Early Photocycle Structural Changes in a Bacteriorhodopsin Mutant Engineered to Transmit Photosensory Signals*

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Bacteriorhodopsin (BR) and sensory rhodopsin II (SRII) function as a light-driven proton pump and a receptor for negative phototaxis in halolarchaeal membranes, respectively. SRII transmits light signals through changes in protein-protein interaction with its transducer HtrII. Recently, we converted BR by three mutations into a form capable of transmitting photosignals to HtrII to mediate phototaxis responses. The BR triple mutant (BR-T) provides an opportunity to identify structural changes necessary to activate HtrII by comparing light-induced infrared spectral changes of BR, BR-T, and SRII. The hydrogen out-of-plane (HOOP) vibrations of the BR-T were very similar to those of SRII, indicating that they are distributed more extensively along the retinal chromophore than in BR, as in SRII. On the other hand, the bands of the protein moiety in BR-T are similar to those of BR, indicating that they are not specific to photosensing. The alteration of the O–H stretching vibration of Thr-215 residue, and a distorted α-helix for the signal generation. We also succeeded in measurements of L minus initial state spectra of BR-T, which are the first FTIR spectra of L intermediates among sensory rhodopsins.

Many photochemically reactive proteins are used to capture energy by light-driven ion transport in prokaryotes and plants, whereas others are used for photosensory signaling. Photosensory functions are myriad, including light regulation of motility (phototaxis) in microorganisms, vision in higher animals, and light control of gene expression in all domains of life (e.g. carotenogenesis, circadian entrainment, plant morphogenesis) (1).

Rhodopsins, 7-transmembrane receptors using retinal as a chromophore, are widespread among microorganisms and animals (2). The microbial rhodopsin family is unusual in that many are light-driven ion transporters, whereas other homologous members of the family are sensory receptors that interact with transducer proteins to activate signal transduction pathways (3). The archaeal halophile Halobacterium salinarum contains four different rhodopsins in the membrane. Two, bacteriorhodopsin (BR)3 and halorhodopsin (HR), are light-driven ion pumps, and the other two, sensory rhodopsin I (SRI) and sensory rhodopsin II (SRII, also called phoborhodopsin, pR), are phototaxis receptors controlling motility (3–5). Crystal structures of BR (6) and SRII (7) show close similarities in architecture, helix positions, and location of the retinal-binding pocket (see Fig. 1). However the detailed structures are very different, because 74% of their residues differ.

SRII forms a 2:2 signaling complex with its cognate transducer protein HtrII in the membrane (8), and signals transmitted from SRII to HtrII modulate a phototransfer pathway controlling the flagellar motor switch. Light absorbed by SRII triggers trans-cis isomerization of the retinal chromophore (9). This photoexcitation results in the sequential appearance of photointermediates K, L, M, and O, followed by return to the unphotolyzed (i.e. dark) form of the protein (4). Protein structural changes in these intermediate states alter the structure of the bound HtrII, a process called signal relay. The mechanism of signal relay, i.e. the nature of these structural changes, is a central question in current studies of sensory receptors (3). The signal relay mechanism from SRII receptors to their cognate Htr transducers has become a focus of interest in part because of its importance to the general understanding of communication between integral membrane proteins, about which little is known.

To determine the minimal core of the signaling mechanism, we worked on defining the minimal modifications necessary to convert BR into a protein that would mimic SRII and relay the photosignal to HtrII. We recently found that just three residues in BR replaced by the corresponding residues in SRII enable BR to efficiently relay the retinal photosensorization signal to...
FTIR Spectroscopy of Signal-competent Bacteriorhodopsin

FTIR Spectroscopy—Low temperature FTIR spectroscopy was as described previously (15, 21). The samples were washed three times with a buffer at pH 7.0 (2 mM phosphate) for spectral measurements of K- and L intermediates. 90 μl of the sample was dried on a BaF2 window with a diameter of 18 mm. After hydration by H2O, D2O, or D18O, the sample was placed in a cell, which was mounted in an Oxford DN-1704 cryostat placed in the Bio-Rad FTS-40 spectrometer. The light-adsorbed state was obtained by illumination of the film with >500 nm of light for 1 min at 273 K.

