 1356 (+), 1374 (+), 1397 (+), and 1450 cm^{-1} (+) (note that +/− refers to the difference bands with positive or negative intensity). Although some of these bands are likely to arise from protein vibrations, many of them have been previously assigned on the basis of resonance Raman studies to vibrations of the retinylidene chromophore of light-adapted bacteriorhodopsin (BR_{568}) and its O_{640} intermediate, both of which have an all-trans chromophore structure (*24, 25*). For example, the negative band at 1200 cm^{-1} is assigned to the C_{14}−C_{15} stretch mode of the *all-trans*-retinal in BR_{568}, while an intense positive band near 1170 cm^{-1} is assigned to the C_{10}−C_{11} stretch mode of the *all-trans*-retinal in O_{640} (*25, 26*). A second negative band near 1246 cm^{-1} is most likely due to a combination of the C_{12}−C_{13} stretch and C_{15}H rock in analogy with a similar band measured at 1254 cm^{-1} in BR. In the hydrogen-out-of-plane (HOOP) mode region,

which is also sensitive to the chromophore configuration, positive bands previously assigned to the O_{640} chromophore appear at 953 and 974 cm^{-1}. A positive band also appears near 1001 cm^{-1} close to the methyl rock mode assigned at 1004 cm^{-1} in the time-resolved resonance Raman spectrum of O_{640} (*26*). On this basis, we conclude that the SRII dark state ($SRII_{487}$)[2] has an all-trans chromophore structure similar to BR and that, under the conditions of the measurements, SRII accumulates an O-like intermediate (SII_{540}) with an *all-trans*-retinylidene chromophore structure similar to the O_{640} intermediate of BR. A similar conclusion can also be reached by comparing FTIR differences from SRII with those obtained using global analysis to extract the BR→O contributions in the native BR photocycle (*27*).

Note that positive contributions in the fingerprint region from the M-like SII_{360} intermediate may not be easily detected. In particular, the intensities of vibrations from retinals with an unprotonated Schiff base, such as expected to be present in SRII, are normally weak compared to those of a protonated Schiff base (*28*). However, comparison with the FTIR difference spectrum recorded for pSRII reconstituted in *H. salinarum* lipids (our unpublished data) (see also ref *10*), which reflects accumulation of mainly the M-like intermediate, shows that bands associated with this intermediate, such as at 1571 cm^{-1}, do not contribute significantly to the SRII→SII_{540} difference spectrum.

Asp73 Undergoes Protonation by Formation of the SII_{540} Intermediate. In addition to bands from vibrations of the chromophore, many of the bands in the SRII→SII_{540} difference spectrum can be assigned to protein vibrations. Of special interest are bands arising from the C═O stretch of carboxylic acid groups in the region between 1700 and 1800 cm^{-1}. In the case of BR, a positive band at 1762 cm^{-1} in the BR→M FTIR difference spectrum (*11*) was assigned to the protonation of Asp85 on the basis of a 40 cm^{-1} downshift when Asp85 is replaced with Glu85 (*6*).

In the case of SRII, a prominent positive band appears at 1761 cm^{-1} along with several other smaller bands at 1749 (shoulder, +), 1739 (−), and 1730 (+) cm^{-1}. As shown in Figure 2, the 1761 cm^{-1} band disappears and positive intensity appears at 1720 cm^{-1} when Asp73 is substituted with Glu73 (D73E). On this basis, the 1761 cm^{-1} band in SRII is assigned to the carboxylic C═O stretch vibration of Asp73. The fact that it is positive indicates that Asp73 becomes protonated by formation of the SII_{540} intermediate. These results are analogous to an earlier FTIR study on the homologous residue, Asp85, in BR which helped establish its role as the Schiff base proton acceptor (*6*) An almost identical 40 cm^{-1} downshift occurs when a Glu substitutes for Asp85. Furthermore, the stretching frequencies of Asp73 (SRII) and Asp85 (BR) are very similar, and at a higher frequency than normally found for an aspartic carboxylic acid (C═O) stretch mode (*11*). This finding, along with almost identical D/H exchange-induced isotope shifts (see below), indicates that the carboxylic acid groups of Asp73 in SRII and its homologous residue in BR have very similar environments.

[2] No evidence has been found on the basis of FTIR for light−dark adaptation in SRII (our unpublished results) as compared to BR which undergoes a partial all-trans →13-cis isomerization of the retinylidene chromophore when left in the dark.

