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Comparative FTIR Study of a New Fungal Rhodopsin

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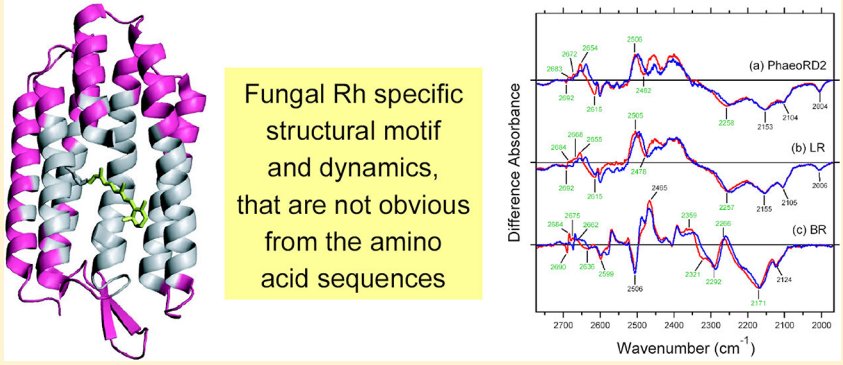
Supporting Information



ABSTRACT: Bacteriorhodopsin (BR) is a light-driven proton pump of halophilic *Archaea*, and BR-like proton-pumping rhodopsins have been discovered in *Bacteria* and *Eucarya* as well. *Leptosphaeria* rhodopsin (LR) and *Phaeosphaeria* Rhodopsin 2 (PhaeoRD2) are both fungal rhodopsins in such a functional class, even though they belong to different branches of the phylogenetic tree. In this study, we compared light-induced structural changes in the K, L, and M photointermediates for PhaeoRD2, LR, and BR using low-temperature Fourier transform infrared (FTIR) spectroscopy. We observed a strongly hydrogen-bonded water molecule in PhaeoRD2 (water O−D stretch in D_2O at 2258 cm^{-1}) as well as in LR and BR. This observation provided additional experimental evidence to the concept that strongly hydrogen-bonded water molecule is the functional determinant of light-driven proton pumping. The difference FTIR spectra for all the K, L, and M states are surprisingly similar between PhaeoRD2 and LR, but not for BR. PhaeoRD2 is more homologous to LR than to BR, but the difference is small. The amino acid identities between PhaeoRD2 and LR, and between PhaeoRD2 and BR are 34.5% and 30.2%, respectively. In addition, the amino acids uniquely identical for the fungal rhodopsins are located rather far from the retinal chromophore. In fact, the amino acid identities within 4 Å from retinal are the same among PhaeoRD2, LR, and BR. For more than 100 amino acids located within 12 Å from retinal, the identities are 48.7% between PhaeoRD2 and LR, 46.0% between PhaeoRD2 and BR, and 47.8% between LR and BR. These results suggest that protein core structures are equally different among the three rhodopsins. Thus, the identical FTIR spectra between PhaeoRD2 and LR (but not BR), even for the K state, indicate that fungal rhodopsins possess some common structural motif and dynamics not obvious from the amino acid sequences.

1. INTRODUCTION

Microbial rhodopsins (Type I rhodopsins) contain all-*trans*-retinal bound to a lysine side chain roughly in the middle of the seventh transmembrane helix via a protonated Schiff base, but are not otherwise homologous to visual rhodopsins (Type II rhodopsins).[1−4] The retinal chromophore experiences all-*trans* to 13-*cis* photoisomerization followed by a chain of thermal relaxations called the photocycle. The protein moiety of microbial rhodopsins responds to the changes in the retinal geometry with its own conformational changes, which serve the purpose of ion transport or signaling. The first major function of microbial rhodopsins is ion transport as exemplified by light-driven pumping of protons or chloride. The second major function is photosensory transduction, where optical signals are transformed into conformational changes and communicated to a transducer protein mediating phototaxis or other responses to light. There is also a special case of rhodopsins from *Chlamydomonas* and other algae, where the microbial rhodopsin domain is part of a much larger protein in which it regulates passive transport of cations or enzyme activity.[4]

Microbial rhodopsins were first discovered in halobacteria, and halobacterial rhodopsins are very well studied. Many halobacterial genomes contain genes coding for four related proteins: bacteriorhodopsin (BR), halorhodopsin (HR), sensory rhodopsin I (SR-I), and sensory rhodopsin II (SR-II, also called phoborhodopsin). BR and HR are light-driven ion

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pumps, which act as an outward proton pump and an inward Cl^- pump, respectively. On the other hand, SR-I and SR-II are photoreceptors of halobacteria mediating attractant and repellent responses in phototaxis, respectively. In addition, recent genome sequencing projects convincingly demonstrated the presence of rhodopsins of haloarchaeal type not only in *Archaea*, but also in *Bacteria* and *Eukaryota*.[5−7]

Among eucaryotes, many fungi possess microbial rhodopsins, the most studied being *Neurospora crassa* rhodopsin (NR) and *Leptosphaeria maculans* rhodopsin (LR).[8−12] Identities of amino acid residues between LR and BR, and between LR and NR are 25.7% and 55.8%, respectively, and it is reasonable that LR is more homologous to NR than BR from the evolutionary point of view. However, it was found that LR pumps protons similar to BR, but NR does not pump protons, suggesting that LR and NR (in comparison to each other and BR) are a good system to reveal the structural elements necessary for proton pumping by rhodopsins.[13,14] Our previous low-temperature FTIR spectroscopy studies showed that the structural changes of LR are closer to those of BR than of NR in terms of details of vibrational bands of retinal and protein.[15,16] The most prominent difference was seen for the water O−D stretching vibrations (measured in D_2O). LR exhibits an O−D stretch of water at 2257 cm^{-1}, indicating the presence of a strongly hydrogen-bonded water molecule. Such strongly hydrogen-bonded water molecules (O−D stretch at <2400 cm^{-1}) were observed for BR, but not for NR. Comprehensive studies of BR mutants and archaeal rhodopsins have revealed that strongly hydrogen-bonded water molecules are only found in the proteins exhibiting proton-pumping activity.[17,18] It was suggested that the strongly hydrogen-bonded water, the determinant of proton pumping, has been preserved between BR and LR during the evolution, whereas the water-containing hydrogen-bonding network in NR has been altered.