The BR-TK minus BR-T difference spectra were measured at 77 K as follows. To convert BR-T to BR-TK, the sample was irradiated for 2 min with 500 nm light; subsequent illumination with >640 nm light for 60 s reconverted BR-TK into BR-T. The difference spectrum was calculated from the spectra constructed with 128 interferograms collected before and after the photoinitiation. Twenty-four spectra obtained in this way were averaged for the BR-TK minus BR-T spectrum.

The BR-TL minus BR-T difference spectra were measured at 170 K as follows. Illumination with >580 nm of light at 170 K for 1 min converted BR-T to the L intermediate. The spectrum by photoreversion of BR-TL to BR-T was not a mirror image of the BR-TL minus BR-T spectrum (forward photoreaction). Therefore, four spectra obtained by illumination were averaged to obtain the BR-TL minus BR-T spectrum.

RESULTS

Infrared Spectral Changes of BR-T upon Formation of the K Intermediate—Fig. 2 shows the K minus initial state infrared difference spectra of BR (21), BR-T, and SRII (Fig. 2, a–c) (15) measured at 77K and pH 7. In Fig. 2, a and c, the negative and positive bands at 1530(−)/1514(+1) and 1554(−)/1544(+) cm−1 correspond to ethylenic C=C stretching vibrations of the retinal chromophore in BR and SRII, respectively. The bands at 1203(−)/1194(+) cm−1 for BR and 1204(−)/1198(+1) cm−1 for SRII correspond to the C=C stretching vibration of the chromophore at position C14–C15. It is well known that these bands indicate the formation of the K intermediate (15). The similar frequency shifts, 1533(−)/1524(+) cm−1 for C=C stretching vibration and 1202(−)/1195(+1) cm−1 for C=C stretching vibration, were observed in BR-T, indicating the formation of the K intermediate. The bands at 1533(−)/1524(+) cm−1 in BR-T are relatively small. This appears to be due to the smaller frequency shift (9 cm−1) than in BR (16 cm−1), resulting in mutual cancellation of the positive and negative bands. Also in SRII, the bands at 1554(−)/1544(+) cm−1 (frequency shift = 10 cm−1) are smaller than in BR (frequency shift = 16 cm−1). One of the differences between BR and BR-T is the appearance of a positive band at 1157 cm−1 in BR-T. The band was also observed in SRII at 1159 cm−1, suggesting similar structural changes of some C=C stretching vibration between BR-T and SRII upon formation of the K intermediate, not occurring in BR.

HOOP Vibrations of the Retinal Chromophore in BR-T—A previous FTIR study (15) showed remarkable spectral differences in the region of hydrogen out-of-plane (HOOP) vibrations of the retinal chromophore (1000–800 cm−1) between BR and SRII, which provide information about the chromophore
and 861 cm\(^{-1}\) (22). The SRIIK minus SRII spectrum exhibits which have been assigned as HOOP vibrations of C15–H and C14-HOOP vibration. These bands were also observed in BR-T sample was hydrated with H\(_2\)O. Spectra in previous papers. One division of the y-axis corresponds to 0.013 of an absorbance unit. The solid lines are H\(_2\)O, and the dotted lines are D\(_2\)O.