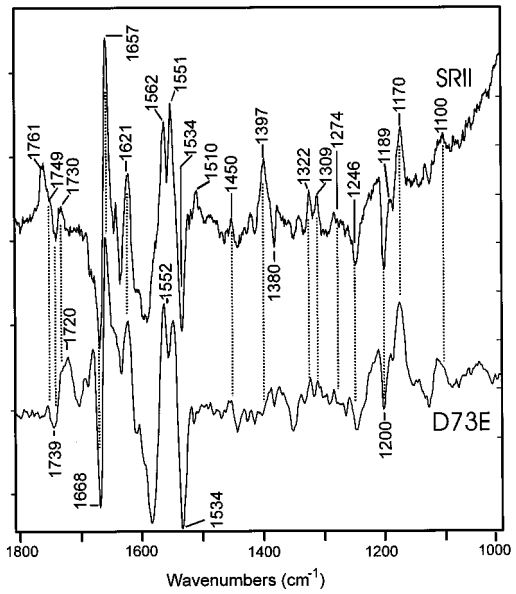

FIGURE 2: Comparison of SRII→SII_{540} difference spectra obtained from WT and D73E SRII. (Top) Wild-type SRII recorded using the same conditions described for Figure 1, top. Spacing of *Y*-axis (absorbance) markers corresponds to 5×10^{-5} OD. (Bottom) FTIR difference spectrum of the SRII mutant D73E recorded at 20 °C and 2 cm^{-1} resolution. Since D73E exhibited a long SII_{540} decay at room temperature, SII_{540} was formed using light from a short-pass filter (<500 nm) and then photoreversed using light from a long-pass filter (>530 nm). Similar results could be obtained by allowing the sample to decay after excitation in dark for over 15 min. The difference spectrum consists of subtracting these two spectra, each recorded with 1400 scans (~15 min), and smoothing with a smoothing factor of 60 using the Grams Fourier smoothing algorithm from Galactic Software (Nashua, NH). Spacing of *Y*-axis (absorbance) markers (right) corresponds to 2×10^{-5} OD.

In contrast to the 1761 cm^{-1} band, the bands at 1749 (+) cm^{-1}, 1739 (−) cm^{-1}, and 1730 (+) cm^{-1} are not affected by the Asp73→Glu73 substitution, indicating that they arise from other Asp and/or Glu residues in SRII. Interestingly, negative/positive bands near 1739/1730 cm^{-1} also appear in the BR→M difference spectrum of BR, assigned on the basis of site-directed mutagenesis to changes in hydrogen bonding of Asp115 (*6*). In analogy, these bands may arise from the homologous residue Asp103.

The Asp73→Glu73 substitution (D73E) also causes the disappearance of bands at 1397(+) and 1380 (−) cm^{-1}. Since the C═O symmetric stretch mode of carboxylate groups (*29*) falls in this region, these bands might reflect a change in environment of an ionized carboxylate form of Asp73. However, in view of the assignment of the 1761 cm^{-1} band to the Asp73 carboxylic group, there would also have to exist a population of Asp73 which remains unprotonated but undergoes a change in environment. Alternatively, these bands may reflect carboxylate groups from a different Asp/Glu residue(s) whose environment and/or conformational changes are perturbed by the Asp73→Glu73 substitution. Site-directed mutagenesis, isotope labeling, and site-directed isotope labeling in conjunction with FTIR difference spectroscopy can be used to further explore these possibilities.

We also examined the effect of D/H exchange on the frequency of the carboxylic acid assigned bands. In general, D/H exchange is expected to cause a downshift in the C═O stretching frequency. As seen in Figure 3, the 1761 cm^{-1} band downshifts 11 cm^{-1} to 1750 cm^{-1}, almost identical to