In this article, we report a low-temperature Fourier transform infrared (FTIR) spectral comparative study of another fungal rhodopsin from *Phaeosphaeria* (*Stagonospora*) *nodorum* (PhaeoRD2).[19] On the basis of time-resolved spectroscopic and mutagenesis evidence, PhaeoRD2 was suggested to pump protons like LR and BR. The identities of amino acid residues between PhaeoRD2 and LR, and between PhaeoRD2 and BR are 34.5% and 30.2%, respectively. Therefore, PhaeoRD2 is more homologous to LR than BR, but the overall difference is small. This is reasonable, because PhaeoRD2 and LR belong to the distinctly different branches in the phylogenetic tree of fungal rhodopsins, which could be the result of early duplication of the ancestral BR-like gene with the subsequent divergence. It should be particularly noted that the local environment of the retinal chromophore is very similar among the three proton-pumping rhodopsins. For instance, there are 16 amino acid residues within 4 Å from the retinal chromophore in the BR structure, where 12 amino acid residues are identical between PhaeoRD2 and LR, between PhaeoRD2 and BR, and between LR and BR. Among 45 amino acid residues within 7 Å from the retinal chromophore in the BR structure, the identities are 60.0% between PhaeoRD2 and LR, 62.2% between PhaeoRD2 and BR, and 64.4% between LR and BR. Even within 12 Å from the retinal chromophore, the identities of the 113 amino acid residues are 48.7% between PhaeoRD2 and LR, 46.0% between PhaeoRD2 and BR, and 47.8% between LR and BR. These results suggest that protein core structures are equally different among the three rhodopsins. However, the difference FTIR spectra between PhaeoRD2 and LR are surprisingly similar for the K, L, and M intermediates, but they are different from those of BR. The present observation suggests that fungal rhodopsins possess the unique properties that must be conserved during evolution, but are not obvious from the amino acid sequences.

2. EXPERIMENTAL SECTION

Materials. PhaeoRD2 was prepared as described previously.[19] Briefly, it was heterologously expressed in methylotrophic yeast *Pichia pastoris*, strain GS115, using a pPICZαA vector. The coding sequence of *Phaeosphaeria* rhodopsin gene *ops2* (SNOG_00341, Gene ID: 5967674, renamed PhaeoRD2, the DNA kindly provided by P. Solomon and R. Oliver) was truncated to 822 bp to remove most of the putative extramembrane part of the N terminus and replaced with the yeast signal sequence for membrane targeting, and a 6-His-tag coding sequence was added at the C-terminus. The pPICZαA-PhaeoRD2 vector was transformed into *P. pastoris* GS115 cells by electroporation, and the transformed colonies were isolated from the YPDS/zeocin plates and screened for high expression levels of rhodopsins in small-scale cultures (25 mL), similar to what was done with LR.[14] The large-scale protein expression followed the established shake-flask protocol of the *Pichia* expression kit (Invitrogen) with small modifications, using BMGY and BMMY media at 29−30 °C.[19] The rhodopsin expression was induced by methanol (final concentration 0.7%), and all-*trans*-retinal (Sigma, final concentration 5 μM) was added to the growth medium after 24 h of induction for rhodopsin reconstitution. The red-colored cells were collected by centrifugation after 40 h of induction and washed with Milli-Q water twice. The cells were incubated with lyticase (from *Arthrobacter luteus*, Sigma) for digestion of the cell walls and disrupted using glass beads. The membrane fraction was collected using differential centrifugation and used for the protein purification.

To purify PhaeoRD2 for reconstitution into liposomes needed for FTIR spectroscopy, we used 6-His tag affinity resin (Ni-NTA agarose, Qiagen). The membrane pellets of PhaeoRD2 were resuspended in solubilization buffer (1% Triton X-100, 20 mM KH_2PO_4, 0.3 M NaCl, 10 mM β-mercaptoethanol, 1 mM PMSF, pH 7.5) and stirred in the dark at 4 °C overnight, then centrifuged at 38 000 rpm for 50 min at 4 °C. Solubilized rhodopsin was mixed with 6-His tag affinity resin and incubated in the dark at room temperature with gentle agitation to allow complete binding (usually 3 h). The clear supernatant containing other solubilized proteins was removed after centrifugation at 4000*g* at 4 °C for 2 min. The resin was washed with increasing concentrations of imidazole (0.25% Triton-X100, 50 mM KH_2PO_4, 400 mM NaCl, up to 35 mM imidazole, 1 mM DTT, pH 7.5) until the spectral cytochrome band at 410 nm disappeared from the wash spectrum. The purified protein was eluted from the resin with the elution buffer of the same composition as the wash buffer, but with 250 mM imidazole. Addition of *Pichia* lipid extract (at 0.2 mg/mL) was needed to stabilize solubilized PhaeoRD2, similar to what was found for NR.[10,15]

The lipid reconstitution protocol followed that used for LR.[12,14] The dry powder lipids (1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine (DMPC):dimyristoyl-l-α-phosphatidic acid (DMPA) = 9:1 w/w, Avanti lipids) were first dissolved and mixed in warm chloroform, which was thoroughly removed by evaporation under vacuum to yield a thin lipid film. The dry lipids were rehydrated by 50 mM KH_2PO_4, 100 mM NaCl, pH

7.5 and agitated to obtain lipid suspension at high concentration (usually, 10 mg/mL). Purified solubilized rhodopsin was added to the preformed liposomes, which were semisolubilized (as judged by the drop in turbidity) with Triton X-100 at protein/lipids/detergent (w/w/w) ratio of 1:3:1.5, and stirred for 15 min at room temperature. The resultant semitransparent mixture became turbid after removal of detergent by adding 400 mg of Biobeads SM-2 (Biorad) per 1 mL of the mixture and incubation with stirring at 4 °C in the dark. The proteoliposomes were collected by centrifugation at 20 000*g* for 30 min at 4 °C. The absorption maxima of PhaeoRD2 are located at 535 and 538 nm in detergent and membrane, respectively.[19]

Methods. *FTIR Spectroscopy.* FTIR spectroscopy was performed as described previously.[15,16,20] The PhaeoRD2 sample reconstituted into DMPC/DMPA liposomes was washed three times with 2 mM phosphate buffer (pH 7). The pellet was resuspended in the same buffer, and the concentration was adjusted to ~1 mg/mL. An 80 μL aliquot was deposited on a BaF_2 window with a diameter of 18 mm and dried in a glass vessel that was evacuated with an aspirator. The PhaeoRD2 sample was hydrated with H_2O, D_2O, or $D_2{}^{18}O$. The sample was then placed in a cell in an Oxford DN-1704 cryostat mounted in the Bio-Rad FTS-40 spectrometer. The cryostat was equipped with an Oxford ITC-4 temperature controller, and the temperature was regulated with 0.1 K precision.

Illumination with 501 nm light at 77 K for 2 min converted PhaeoRD2 to PhaeoRD2$_K$.[16] Since PhaeoRD2$_K$ was completely reconverted to PhaeoRD2 upon illumination with >600 nm light for 1 min, as evidenced by the spectral shape and amplitude, which is a mirror image of that for the PhaeoRD2-to-PhaeoRD2$_K$ transition, cycles of alternating illumination with 501 and >600 nm light were repeated a number of times. The difference spectrum was calculated from two spectra constructed from 128 interferograms taken before and after the illumination; 24 difference spectra obtained in this way were averaged to produce the PhaeoRD2$_K$ minus PhaeoRD2 spectrum.

Illumination with >560 nm light at 170 K for 2 min converted PhaeoRD2 to PhaeoRD2$_L$, and LR to LR$_L$. The L intermediate was converted back to the original state by warming. The difference spectrum was calculated from two spectra constructed from 264 interferograms taken before and after the illumination; 3−5 difference spectra obtained in this way were averaged to produce the PhaeoRD2$_L$ minus PhaeoRD2, and LR$_L$ minus LR spectra.