Vibrational Bands of C= N Stretching Vibrations of the Retinal Schiff Base and the Protein Moiety in BR-T—Fig. 4 shows spectral changes in the 1780-1560 cm\(^{-1}\) region, where most signals originate from protein vibrations. One exception is the C=N stretching vibration of the retinylidene Schiff base that appears in the 1650-1600 cm\(^{-1}\) region (25). The overall spectral shape of BR-T looks similar to that of BR, not SRII. The C=N stretching vibrations of BR are located at 1641(–)/1608(+) cm\(^{-1}\) in H\(_2\)O and at 1628(–)/1606(+) cm\(^{-1}\) in D\(_2\)O (Fig. 4a), whereas those of SRII are located at 1657(–)/1600(+) cm\(^{-1}\) in H\(_2\)O and at 1633(–)/1597(+) cm\(^{-1}\) in D\(_2\)O (Fig. 4c). The bands at 1641(–)/1612(+) cm\(^{-1}\) in H\(_2\)O and 1628(–)/1608(+) cm\(^{-1}\) in D\(_2\)O appeared in the case of BR-T, which are similar frequencies to BR, implying that structure and structural changes of the C=N stretching vibrations in BR-T are almost the same as those in BR.

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Fig. 4 also shows vibrational bands of the protein. The intense peaks at 1704(–)/1700(+) cm\(^{-1}\) (Fig. 4e) originate form the C=O stretching vibrations of the side chain of Asn-105 in SRII (26). Because the Asn is replaced by aspartate both in BR and BR-T, the spectrum lack the bands, but contain the D\(_2\)O-sensi-
tive bands at 1742(−)/1732(+) cm\(^{-1}\) for BR (27) and at 1740(−)/1731(+) cm\(^{-1}\) for BR-T, respectively (Fig. 4, a and b), showing similar structural changes of the aspartic acid (Asp-115) between BR and BR-T. The band pair at 1623(+)/1617(−) cm\(^{-1}\) in BR (Fig. 4a) was assigned to the C=O stretching vibrations of amide I of Val-49 (28). In BR-T, a band pair was also observed at 1623(+)/1618(−) cm\(^{-1}\), indicating the similar structural changes of Val-49 between BR and BR-T. In BR, the 1668(−)/1664 cm\(^{-1}\) bands correspond to the frequency region typical for the amide I vibration of the highly dichroic \(\alpha\)\(_{II}\)-helix (29). In contrast, the intensity of the bands was markedly decreased in SRII and BR-T. Because the \(\alpha\)\(_{II}\)-helix possesses considerably distorted structure (29), the structural changes of distorted \(\alpha\)-helix did not occur upon formation of the K intermediates in both BR-T and SRII. Except for the changes in the \(\alpha\)\(_{II}\)-helix region, the spectra of BR-T is likely similar to BR in the typical amide I vibrational region (1670-1600 cm\(^{-1}\)), suggesting that protein structural changes in BR-T are similar to that in wild-type BR.