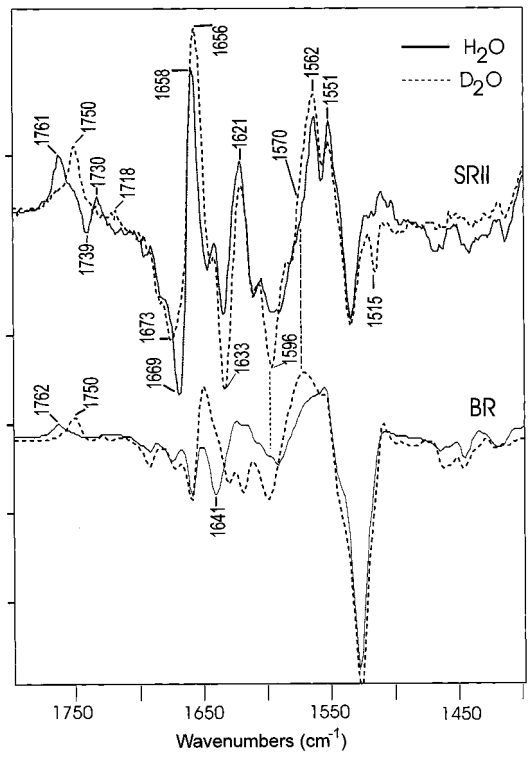

FIGURE 3: Comparison of the effects of D/H exchange on the FTIR difference spectrum for SRII→SII_{540} and BR→M. (Top) Solid trace, same as SRII difference spectrum shown in Figure 1; dashed trace, spectrum recorded with SRII sample hydrated with D_2O. Spectra were recorded using 440 scans (5 min) and light with $500 > \lambda > 420$ nm at 20 °C (see Materials and Methods). (Bottom) Solid (dashed) traces, BR→M difference spectrum recorded at −20 °C for samples hydrated with H_2O (D_2O) (see reference *59* for further details).

the downshift of the Asp85-assigned 1762 cm^{-1} band in BR. Since the frequency and extent of D/H exchange-induced downshift of the C═O stretch mode are sensitive to the hydrogen bonding strength (*30*), Asp73 appears to have a similar environment as Asp85 in bacteriorhodopsin. D/H exchange also produces a ∼12 cm^{-1} downshift of the 1730 (+) cm^{-1} and possibly the 1739 (−) cm^{-1} bands.

Assignment of Bands Associated with Alterations in the SRII Peptide Backbone. D/H-induced spectral shifts also occur in the amide I region (1600−1700 cm^{-1}) associated with vibrations of the polypeptide backbone (*31*). As seen in Figure 3 (upper traces), an intense negative band at 1669 cm^{-1} undergoes a drop in intensity and upshift to 1673 cm^{-1} in frequency due to D/H exchange. Interestingly, an intense negative band at the same frequency appears in the BR_{568}→N difference spectrum of BR (*32*−*34*) but not in the BR→O difference spectrum shown in Figure 1. However, in contrast to SRII, a D/H exchange induced effect is only detected when the protein is refolded in the presence of D_2O (*35*) (our unpublished data). Thus, the peptide groups, which give rise to the 1669 cm^{-1} band in SRII, appear to be more accessible to H/D exchange compared to BR. The exact conditions which facilitate this D/H exchange are currently under investigation.

D/H exchange also results in an increase in intensity and slight downshift in frequency of an intense positive band near 1658 cm^{-1} along with an increase in intensity of the negative band near 1633 cm^{-1}. These changes may reflect the D/H exchange induced downshift to 1633 cm^{-1} of a hidden negative band near 1650−1655 cm^{-1}, which arises from the C═N stretching frequency of the protonated Schiff base in $SRII_{487}$ and is canceled by the intense positive band at 1658 cm^{-1} near the same frequency. A similar downshift (∼17 cm^{-1}) occurs in the case of bacteriorhodopsin, although the C═N stretch frequency is at 1641 cm^{-1}. The higher frequency of the C═N stretch frequency in SRII can be accounted for by the observed inverse correlation of C═N stretch frequency with λ_{max} (*36*) along with the lower λ_{max} of SRII relative to BR (487 nm vs 568 nm). On the other hand, the extent of the D/H-induced downshift depends largely on the local environment of the Schiff base and the coupling between the C═N stretch and NH bend modes (*37*, *38*). The similar D/H exchange-induced downshifts of the C═N stretch modes in SRII and BRII indicate that the respective Schiff base environments are similar. Note that a more definitive assignment of the SRII Schiff base C═N stretch mode will require either isotope labeling of the SRII retinylidene chromophore or resonance Raman measurements.

D/H exchange-induced alterations are also observed in the region below 1630 cm^{-1} including a change in intensity of bands at 1621 cm^{-1} (+), 1596 cm^{-1} (−), 1570 cm^{-1} (shoulder), and 1515 cm^{-1} (−). Assignment of these bands is complicated by contributions in this region from amide I, amide II, histidine, tyrosine, and retinal ethylenic vibrations (C═C) stretch modes, all of which may be affected by D/H exchange. Definitive assignment will require more complete analysis utilizing isotope labeling of backbone peptide groups, retinal labels, and resonance Raman spectroscopy. However, experiments at low temperature (see below) and comparison to results obtained on pSRII (*10*) indicate that the ethylenic stretch (C═C stretch) of the $SRII_{487}$ chromophore is most likely located near 1547 cm^{-1} in the SRII→SII_{540} difference spectrum but is hidden by other bands. We also note that there is a striking similarity with D/H-induced changes observed in bands at very similar frequency in the BR→M difference spectrum of BR (Figure 3, lower traces), further indicating the similarities in the conformational changes which SRII and BR undergo through their respective photocycles.