Illumination with >480 nm light at 240 K for 2 min converted PhaeoRD2 to PhaeoRD2$_M$, and LR to LR$_M$. Since the formed M intermediate was reconverted to the original state upon illumination with 420 nm light for 2 min, as evidenced by the spectral shape and amplitude, which is a mirror image of that for the PhaeoRD2 to PhaeoRD2$_M$ (LR to LR$_M$) transition, cycles of alternating illumination with >480 and 420 nm light were repeated a number of times. Under the present conditions, branching photoreactions from the M state were negligible for PhaeoRD2 and LR. The difference spectrum was calculated from two spectra constructed from 128 interferograms taken before and after the illumination; 24 difference spectra obtained in this way were averaged to produce the PhaeoRD2$_M$ minus PhaeoRD2, and LR$_L$ minus LR spectra.

As linear dichroism experiments revealed the random orientation of PhaeoRD2 and LR molecules in the liposome film, an IR polarizer was not used. LR$_K$ minus LR, and BR$_K$ minus BR difference spectra were taken from Sumii et al.,[16] and BR$_L$ minus BR, and BR$_M$ minus BR difference spectra were taken from Tanimoto et al.[20] Since BR molecules are highly oriented in the film, unlike PhaeoRD2 and LR, the data with a window tilting angle of 53.5° in polarized FTIR spectroscopy were used for comparison.

Sequence Analysis. Amino acid sequences of PhaeoRD2, LR, and BR were manually aligned to show common amino acid residues (Figure S1). In the present study, identities are calculated only for the amino acid positions present in all three rhodopsins. This means that if there was a gap in the alignment for one of the three rhodopsins, such amino acid residues were not taken into the account in the analysis. It should be noted that all transmembrane helical segments were aligned without gaps.

3. RESULTS

Comparison of the Vibrational Bands of the Retinal Chromophore in the K Intermediates of PhaeoRD2, LR, and BR. Figure 1 compares light-induced difference FTIR spectra of PhaeoRD2 (a), LR (b), and BR (c) at 77 K. The ethylenic stretching vibration at 1537 (−)/1526 (+) cm^{-1} in Figure 1a implies that PhaeoRD2 is converted to a red-shifted intermediate, PhaeoRD2$_K$, as in the case of LR (Figure 1b) and BR (Figure 1c). There are negative bands at 1256, 1204, and 1168 cm^{-1} in the C−C stretching region and at 1008 cm^{-1} in

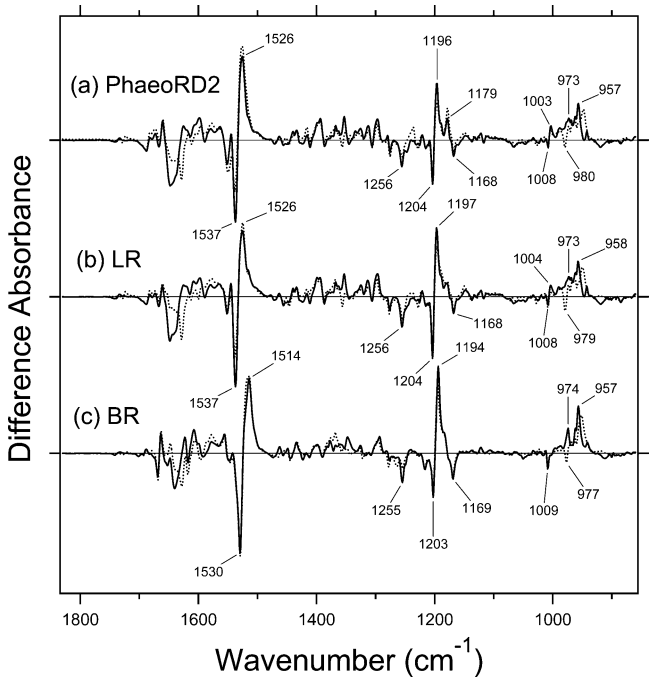

Figure 1. Difference FTIR spectra between the K intermediates and unphotolyzed states of PhaeoRD2 (a), LR (b), and BR (c) in the 1830−860 cm^{-1} region. Solid and dotted lines represent the samples measured at 77 K under H_2O and D_2O hydration, respectively. The spectra of LR and BR are normalized to those of PhaeoRD2 by using the averaged intensities of the C=C stretch at 1540−1510 cm^{-1} and the C−C stretch at 1205−1190 cm^{-1}. Spectra b and c are reproduced from Sumii et al.[16] One division of the *y*-axis corresponds to 0.009 absorbance units.

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the HOOP region (Figure 1a), which were also detected in the Raman spectra reported earlier.[19] These spectral features are similar to those of LR (Figure 1b) and BR (Figure 1c), suggesting that the PhaeoRD2 to PhaeoRD2$_K$ conversion is accompanied by the retinal photoisomerization from the all-*trans* to the 13-*cis* form. While the overall spectral features are very similar among the three rhodopsins, those of PhaeoRD2 are closer to LR than BR, which is described in detail below.

The negative frequencies in the C−C stretching region are identical between PhaeoRD2 and LR, but different by 1 cm^{-1} for BR. Similar spectral shape between PhaeoRD2 and LR is more remarkable in the HOOP region, where broad positive bands at 1005−950 cm^{-1} are identical between PhaeoRD2 and LR (Figure 1a,b). By contrast, BR possesses sharp peaks at 974 and 957 cm^{-1} (Figure 1c). Our previous report showed that the spectral features of the HOOP bands for NR are different from those of LR and BR,[16] although the sequence identity between LR and NR (55.8%) is much higher than that between LR and BR (25.7%).

Figure 2 shows the PhaeoRD2$_K$ minus PhaeoRD2 (a), LR$_K$ minus LR (b), and BR$_K$ minus BR (c) spectra in the 1750−1580 cm^{-1} region, where most of the bands originate from vibrations of the protein. One exception is the C=N stretching vibration of the retinal Schiff base that appears in the 1650−1600 cm^{-1} region. In BR, the C=N stretch has been observed at 1641 cm^{-1} in H_2O and at 1628 cm^{-1} in D_2O (Figure 2c). This frequency upshift in H_2O is caused by coupling to the N−H bending vibration of the Schiff base, and the difference in frequency between H_2O and D_2O has been regarded as a measure of the hydrogen-bonding strength of the Schiff base. The small difference in BR$_K$ (1608 cm^{-1} in H_2O vs 1606 cm^{-1} in D_2O) has been interpreted in terms of the lack of a hydrogen bond for the Schiff base nitrogen after photoisomerization. The corresponding bands of PhaeoRD2 were observed at 1648 and 1629 cm^{-1} in H_2O and D_2O, respectively (Figure 2a). The D_2O-sensitive negative band was also observed for LR (Figure 2b). These IR bands are close in frequency to the Raman bands of LR at 1648 and 1627 cm^{-1}, which were previously assigned to the C=N stretches of the Schiff base in H_2O and D_2O, respectively.[12] The IR frequency shift in PhaeoRD2 (19 cm^{-1}) and LR (19 cm^{-1}) is larger than that in BR (13 cm^{-1}), suggesting that the hydrogen bond of the Schiff base is stronger in PhaeoRD2 and LR than in BR. It should, however, be noted that the negative 1648 cm^{-1} band in PhaeoRD2 (Figure 2a) and LR (Figure 2b) have a shoulder at the lower-frequency side, which makes the estimate less accurate. We reexamined the hydrogen-bonding strength of the Schiff base by the analysis of the N−D stretch vibrations, believed to be a more direct reporter than the C=N stretch (see below). Because it is also difficult to locate the C=N stretching mode in PhaeoRD2$_K$ (Figure 2a) and LR$_K$ (Figure 2b), the spectral analysis of the X−D stretching frequencies becomes even more relevant for providing clear information about the Schiff base environment.