\(X\)–\(D\) (X=O,N) Stretching Vibrations in BR-T—Fig. 5 shows a spectral comparison in the 2780-1850 cm\(^{-1}\) region between hydration with D\(_2\)O (red lines) and D\(_2\)\(^{18}\)O (blue lines) for BR (Fig. 5a) (21), BR-T (Fig. 5b) and SRII (Fig. 5c) (30), where isotope shifts were observed for many bands. We assign the green-labeled bands to the O–D stretching vibrations of water because of the isotope shift. In BR, negative peaks at 2690, 2636, 2599, 2323, 2292, and 2171 cm\(^{-1}\) and positive peaks at 2684, 2675, 2662, 2359, and 2265 cm\(^{-1}\) were earlier assigned to vibrations of water molecules (21) (Fig. 5a). Because the frequencies of the negative peaks at 2321, 2292, and 2171 cm\(^{-1}\) are much lower than those of fully hydrated tetrahedral water molecules (31), the hydrogen bonds of those water molecules must be very strong. The strong hydrogen-bonded water molecules were observed not only in BR but also in BR-T (at 2323, 2292, and 2195 cm\(^{-1}\)) and SRII (at 2307 and 2213 cm\(^{-1}\)). A band of the strongest hydrogen-bonded water molecule at 2171 cm\(^{-1}\) in BR, which was assigned to the O–D stretching vibration of water 402 hydrating Asp-85 (32), shifted to 2195 cm\(^{-1}\) in BR-T. In BR-T, the introduced Thr-215 probably forms a hydrogen bond with Tyr-185 (as discussed below). Because Tyr-185 also forms a hydrogen bond with Asp-212, the hydrogen bond between them becomes weaker. Moreover, Asp-212 directly forms a hydrogen bond with Tyr-185 (as discussed below). Because Tyr-185 also forms a hydrogen bond with Asp-212, the hydrogen bond between them becomes weaker. Moreover, Asp-212 directly forms a hydrogen bond with water 402 (33). The hydrogen bond between Asp-212 and water 402 becomes stronger, the hydrogen bond between Asp-85 and water 402 becomes weaker. As a result, the band at 2171 cm\(^{-1}\) in BR shifts to 2195 cm\(^{-1}\) in BR-T. Supporting this interpretation, the candidate...
band for the O–D stretching vibration of water 402 hydrating Asp-212 (2636 cm\(^{-1}\)) seems to be down-shifted to 2628 cm\(^{-1}\). Recently Fututani \textit{et al.} \cite{34} proposed that the existence of strongly bound water molecules was required for proton pumping, because was was well correlated with proton pumping activity of archaeal rhodopsins. Sudo and Spudich \cite{10} confirmed that BR-T in the absence of bound HtrII does pump protons with similar efficiency as wild-type BR. Therefore, this result is consistent with the correlation between the strongly hydrogen-bonded water molecule and pumping activity.

The frequency region shown in Fig. 5 also contains X–D stretching vibrations other than water molecules. In the BR\(_K\) minus BR spectrum, the bands at 2506(−)/2465(+) cm\(^{-1}\) labeled in purple and the underlined bands at 2171(−) and 2124(−) cm\(^{-1}\) were assigned to the O–D stretching vibrations of Thr-89 \cite{35,36} and the N–D stretching vibrations of the retinal Schiff base \cite{37}, respectively. Thus, the negative 2171 cm\(^{-1}\) band contains both the O–D stretch of water and the N–D stretch of the Schiff base. In the BR-T\(_K\) minus BR-T spectrum, there are similar bands. The bands at 2506(−)/2471(+) cm\(^{-1}\) can be assigned to the O–D stretching vibrations of Thr-89 in analogy to wild-type BR. The O–D frequencies of Thr-89 in BR-T and BR-T\(_K\) (2506 and 2471 cm\(^{-1}\)) are almost identical to those of Thr-89 in BR and BR\(_K\) (2506 and 2465 cm\(^{-1}\)), respectively, indicating that the strength of hydrogen bonding between Thr-89 and Asp-85 is identical to that in BR.

Although not assigned directly by use of the labeled protein, the bands at 2179 and 2128 cm\(^{-1}\) in BR-T (Fig. 5b) are likely to originate from N–D stretching of the Schiff base, whose frequencies are very similar to those in BR (2171 and 2124 cm\(^{-1}\)). This fact indicates similar hydrogen bonding strengths between BR-T and BR. The frequency of the intense band (2179 cm\(^{-1}\) for BR-T and 2171 cm\(^{-1}\) for BR) corresponds to the results obtained for the C=N stretching vibrations as shown in Fig. 4. Intriguingly, a new negative band at 1993 cm\(^{-1}\) \cite{34} appears in BR-T (Fig. 5b). The corresponding band at 2004 cm\(^{-1}\) was also observed in SRII spectra (Fig. 5c) and was not observed in BR spectra (Fig. 5a). It is assignable to an X–D stretching vibration, because there is no band in the corresponding region of the difference spectra measured in H\textsubscript{2}O. However, this band does not originate from O–D stretching vibrations of water, because no isotope shift was observed in the sample hydrated with D\textsubscript{2}O. In addition, the band at 2004 cm\(^{-1}\) in SRII was not affected by [\textsuperscript{15}N]lysine-labeled SRII as reported previously \cite{38}. It may originate from an N–D stretching vibration of the guanidinium group of Arg, which is suggested to form a strong hydrogen bond because of its relatively low frequency. In the case of BR, the bands at 2292(−)/2266(+), 2579(−)/2567(+) cm\(^{-1}\) were assigned to the N–D stretching vibrations of Arg-82 \cite{39}.