SRII Photoactivation at Low Temperature. FTIR difference spectra were also obtained for SRII at low temperature (−20 °C). This was accomplished by first measuring a sample which has been kept in the dark for over 6 h and then during illumination. The difference spectrum obtained under these conditions (Figure 4A) is similar to those measured at 20 °C (Figure 4B), especially in the configurationally sensitive fingerprint region. This indicates that the photoproduct (SII_{540}) which accumulates during steady-state illumination at room temperature also forms at −20 °C. Note, however, that in contrast to room-temperature spectra, a broad band centered near 1224 cm^{-1} is more intense in the low-temperature spectrum and the 1170 cm^{-1} band appears less intense relative to the negative 1200 cm^{-1} band.

Measurement of difference spectra of SRII at −20 °C (Figure 4C) obtained by photocycling of the sample using alternating 15 min periods of dark, similar to the procedure performed using shorter periods at room temperature (Figure 4A), resulted in a significantly altered spectrum compared to Figure 4A and 4B. This indicates that the dominant SII_{540} photointermediate formed by absorption of a photon by SII_{540}

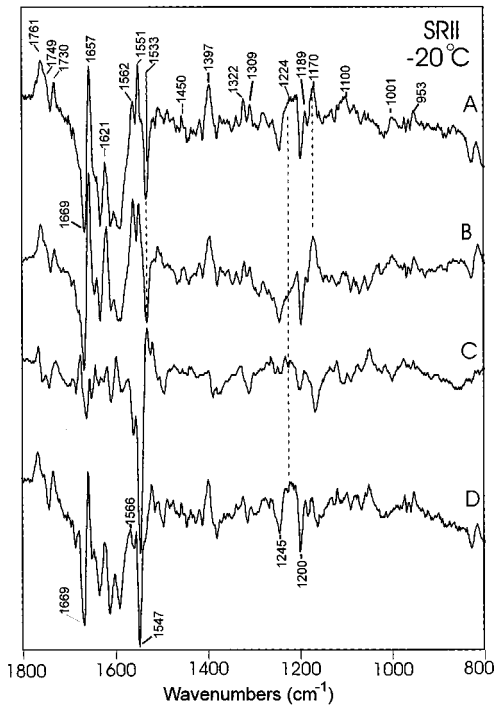

FIGURE 4: SRII FTIR difference spectra recorded at low temperature. (A) SRII→SII_{540} difference spectrum recorded at −20 °C using the "first push" procedure described under Materials and Methods. Each spectrum was recorded using 1400 scans (∼15 min), and light was filtered with λ > 475 nm. (B) Difference spectrum recorded at 20 °C (same as Figure 1) shown for comparison. (C) Difference spectrum recorded at −20 °C consisting of the average of 13 differences obtained by photocycling sample between dark and light (λ > 475 nm) at −20 °C using 1400 scans (∼15 min) for each spectrum (see Materials and Methods) after the first push. (D) Difference spectrum computed by interactive addition of spectra shown in (A) and (C) using visual inspection to minimize contributions from bands at 1170 and 1533 cm^{-1} attributed to the SII_{540} intermediate.

does not fully decay after 15 min and possibly absorbs a second photon, thereby producing photoproduct(s). Alternatively, it is possible that SII_{540} decays into an unknown intermediate, which absorbs a second photon. Such a photoreaction of SII_{540} (or a decay product with a similar chromophore structure) is also indicated by the reversal in sign of several bands associated with formation of SII_{540} including the appearance of bands at 1533(+) and 1170 (−) cm^{-1}.