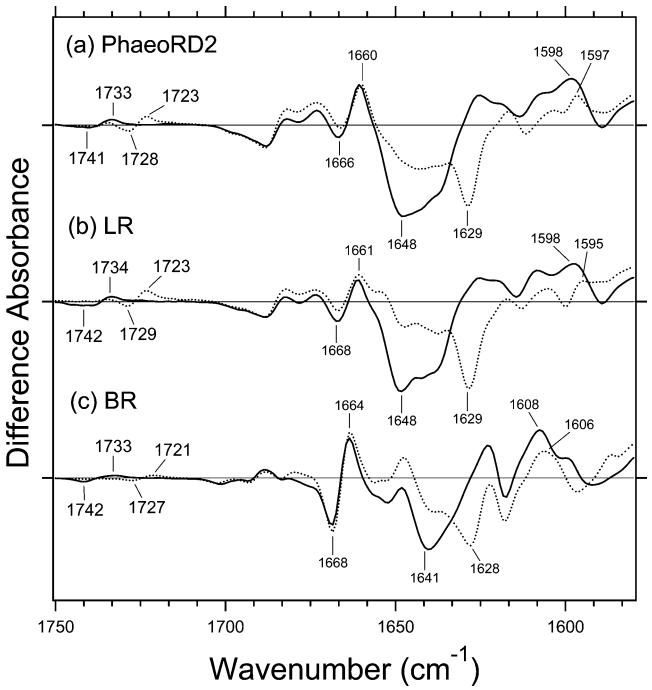

Figure 2. Difference FTIR spectra between the K intermediates and unphotolyzed states of PhaeoRD2 (a), LR (b, and BR (c) in the 1750−1580 cm^{-1} region, which are enlarged from Figure 1. Solid and dotted lines represent the samples measured at 77 K under H_2O and D_2O hydration, respectively. One division of the *y*-axis corresponds to 0.005 absorbance units.

Comparison of Vibrational Bands of the Protein Moiety in the K Intermediates of PhaeoRD2, LR, and BR. The bands in Figure 2 (except for the C=N stretching vibrations of the Schiff base) come from the protein moieties of PhaeoRD2, LR, and BR. In BR, the 1742 (−)/1733 (+) cm^{-1} bands in H_2O are shifted to 1727 (−)/1721 (+) cm^{-1} in D_2O, which was previously assigned to the C=O stretches of Asp115.[21] Corresponding amino acid residues are Asp145 in PhaeoRD2 and Asp169 in LR, and similar bands were observed at 1750−1720 cm^{-1} (Figure 2a and b). This indicates that the aspartic acid at this position is protonated in both PhaeoRD2 and LR, and the hydrogen-bonding alterations upon retinal isomerization are similar. This tendency is consistent with that for the chromophore bands.

The bands at 1668 (-)/1664 (+) cm^{-1} in BR are highly dichroic and appear in the typical frequency region of the amide I vibrations of the α_{II} helix[22] (Figure 2c), which is probably located in the transmembrane region. Similar spectral changes at 1666 (-)/1660 (+) cm^{-1} were also observed for PhaeoRD2 (Figure 2a).

Comparison of the Structural Features of the Schiff Base Region between PhaeoRD2, LR, and BR. Figure 3 shows the PhaeoRD2$_K$ minus PhaeoRD2 (a), LR$_K$ minus LR (b), and BR$_K$ minus BR (c) spectra in the 2770−1970 cm^{-1} region, which contains the X−D stretching vibrations of protein and water molecules. A spectral comparison between the samples hydrated with D_2O and $D_2{}^{18}O$ reveals O−D stretching vibrations of water molecules, which change in frequency upon retinal photoisomerization. The vibrational bands exhibiting isotope-induced downshifts can be assigned to the O−D stretching vibrations of water (labeled in green). In BR, six negative peaks at 2690, 2636, 2599, 2321, 2292, and 2171 cm^{-1} were earlier assigned to vibrations of water molecules.[23] The bands are widely distributed over the possible frequency range for stretching vibrations of water (Figure 3c). Since the frequencies of the negative peaks at 2321, 2292, and 2171 cm^{-1} are much lower than those of fully hydrated tetrahedral water molecules,[24] the hydrogen bonds of those water molecules must be very strong, possibly indicating their association with negative charges. Indeed, previously we assigned the 2171 cm^{-1}

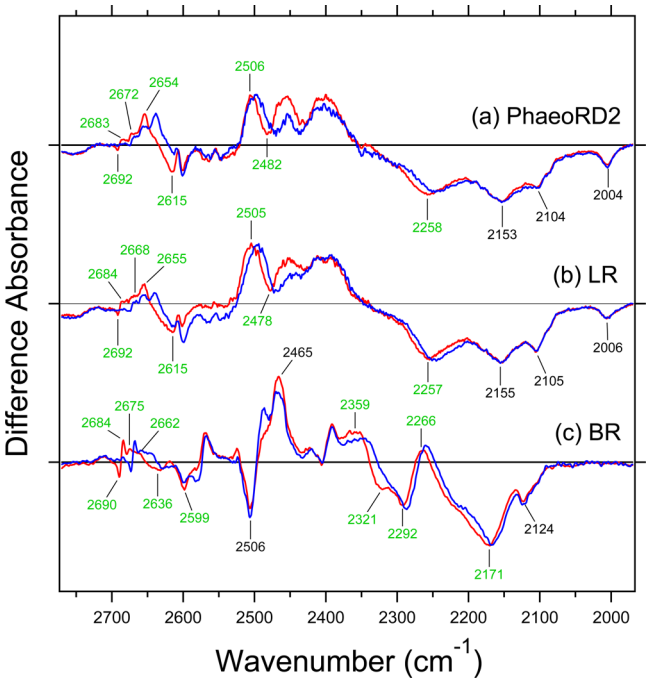

Figure 3. Difference FTIR spectra between the K intermediates and unphotolyzed states of PhaeoRD2 (a), LR (b) and BR (c) in the 2770−1970 cm^{-1} region. Red and blue lines represent the samples measured at 77 K under D_2O and $D_2{}^{18}O$ hydration, respectively. Green-labeled frequencies correspond to those identified as water stretching vibrations. Spectral normalizations are the same as in Figure 1. Spectra b and c are reproduced from Sumii et al.[16] One division of the *y*-axis corresponds to 0.001 absorbance units.

band to the O−D group of a water molecule associated with deprotonated Asp85.[23] In addition, we concluded that six vibrational bands can be assigned to six O−D stretching vibrations of the three water molecules present in the Schiff base region.[23,25]

In the case of PhaeoRD2, three negative bands were observed at 2692, 2615, and 2258 cm^{-1} (Figure 3a). In addition, another negative peak of water at 2482 cm^{-1} is probably obscured by large positive bands. Corresponding positive bands are located at 2683, 2672, 2654, and 2506 cm^{-1}. The spectral features observed for PhaeoRD2 are surprisingly similar to those of LR (Figure 3b), where all water vibrations are reproduced within only 5 cm^{-1} frequency difference. By contrast, water vibrations of PhaeoRD2 are largely different from those of BR.