Spectral Differences Caused by Association with HtrII in Vibrations in the Low Frequency Region—Fig. 6, \textit{a} and \textit{b}, shows BR-T\(_K\) minus BR-T and SRII\(_K\) minus SRII infrared difference spectra in the absence (dotted lines) and presence (solid lines) of HtrII. The right panels show the spectra in the 1320-900 cm\(^{-1}\) region. Both dotted and solid lines in BR-T look very similar in this frequency region, implying that complex formation of BR-T with HtrII has almost no effect on the spectral changes in the 1320-900 cm\(^{-1}\) region upon retinal photoisomerization. It should be noted that BR-T is almost completely in complex with HtrII, because the BR-T: HtrII complex does not show measurable photoinduced proton transport activity \cite{10}. Therefore the lack of effect in this spectral region is not due to lack of binding. Furthermore, in BR-T, a spectral difference was observed in the frequency region of the amide I vibration of \(\alpha\)-helix (1664(−)/1670(+), cm\(^{-1}\)) \cite{6a, left panel}. The new bands appear at 1663(−)/1671(+) cm\(^{-1}\) in an HtrII-dependent manner. As described above, because the \(\alpha\)-helix possesses considerably distorted structure, the results suggest that the structural changes of distorted \(\alpha\)-helix are caused by association with HtrII both in the case of BR-T and SRII. Another difference due to HtrII is that the negative band at 1653 cm\(^{-1}\) in BR-T without HtrII completely disappeared in BR-T with HtrII, and a new band appeared at 1655 cm\(^{-1}\) (Fig. 6a), suggesting that amide-I vibration of the peptide backbone of BR and/or HtrII was greatly altered by association with HtrII.

\textbf{The O–H Stretching Vibrations of the Introduced Thr Residue into BR—Fig. 7, \textit{a} and \textit{b}, shows the BR-T\(_K\) minus BR-T and SRII\(_K\) minus SRII infrared difference spectra in the absence and presence of HtrII. Also shown is the BR\(_K\) minus BR infrared difference spectrum. Previously we reported that the hydrogen bond between Thr-204\textsuperscript{SRII} and Tyr-174\textsuperscript{SRII} was greatly altered by formation of the K intermediate in a HtrII-dependent manner (Fig. 7b), and the O–H stretching vibration of the hydroxyl...
group of Thr-204 exhibited a frequency downshift of 110 cm\(^{-1}\) (40). This result suggested that a specific hydrogen bonding alteration between Thr-204 and Tyr-174 takes place upon retinal photoisomerization, because it has been reported (35, 36) that the frequency shifts are very small in BR at 77 K (18 cm\(^{-1}\) for Thr-17, 13 cm\(^{-1}\) for Thr-121, and 60 cm\(^{-1}\) for Thr-89 in BR). The BR-T used in this study has the Thr residue as A215T (Ala-215 corresponds to Thr-204 in SRII) as described above.