To determine the vibrational contributions from the SII_{540} photoproduct, we interactively added spectra in Figure 4A and 4C in order to cancel bands associated with the formation of SII_{540} such as at 1170 cm^{-1} (+). Assuming that these difference spectra represent SRII→SII_{540} and SII_{540}→X, the SRII→X difference spectrum can be obtained using this procedure. Interestingly, the resulting difference spectrum (Figure 4D) resembles the SRII→SII_{360} (SRII→M) difference spectrum reported for pSRII (*10*), which has slower M decay kinetics compared to pSRII. pSRII and SRII share similar amino acid sequences and exhibit very similar functions and photocycles, thus justifying spectroscopic comparison (*9, 10, 12*). Negative bands are found at 1547, 1245, and 1200 cm^{-1} which correspond to the expected frequency of the C═C ethylenic and C−C stretch modes of the $SRII_{487}$ retinylidene chromophore. A positive band also appears near 1566 cm^{-1} which may correspond to the ethylenic stretch of the SII_{360} chromophore. Only very weak positive bands are observed in the fingerprint region consistent with a deprotonated Schiff base chromophore expected in SII_{360}. One exception is the 1224 cm^{-1} band which is also present in the SRII →SII_{540} spectrum recorded at low temperature. The C═O stretch frequency of Asp73 now appears at 1764 cm^{-1} at a slightly higher frequency than in SII_{540}, similar to an upshift in frequency observed for the C═O stretch of Asp85 in the M compared to O intermediates of BR (*20*).

On this basis we conclude that light absorption by SII_{540} produces a slowly decaying photoproduct of SII_{540} which is similar to the M-like photoproduct of the normal SRII photocycle. The presence of intense bands at 1669 cm^{-1} (Figure 4D) also indicates that the conformational switch which occurs between $SRII_{487}$ and formation of SII_{540} is also still present in this photointermediate of SII_{540}. However, this may not be true of the normal SII_{360} intermediate formed in the SRII photocycle. For example, much smaller bands are observed in the amide I region in the FTIR difference spectrum of the pSRII photocycle which reflects mainly the transition to the M-like intermediate (our unpublished results).

DISCUSSION

SRII and BR share several homologous residues identified as important for proper proton pumping in BR, including the existence of three buried Asp groups (Asp73, Asp103, and Asp198) located on the C, D, and G helices. Because of these homologies, it is possible that SRII and BR share common mechanistic features involving movement of protons, despite their different function. In this work, we have used FTIR difference spectroscopy to explore such possible similarities. FTIR difference spectroscopy is an effective method since it can detect very small conformational changes involving both the protein and the retinal chromophore structure (*4, 11, 39*). We have focused on SII_{540}, the longest lived intermediate in the SRII photocycle. Evidence indicates that it is an active signaling intermediate of SRII (*12*) and that it generates the photorepellent response. Furthermore, its red-shifted λ_{max} (540 nm) and appearance as the last intermediate in the SRII photocycle suggest that it may have properties similar to the O intermediate in the BR photocycle.

Our findings reveal that there are several similarities between the late photocycles of BR and SRII photocycles including:

1. Similar Chromophores in the BR and SRII Photocycle. The SRII→SII_{540} and BR→O_{640} difference spectra display very similar vibrational bands in the configurationally sensitive retinal fingerprint region. In the case of BR, it has been conclusively established through resonance Raman measurements of BR regenerated with isotopically labeled retinals that both BR_{568} and O_{640} have all-trans configurations (*24, 25*). The close similarity of the difference spectra supports the conclusion that $SRII_{487}$ and SII_{540} also have all-trans chromophore configurations. However, resonance Raman spectroscopy and chromophore isotope labeling will be necessary to explore in more detail the exact structure of these chromophores.

2. Similar Structure of Protonated Schiff Bases. The C═N stretching frequency is very sensitive to the electrostatic and hydrogen-bonding interactions of the Schiff base (*40*−

42). The Schiff bases of both BR and SRII must have similar environments since they undergo similar D/H-induced downshifts and have C═N stretch frequencies consistent with their respective λ_{max}. In contrast, the C═N stretch frequency is lower (1628 cm^{-1}), and H/D exchange shift is much less ($\sim$8 cm^{-1}) in SRI (*43*), indicating hydrogen bonding weaker than in BR (*44*) consistent with the neutralization of Asp76 (*45*).

3. Similar Structure and Behavior of Schiff Base Counterions. Like Asp85, the homologous residue in SRII (Asp73) appears to function as a proton acceptor. This contrasts with the Asp85 homologue in SRI (Asp76) which remains protonated throughout the photocycle (*46*). In particular, FTIR studies on SRI (*46*) reveal that Asp76 is already protonated in the initial ground state ($SRII_{587}$), accounting for the red-shifted λ_{max} of 587 nm compared to light-adapted BR (568 nm). In the case of halorhodopsin, a homologous residue for Asp85 is not present (*47*).