Among various water vibrations, a negative peak at 2258 cm^{-1} for PhaeoRD2 (Figure 3a) is particularly notable. The frequency is considerably low for the water O−D stretch, indicating that this water forms a strong hydrogen bond in PhaeoRD2.[24] A similar band is observed at 2257 cm^{-1} for LR (Figure 3b). It should be emphasized that there are no water bands in the <2400 cm^{-1} region for NR,[15] which does not pump protons. Through the vibrational studies of various rhodopsins, we have discovered such water vibrations as the signature of proton pump, because proton-pumping rhodopsins possess strongly hydrogen-bonded water molecules (water O−D stretch at <2400 cm^{-1}).[17,18] Therefore, we concluded that strongly hydrogen-bonded water molecules are the functional determinants for light-driven proton pump, and the present results for PhaeoRD2 provide additional support to that idea.

The frequency region shown in Figure 3 also contains X−D stretching vibrations other than those of water molecules. In the BR_K minus BR spectrum, the bands at 2506 (−)/2465 (+) cm^{-1} and the bands at 2465 (+), 2171 (−), and 2124 (−) cm^{-1} were assigned to the O−D stretching vibrations of Thr89,[26] and the N−D stretching vibrations of the retinal Schiff base,[27] respectively (Figure 3c). Thus, the negative 2171 cm^{-1} band contains both the O−D stretch of water and the N−D stretch of the Schiff base. The bands at 2292 (−)/2266 (+) cm^{-1} also contain N−D stretching vibration of Arg82.[28] Although not assigned directly by use of isotopic labeling for PhaeoRD2 (Figure 3a), the bands at 2153 and 2104 cm^{-1} are likely to originate from N−D stretching of the Schiff base, where similar bands are observed for LR (Figure 3b). These frequencies for PhaeoRD2 and LR are lower than in BR (2171 and 2124 cm^{-1}). Therefore, hydrogen-bonding strength of the Schiff base is stronger in PhaeoRD2 and LR than in BR, which is consistent with the results obtained for the C=N stretching vibrations shown above (Figure 2). The spectral shape of the $PhaeoRD2_K$ minus PhaeoRD2 (Figure 3a) clearly shows that the N−D stretch of the Schiff base is located at the higher frequency side in $PhaeoRD2_K$ (2500−2400 cm^{-1}), indicating its weakened hydrogen bond. This is also the case for LR (Figure 3b). It is likely that the hydrogen bond is disrupted upon retinal isomerization in PhaeoRD2, similar to LR and BR.

Comparison of the Vibrational Bands in the L Intermediates of PhaeoRD2, LR, and BR. Figure 4 compares light-induced difference FTIR spectra of PhaeoRD2

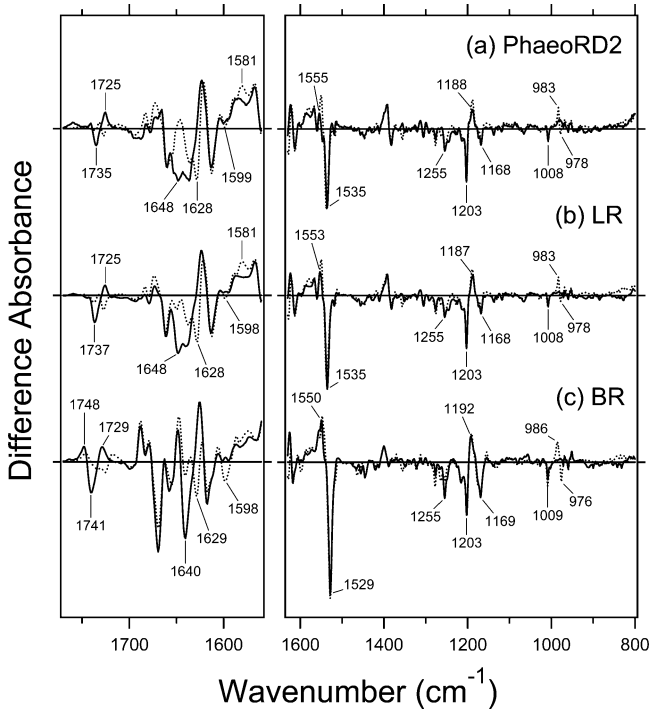

Figure 4. Difference FTIR spectra between the L intermediates and unphotolyzed states of PhaeoRD2 (a), LR (b) and BR (c) in the 1760−1560 cm^{-1} region (left) and the 1630−800 cm^{-1} region (right). Solid and dotted lines represent the samples measured at 170 K under H_2O and D_2O hydration, respectively. The spectra of LR and BR are normalized to that of the PhaeoRD2 according to the intensities of the C−C stretching vibration of the retinal at 1203 cm^{-1}. One division of the *y*-axis corresponds to 0.005 (left) and 0.01 (right) absorbance units.

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(a), LR (b), and BR (c) at 170 K, which correspond to the difference between the L intermediates and unphotolyzed states. While the spectra are already known for BR, we report the data not only for PhaeoRD2, but also for LR, for the first time. The right panel of Figure 4 shows characteristic spectra for the L intermediate, where vibrational bands of retinal are predominantly observable. The spectra are similar among PhaeoRD2, LR and BR, suggesting that structural changes between the unphotolyzed and the L intermediate states are similar. In contrast, the left panel of Figure 4 emphasizes protein bands such as amide-I (1700−1600 cm^{-1}) and those of protonated carboxylic acids (1770−1700 cm^{-1}). It is obvious that the spectra are very similar between PhaeoRD2 (Figure 4a) and LR (Figure 4b), but different for BR (Figure 4c). The similarity of the spectral features is remarkable for the amide-I vibration, suggesting that secondary structure alterations are similar between PhaeoRD2 and LR. While structural changes in the K intermediate are local, situated around the retinal chromophore, they are possibly extended farther in the L intermediate because of the higher temperature of stability (170 K) corresponding to the later times in the photocycle. However, the present FTIR spectral comparison exhibits surprising similarity between PhaeoRD2 and LR for both K and L intermediates.