Comparison of wild-type BR (Fig. 7a, solid lines) with BR-T (Fig. 7a, dotted lines) revealed that enhanced bands at 3518, 3413, 3390, and 3368 cm\(^{-1}\) appeared in BR-T without HtrII. The complex formation between BR-T and HtrII (Fig. 7a, solid lines) has essentially no effect on these bands, unlike in SRII. In BR, the bands at the 3480/3462 and 3415/3402 cm\(^{-1}\) were assigned to the O–H stretching vibrations of Thr-17 and Thr-121, respectively (35, 36). Corresponding bands seem to be present in BR-T. We tentatively assigned the band at 3518 cm\(^{-1}\) as the O–H stretch of introduced Thr-215 in BR-T. Although we cannot identify which positive band originates from Thr-215, in all cases, the results suggest that the hydrogen bond of the introduced Thr residue strengthens by formation of the K intermediate due to the low frequency shifts. These frequency changes in BR-T (105, 128, or 150 cm\(^{-1}\)) are similar to those in SRII (110 cm\(^{-1}\)).

Infrared Spectral Changes of BR-T upon Formation of the L Intermediate—The illumination of rhodopsins elicits a linear and cyclic sequence of spectrally distinct transitions (photocycle), during which these pigments function (4). The photocycle intermediates in SRII are denoted as K, L, M, N, and O and are analogous to those of BR. We can trap and measure the BR\(_L\) minus BR spectra at 170 K using FTIR spectroscopy (21). However, in the case of SRII, the high thermal stability of the K intermediate makes it difficult to trap and measure the SRII\(_L\) minus SRII spectrum (15).

In this study, we succeeded in measurement of the L intermediate of BR-T, which is the first report among archaeal sensory-competent rhodopsins. Fig. 8b, solid lines, shows the L minus initial state infrared difference spectra of BR, transducer-free BR-T, and BR-T-HtrII complex measured in D\(_2\)O condition. The C–C stretching vibrational bands at 1555/1533 cm\(^{-1}\), the C–C stretching bands at 1202/1194 cm\(^{-1}\), and the C15-HOOP vibration at 986 cm\(^{-1}\), which are characteristic of the BR→L transition, appeared in the BR-T\(_L\) minus BR-T spectra (Fig. 8a, solid lines), implying the formation of an L intermediate in BR-T. In contrast to the BR-T\(_K\) minus BR-T spectra, the difference spectra for L intermediates of BR and BR-T are identical in the 1600-800 cm\(^{-1}\) region, indicating that the HOOP vibrations of the retinal chromophore are restored upon formation of L intermediate. The wagging vibrations of the retinal chromophore are also restored upon formation of L intermediate. In addition, the other bands in the 1600-800 cm\(^{-1}\) region were not perturbed by association with HtrII (Fig. 8b), implying no HtrII-effect on BR-T.
FTIR Spectroscopy of Signal-competent Bacteriorhodopsin

FIGURE 9. BR, minus BR (dotted lines (a)), BR-T, minus BR-T (solid lines (a) and dotted lines (b)), and BR-T, complex with HtrII minus BR-T complex with HtrII (solid lines (b)) spectra measured in the 1720-1640 cm⁻¹ region at 170 K, reproduced and expanded from Fig. 8. Most of the bands in this region are attributable to vibrations of the protein moiety. One division of the y-axis corresponds to 0.005 of an absorbance unit.

On the other hand, the amide I vibrational region (1720-1640 cm⁻¹) in wild-type BR (Fig. 9a, dotted line) was quite different from that in BR-T (Fig. 9a, solid line). The bands at 1688(+) and 1679(+) cm⁻¹ and at 1674(+), 1667(-), 1657(-), and 1648(+) cm⁻¹ disappeared and appeared in BR-T, respectively (Fig. 9a). Because the bands are nearly H-D exchange-independent (data not shown), this suggests the perturbation of the peptide backbone upon formation of the L intermediate of BR-T. In the complex of BR-T with HtrII, new positive and negative peaks appeared at 1672 and 1665 cm⁻¹, indicative of a distorted α-helix; (iii) formation of a strong hydrogen bond in the earliest intermediate (K), between Thr-215 and Tyr-185 in BR-T, corresponding to the Thr-204 and Tyr-174 hydrogen bond in SRII, shown to be essential for signaling in the SRII-HtrII complex (13); and (iv) a negative band at 1993 cm⁻¹ appearing in BR-T, corresponding to a band at 2004 cm⁻¹ in SRII, and not observed in the BR spectra. This band may originate from an N-D stretching vibration of Arg.