The environments of the protonated Asp residue in SRII and BR (Asp73 and Asp85, respectively) in their late photocycle intermediates are also very similar. In particular, an almost identical D/H-induced downshift of C═O is found for both residues based on comparison of M in BR and SII_{540}. Previously, the extent of D/H-induced downshift of the carboxylic C═O stretch mode of Asp and Glu amino acid residues was shown to decrease with stronger hydrogen bonding, reflecting the coupling between the OH in-plane bend and C═O stretch modes (*30*).

4. Similar Structural Changes in Protein Conformation. Both BR and SRII difference spectra exhibit intense bands near 1669 (−) cm^{-1} and 1650 (+) cm^{-1} in the amide I region, indicating similar changes in the peptide backbone conformation. However, unlike BR where the bands decay during formation of the O intermediate (see below), the amide I related bands persists in the late O-like intermediate, SII_{540} (see, for example, Figure 1). A second difference is the increased accessibility of the SRII peptide groups to H_2O as indicated by the drop in intensity of these bands upon exposure to D_2O. In the case of BR, only delipidation of the protein and reconstitution with *H. salinarum* lipids in the presence of D_2O appear to alter these bands (*35*). One possible explanation is that the absence of the transducer (HtrII), which is normally present in a molecular complex with SRII, provides a pathway for H/D exchange which does not exist in BR.

Implications for SRII Signal Transduction. In the case of BR, evidence indicates that a conformational change occurs in the protein during the photocycle which may be associated with a necessary switching of the accessibility of the Schiff base from an extracellular to a cytoplasmic pathway. Diffraction studies show that this change is present in the N intermediate in BR and involves a tilting of the F and G α-helical segments on the cytoplasmic side of the protein (*48*). Since the change is also found in the M intermediate of D96N (M_N) (*49*) which has a slow M decay, it may occur prior to formation of N (e.g., during $M_1 \rightarrow M_2$). Electron diffraction of L93A, which has a slowed O decay, also indicates that the conformational switch is partially reversed by formation of the O intermediate (*49*). Interestingly, the same pattern is found by FTIR difference spectroscopy. Intense negative bands in the amide I region at 1669 (−) and 1650 (+) cm^{-1} have been associated with a change in orientation of buried α-helices (*50, 51*). Furthermore, these bands appear during the M→N transition (*32*−*34*) and disappear during the N→O transition (*20*).

Although diffraction studies have not yet been performed on SRII, the appearance of intense bands in SRII at 1669 and 1658 cm^{-1} indicates that conformational switching occurs similar to BR. However, unlike BR where the amide I bands decay along with the N intermediate, they persist at the SII_{540} stage in the SRII photocycle. Recent FTIR difference measurements of pSRII reconstituted in *H. salinarum* lipids using an identical procedure as for SRII (our unpublished results) reveal that much weaker negative bands are observed in the amide I region under conditions where SII_{360} accumulates. These findings may have important implications for the mechanism of SRII function. Since the SII_{540} intermediate is the longest-lived active repellent signaling state (*52*), the occurrence of a conformational switch at this stage is consistent with the proposal that it is associated with transmitting the signal to the HtrII transducer (*13*). As previously discussed in the case of SRI, such a signal transmission could occur by the outward tilting of helices F and G interacting with TM2 of HtrI (*13, 53*)

It will be important for future studies to explore possible coupling between Asp73 protonation and other downstream mechanistic events in the SRII photocycle. For example, in the case of BR, Asp85 protonation has been related to structural changes in the protein (*8*,) proton release, and chromophore isomerization (*54*). Additional information can be obtained about conformational changes in the SRII photocycle through the combined use of isotope labeling, site-directed mutagenesis, and FTIR difference spectroscopy. For example, bands can be assigned to the vibrations of specific amino acids by the introduction of isotopically labeled amino acids into the growth medium (*55*). More specific assignments can be made by site-directed mutagenesis (*56*) or site-directed isotope labeling (*57*) of individual residues. It should also be pointed out that the above conclusions are based on FTIR measurements of light-induced steady-state mixtures of various SRII photointermediates. While the O intermediate appears to be the predominant intermediate in these mixtures under the conditions used, it is likely that other photointermediates such as M and N are also present which contribute to the difference spectra. A more accurate assignment of vibrational bands to specific photointermediates will be possible in the future using FTIR time-resolved techniques which allow measurements to be made at physiological temperatures (*58*).

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