In the protonated carboxylic stretching region, BR shows spectral changes of Asp96 (1742 to 1748 cm^{-1}) and Asp115 (1734 to 1729 cm^{-1}),[29] and consequently, two positive and one negative band are observed (Figure 4c). By contrast, PhaeoRD2 and LR show only a pair of peaks at 1735 (−)/1725 (+) cm^{-1}, and at 1737 (−)/1725 (+) cm^{-1}, respectively, which presumably correspond to Asp115 in BR. This suggests the absence of structural perturbation of the homologue of BR's Asp96 in the L intermediate. A previous time-resolved FTIR study of PhaeoRD2 observed a negative band at 1745 cm^{-1} in the N-like state.[19]

Figure 5 shows the PhaeoRD2$_L$ minus PhaeoRD2 (a), LR$_L$ minus LR (b), and BR$_L$ minus BR (c) spectra in the X−D stretching region. Again, spectral features are very similar between PhaeoRD2 and LR, but different for BR. Unlike in the spectra of the K state, no isotope effect of water was observed at <2300 cm^{-1} (right panel in Figure 5), which is common among PhaeoRD2, LR, and BR. This fact implies that the water molecules in the Schiff base region reform strong hydrogen bonds with the negative charge(s). By contrast, several water bands are observed at 2700−2450 cm^{-1}, the frequency region of weak and moderate hydrogen bonding, where the spectral features are similar between PhaeoRD2 and LR, but entirely different for BR (left panel in Figure 5). In BR$_L$, broad positive features at 2650−2550 cm^{-1} (Figure 5c) are identified as the water O−D stretches in the cytoplasmic region.[24,30] Such water bands are absent for PhaeoRD2 (Figure 5a) and LR (Figure 5b), and the lack of the broad positive band may be correlated with the lack of structural perturbation of the homologue of BR's Asp96. In the case of BR, formation of the L intermediate accompanies structural perturbation of the cytoplasmic region that connects to Asp96, where protein-bound water molecules alter their hydrogen bonds.[24] Such structural changes are likely to be absent in fungal rhodopsins such as PhaeoRD2 and LR.

The PhaeoRD2$_K$ minus PhaeoRD2 spectra exhibit three negative water bands at 2700−2450 cm^{-1} (Figure 3a), among which two (2692 and 2482 cm^{-1}) are preserved in the PhaeoRD2$_L$ minus PhaeoRD2 spectra (Figure 5a). On the other hand, the negative band at 2615 cm^{-1} in the PhaeoRD2$_K$

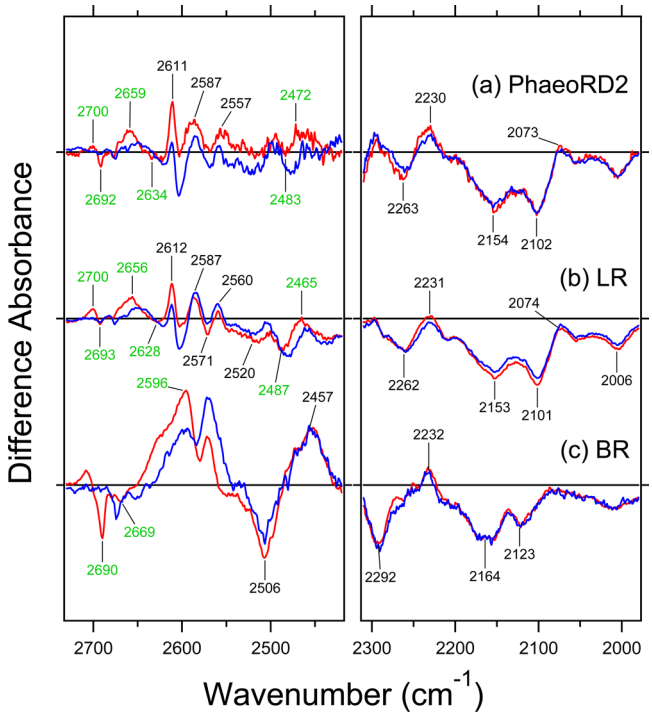

Figure 5. Difference FTIR spectra between the L intermediates and unphotolyzed states of PhaeoRD2 (a), LR (b), and BR (c) in the 2730−2420 cm^{-1} region (left) and the 2310−1980 cm^{-1} region (right). Red and blue lines represent the samples measured at 170 K under D_2O and $D_2{}^{18}O$ hydration, respectively. Green-labeled frequencies correspond to those identified as water stretching vibrations. Spectral normalizations are the same as in Figure 4. One division of the *y*-axis corresponds to 0.001 (left) and 0.0007 (right) absorbance units.

minus PhaeoRD2 spectra is relaxed in PhaeoRD2$_L$, while a new band appears at 2634 cm^{-1}. Thus, among four water vibrations in PhaeoRD2, three change in PhaeoRD2$_K$ and PhaeoRD2$_L$, where two water bands are common. Regarding the positive water bands of PhaeoRD2$_K$ and PhaeoRD2$_L$, only a band at about 2655 cm^{-1} seems to be common, suggesting that the K-L transition accompanies hydrogen-bonding alteration of protein-bound water molecules. Since the spectral features are almost identical, similar events take place for LR, LR$_K$, and LR$_L$ (Figure 5b).

It should be noted that there are also spectral changes in the 2300−2000 cm^{-1} region typical for the strongly hydrogen-bonded waters that do not originate from water O−D stretches. In particular, negative features for the K intermediate at 2200−2050 cm^{-1} (Figure 3a and b) are reproduced in the L intermediate (Figure 4a and b), suggesting that these bands originate from the N−D stretch of the Schiff base.

Comparison of the Vibrational Bands in the M Intermediates of PhaeoRD2, LR, and BR. Figure 6 compares light-induced difference FTIR spectra of PhaeoRD2 (a), LR (b), and BR (c) at 230 K, which correspond to the difference between the M intermediates and unphotolyzed states. While the spectra are already known for BR, we report the data not only for PhaeoRD2, but also for LR, for the first time. The right panel of Figure 6 shows characteristic spectra for the M intermediates, similar among PhaeoRD2, LR and BR. By contrast, the left panel of Figure 6 emphasizes protein bands such as amide-I (1700−1600 cm^{-1}) and those of protonated carboxylic acids (1770−1700 cm^{-1}). Once again, the spectra

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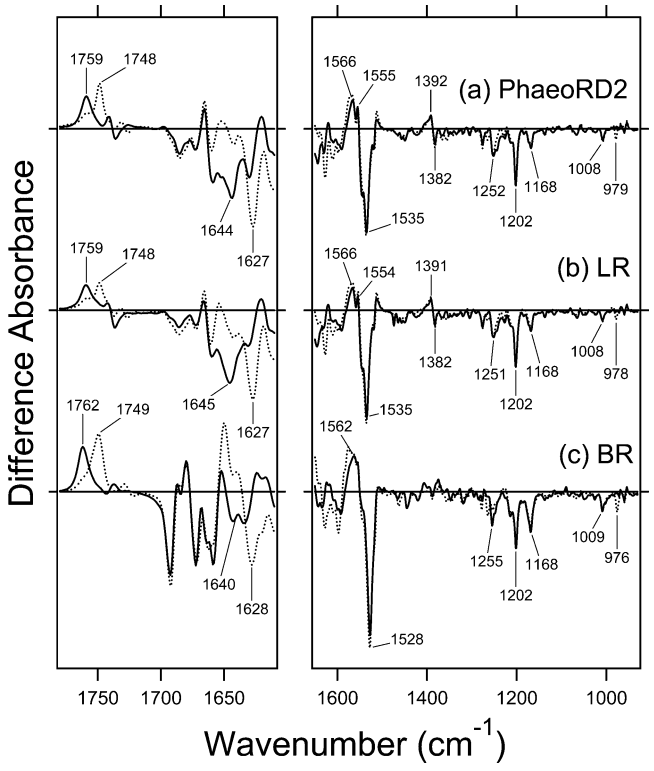