Therefore the structural changes of the HOOP vibrations, the O–H stretching vibration of the introduced Thr residue (A215T), and a distorted α-helix appear to be important for signal generation. The results from several different methods show that light-induced structural changes occur all along the SRII-HtrII interface, which includes the region on the periplasmic side of the membrane (41), the membrane-embedded domain (42–44), and the cytoplasmic membrane-proximal domain (45, 46). The structural changes detected in this study further suggest that the signal relay occurs within the membrane-embedded contact surfaces.

The vibrational bands of the C=O stretching vibrations of the retinal Schiff base, the protein moiety, and X–D stretching vibrations in BR-T are more similar to BR than SRII, indicating that the spectral differences between BR and SRII in these regions are not important for the signal transfer to HtrII. In contrast, HOOP vibrations of the retinal chromophore in BR-T are very similar to those of SRII. It is notable that SRII, another phototaxis sensor in H. salinarum, also exhibits more vibrational bands in the HOOP region than does BR.⁴

In BR-T, the changes of HOOP vibrational bands are presumably caused by the A215T mutation, which is located near the retinylidene Schiff base, because the spectrum of the K intermediate in BR-P200T/V210Y is almost identical to that of wild-type BR (data not shown). In SRII, by using all monodeuterated retinal analogues, we reported a local steric constraint between the C₁₄-H of the retinal chromophore and protein caused by C₁₃=C₁₄ double bond rotation (12). The protein conotrigger to this steric conflict of the C₁₄-H group in SRIIₖ may be Thr-204, because the structure of SRIIₖ shows that the distance to Thr-204 is significantly reduced upon isomerization from 4.1 to 3.3 Å. A similar structural change appears to be induced in BR-T. In SRII, Thr-204 and its hydrogen-bonded partner Tyr-174 are crucial residues for photosignal relay (13). The hydrogen bond between them is greatly strengthened upon the formation of the earliest SRII photointermediate (SRIIₖ) only when SRII is complexed with HtrII (40). The O–H frequency differences at the 204 position are 110 cm⁻¹ (wild type

⁴ Y. Sudo, Y. Furutani, J. L. Spudich, and H. Kandori, unpublished data.
The high thermal stability of SRIII makes it very difficult to measure the SRIII minus SRII FTIR spectra (15). BR-T, we observed D2O-insensitive new bands at 3518 (−), 3413 (+), 3390 (+), and 3368 (+) cm−1 in the X–H frequency region. These bands are not HtrII-dependent as in SRII, because of no O–H groups, and 28 cm−1 (13). In BR-T, we observed D2O-insensitive new bands at 1670 (−), 1640 cm−1, which originate from amide I vibrational region (1720–1760 cm−1) upon formation of the L intermediate (Fig. 9g).

Our results also demonstrate HtrII-binding effects on the spectral changes probably originate from F and/or G helices. 13C labeling of SRII or HtrII revealed that these spectral changes originate from the SRII helices and not those of HtrII (17). SRII binds to TM1 and TM2 of HtrII via the F and G helices of SRII (8, 47). Therefore, the spectral changes probably originate from F and/or G helices.

The comparative study of BR, BR-T, and HtrII reveal the functional importance of the structure and structural changes of HOOP vibrations, αH-H, and an O–H stretching vibration. The next steps in signal relay and signal propagation through HtrII require further study. According to data from several laboratories (3), these structural changes may induce outward F-helix tilting of SRII (48), structural changes of binding surfaces between SRII and HtrII (16, 43, 44), rotation of TM2 of HtrII, and structural changes in the membrane proximal domain (HAMP) of HtrII (45, 49) to relay the signal to the phosphorylation cascade controlling the flagellar motor switch.

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