Figure 6. Difference FTIR spectra between the M intermediates and unphotolyzed states of PhaeoRD2 (a), LR (b), and BR (c) in the 1670−1620 cm^{-1} region (left) and the 1640−940 cm^{-1} region (right). Solid and dotted lines represent the samples measured at 230 K under H_2O and D_2O hydration, respectively. The spectra of LR and BR are normalized to that of the PhaeoRD2 according to the intensities of the C−C stretching vibration of the retinal at 1202 cm^{-1}. One division of the *y*-axis corresponds to 0.005 (left) and 0.01 (right) absorbance units.

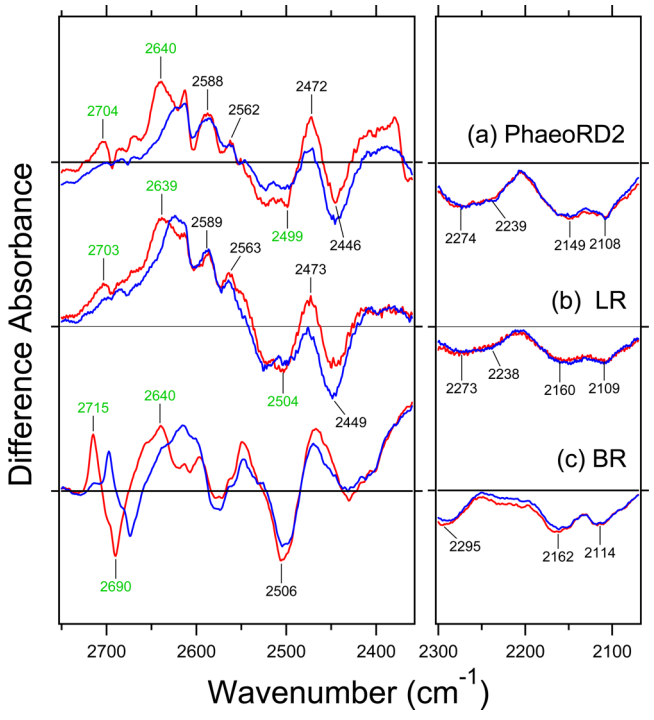

Figure 7. Difference FTIR spectra between the M intermediates and unphotolyzed states of PhaeoRD2 (a), LR (b) and BR (c) in the 2750−2360 cm^{-1} region (left) and the 2200−2070 cm^{-1} region (right). Red and blue lines represent the samples measured at 230 K under D_2O and $D_2{}^{18}O$ hydration, respectively. Green-labeled frequencies correspond to those identified as water stretching vibrations. Spectral normalizations are the same as in Figure 4. One division of the *y*-axis corresponds to 0.0005 (left) and 0.0005 (right) absorbance units.

are very similar between PhaeoRD2 (Figure 6a) and LR (Figure 6b), but different for BR (Figure 6c). The spectral comparison of the amide-I vibrations suggests that secondary structure alterations are similar between PhaeoRD2 and LR. This observation is common for K, L, and M states. The appearance of a positive peak at 1759 cm^{-1} for PhaeoRD2 (Figure 6a) and LR (Figure 6b), and at 1762 cm^{-1} for BR (Figure 6c) shows protonation of the homologue of BR's Asp85.

Figure 7 shows the $PhaeoRD2_M$ minus PhaeoRD2 (a), LR_M minus LR (b) and BR_M minus BR (c) spectra in the X-D stretching region. While spectra are all similar at 2300−2100 cm^{-1} (right panel), spectral features at 2700−2400 cm^{-1} (left panel) are very similar between PhaeoRD2 and LR, but different for BR. Unlike in the spectra of the K states, no isotope effect of water was observed at <2300 cm^{-1} (right panel in Figure 7), which are common among PhaeoRD2, LR, and BR. This fact implies that the water molecules in the Schiff base region reform strong hydrogen bonds with the negative charge(s).[20] In the case of BR, the water band at 2171 cm^{-1} originates from the water being hydrogen-bonded with Asp85, whereas the hydrogen bond of water402 with Asp85 would not be as strong in the M intermediate because of the disappearance of a negative charge at position 85. This requires the formation of an extremely strong hydrogen bond in a different interaction inside BR, and we proposed a hydration switch model upon the proton transfer from the Schiff base to Asp85, where strongly hydrogen-bonded water forms a new hydrogen bond to Asp212.[20,31] Since PhaeoRD2 and LR conserve amino acid residues in the Schiff base region of BR, a similar mechanism must occur.

Spectral features at 2700−2400 cm^{-1}, the frequency region of weak and moderate hydrogen bond, are similar between PhaeoRD2 and LR, but considerably different for BR (left panel in Figure 7). Broad positive feature at 2650−2550 cm^{-1} in BR_L (Figure 5c) is reduced in BR_M (Figure 7c), suggesting relaxation of water-containing hydrogen bonds in the cytoplasmic region of the M intermediate. In contrast, the M intermediates of PhaeoRD2 and LR show broad positive band of water at 2650−2550 cm^{-1} similar to BR_L, although its amplitude in LR_M is larger than that of $PhaeoRD2_M$. This may suggest that the BR_L-like hydrogen-bonding alteration occurs in the M intermediates of PhaeoRD2 and LR.

4. DISCUSSION

In this article, we compared light-induced difference FTIR spectra of two fungal proton-pumping rhodopsins, PhaeoRD2 and LR, with those of BR. The extreme similarity of low-temperature FTIR difference spectra of the early photocycle intermediates of PhaeoRD2 and LR presented above corroborates the somewhat surprising earlier result of the time-resolved studies, which showed highly similar FTIR difference spectra of the late M and N intermediates, but quite different photocycle kinetics.[19] This may suggest that FTIR spectra are dominated by the changes in the protein core, while the photocycle kinetics may be sensitive to the global

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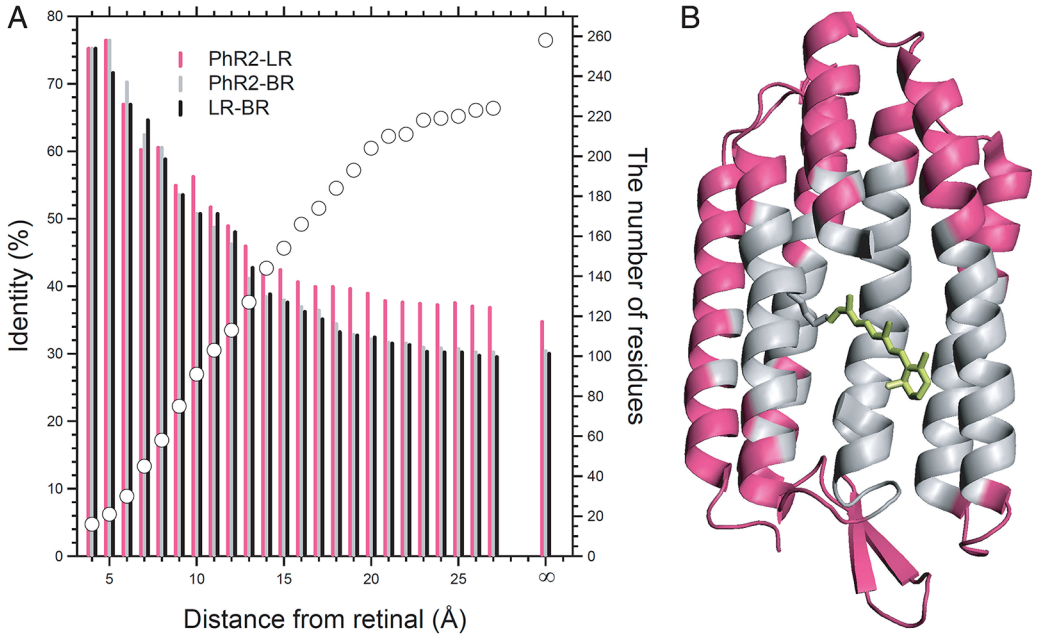

Figure 8. (A) Amino acid identities between PhaeoRD2 and LR (pink bars), PhaeoRD2 and BR (gray bars), and LR and BR (black bars) are plotted versus the distance from the retinal chromophore. Since there are no structures for PhaeoRD2 and LR, distance information was obtained from the BR structure (PDB entry 1IW6). Open circles represent the numbers of the amino acid residues within the given distance, which are taken into account to calculate the identity. Any residues lacking in one or more rhodopsins are not counted in this estimate. (B) Illustration of the spatial proximity of analyzed residues and retinal. If any atom of an amino acid residue is present at ≤12 Å from the retinal chromophore in the X-ray structure of BR (PDB entry 1IW6), such amino acid residues are colored gray, while pink residues are at >12 Å from the retinal chromophore.

protein dynamics affected by the differences in more distant regions.

According to the sequence alignments (Figure S1, Supporting Information), the amino acid identities are 34.5% between PhaeoRD2 and LR, 30.2% between PhaeoRD2 and BR, and 29.8% between LR and BR. PhaeoRD2 is more homologous to LR than BR, but the overall difference is small. The homology of the protein cores and more peripheral regions among the three rhodopsins is illustrated in Figure 8A. Open circles in Figure 8A represent the numbers of amino acid residues for the corresponding distance from the carbon atoms of retinal chromophore in the BR structure, and the identities among three rhodopsins are plotted versus the distance. Figure 8A shows that the higher homology between PhaeoRD2 and LR is not obvious at short distances from the retinal, but becomes more evident on the periphery. In fact, the pairwise identities are all 75.0% at 4 Å. At 10 Å, the identity between PhaeoRD2 and LR (56.0%) is higher than those between PhaeoRD2 and BR (50.5%), and between LR and BR (50.5%). Nevertheless, even at 12 Å, the values do not differ significantly: 48.7% between PhaeoRD2 and LR, 46.0% between PhaeoRD2 and BR, and 47.8% between LR and BR. Thus, the higher homology of fungal rhodopsins, i.e., between PhaeoRD2 and LR, is not clear within 12 Å from retinal, and is only obvious at >12 Å as shown in Figure 8A.

It can be deduced from the distances listed in Figure S1 that there are 113 amino acid residues within 12 Å from retinal, while there are 145 at >12 Å. Figure 8B illustrates the amino acid residues within 12 Å (gray) and at >12 Å (pink) from the retinal chromophore in the BR structure. For the amino acid residues at >12 Å, the identity is 20.7% between PhaeoRD2 and LR, 15.2% between PhaeoRD2 and BR, and 13.8% between LR and BR. Therefore, from the results in Figure 8A, gray regions (within 12 Å) in Figure 8B are equally homologous among PhaeoRD2, LR and BR, and parts of the pink regions (>12 Å) may be specific to fungal rhodopsins. It should be noted that identical FTIR spectra were obtained between PhaeoRD2 and LR (but not BR) even for the K state (stabilized at 77 K), which is supposed to reflect changes in the retinal vicinity. This indicates that fungal rhodopsins possess their common structural motif and dynamics, which is not clear from the amino acid sequences.

The present results suggest that the local structural perturbations in the K state are not necessarily determined by the local structural elements. Rather, distant amino acids and/or whole protein structure are the determinants of the structure and its structural changes. A similar conclusion was previously obtained by the analysis of the FTIR spectra at 77 K for LR, BR, and NR.[16] Identities of amino acid residues between LR and BR, and between LR and NR are 25.7% and 55.8%, respectively, and it is reasonable that LR is more homologous to NR than BR from the evolutionary point of view. In fact, among the 25 amino acid residues around the retinal chromophore, only one amino acid residue is different between LR and NR, but 5 amino acid residues are different between LR and BR. It seems that the local structure around the retinal chromophore in LR is similar to that in NR, but not in BR. However, the FTIR study revealed that the structure of this region and its structural changes upon the retinal photoisomerization in LR are similar to those in BR, and not in NR.[16] The conclusion that the local primary structure does not completely determine properties of the chromophore is not totally surprising, as the long-range effects of amino acid replacements on the retinal and its photoisomerization were convincingly demonstrated for a number of rhodopsins, including bacterial (BR, proteorhodopsin and *Gloeobacter* rhodopsin) and fungal (LR).[13,32−35]

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The demonstrated similarity of photoisomerization mechanism and light-iduced conformational changes between different branches of fungal rhodopsins implies that the auxiliary group of fungal rhodopsins (as represented by PhaeoRD2)[19] has conserved all the main properties of the fungal proton pumps (such as LR). This includes the presence of strongly hydrogen bonded water and specific torsions of the retinal skeleton upon photoisomerization. It is in line with other key properties of proton-pumping rhodopsins recently found for PhaeoRD2, such as the fast photocycle turnover and arrest of the photocycle upon the inactivation of the cytoplasmic proton donor (homologue of BR's D96).[19] Why some of the fungal species need two kinds of light-driven proton pumps remains to be elucidated, but this redundancy is not unique, as it was found in some archaeal and algal species.[36−38]

■ ASSOCIATED CONTENT

Supporting Information

Sequence alignment of PhaeoRD2, LR, and BR (Figure S1). This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS:

BR, Bacteriorhodopsin; LR, *Leptosphaeria* rhodopsin; NR, *Neurospora* rhodopsin; PhaeoRD2, *Phaeosphaeria* Rhodopsin 2; FTIR, Fourier transform infrared

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