Vibrational Frequency and Dipolar Orientation of the Protonated Schiff Base in Bacteriorhodopsin before and after Photoisomerization†

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ABSTRACT: Light-driven proton transport in bacteriorhodopsin (BR) is initiated by photoisomerization of the retinylidene chromophore, which perturbs the hydrogen bonding network in the Schiff base region of the active site. This study aimed to identify the frequency and dipolar orientation of the N−D stretching vibrations of the Schiff base before and after photoisomerization, by means of low-temperature polarized FTIR spectroscopy of [ζ-^{15}N]lysine-labeled BR in D_2O. ^{15}N-shifted modes were found at 2123 and 2173 cm^{-1} for BR, and at 2468 and 2495 cm^{-1} for the K intermediate. The corresponding N−H stretches are at ∼2800 cm^{-1} for BR and 3350−3310 cm^{-1} for the K intermediate. The shift to a 350 cm^{-1} higher frequency upon photoisomerization is consistent with loss of the hydrogen bond of the Schiff base. The N−D stretch frequencies of the Schiff base in BR and the K intermediate are close to the O−D stretch frequencies of strongly hydrogen bonded water and Thr89, respectively. The angles of the dipole moments of the N−D stretches to the membrane normal were determined to be 60−65° for BR and ∼90° for the K intermediate. In the case of BR, the stretch orientation is expected to deviate from the N−D bond orientation due to vibrational mixing in the hydrogen bonding network. In contrast, the data for the K intermediate suggest that the N−D group is not hydrogen bonded and orients along the membrane.

Bacteriorhodopsin (BR),[1] a membrane protein found in *Halobacterium salinarum*, functions as a light-driven proton pump with a retinylidene chromophore (*1*, *2*). Absorption of light by the all-trans form of the chromophore triggers a cyclic reaction that comprises a series of intermediates, designated as the J−O states. From the cytoplasmic side to the extracellular side, the proton transport pathway includes Asp96, the Schiff base between retinal and Lys216, Asp85, and Glu204 (or the Glu204−Glu194 region). Active transport is achieved by a sequence of proton transfers which must be well controlled spatially and temporally. However, previous mutant studies have shown that D96N and E204Q pump protons (*3*), indicating that terminal protonatable groups, such as Asp96 and Glu204, are not essential for the proton pump mechanism. These facts point to the importance of the Schiff base region as the "switch" in the pump function. The crucial role of the Schiff base region is also demonstrated by the conversion of BR into a chloride ion pump by replacement of a single amino acid at position 85 (*4*).

The Schiff base region has a quadrupolar structure with positive charges located at the protonated Schiff base and at Arg82, and counterbalancing negative charges located at Asp85 and Asp212. Along with several water molecules, these groups participate in an extended hydrogen bonding network (*5*, *6*). High-resolution X-ray crystallographic structures of BR crystals show the details of this network (Figure 1) (*7*, *8*). A notable feature is that the two negatively charged carboxylates, Asp85 and Asp212, are located similar distances from the retinal Schiff base. It is thus an interesting and important question why the Schiff base proton is transferred only to Asp85. The structures also show that the Schiff base is connected to Asp85 and Asp212 through hydrogen bonds of a water molecule, water402. In each case, the aspartate oxygen is also hydrogen bonded to another water molecule, water401 and water406 (Figure 1). As a result, the Schiff base region contains a roughly planar pentagonal cluster, composed of three water molecules (water401, -402, and -406) and one oxygen each of Asp85 and Asp212. There are additional hydrogen bonds for the other oxygen of each of the aspartates; one is between Asp85 and the O−H group of Thr89, while the other is between Asp212 and the O−H group of Tyr185. Water406 is also hydrogen bonded to one of the terminal nitrogens of the Arg82 side chain. Structural changes of the hydrogen bonding network must be related to the functional processes of BR.

X-ray diffraction studies of BR crystals have focused on photointermediates such as K (*9*), L (*10*), and M (*11*−*13*). However, these have generated some discrepancies with other techniques. For instance, the structure proposed for the K intermediate (*9*) is inconsistent with FTIR spectroscopic data regarding the interaction between Thr89 and Asp85 (*14*, *15*) and the assumption of an undistorted chromophore confor-

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[1] Abbreviations: BR, bacteriorhodopsin; FTIR, Fourier transform infrared; HOOP, hydrogen out-of-plane.

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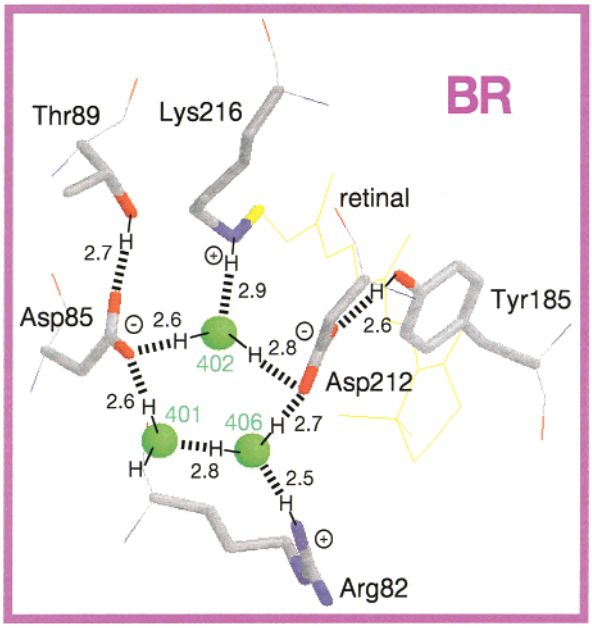

FIGURE 1: Diffraction structure of the Schiff base region in BR from PDB entry 1C3W (*8*). The membrane normal is approximately in the vertical direction of this figure. Upper and lower regions correspond to the cytoplasmic and extracellular sides, respectively. Green spheres (401, 402, and 406) represent water molecules which form a roughly pentagonal cluster with an oxygen from D85 and an oxygen from D212.

mation (*16*, *17*). In the study of the L state (*10*), the visible spectrum suggests the presence of other photocycle intermediates (*18*). Doubts have also been raised about diffraction studies of the M intermediate in view of the changes in the cytoplasmic domain expected from electron microscopy work (*19*). Furthermore, crystallographic structures of the M intermediate (*11*−*13*) are inconsistent with the specific interaction of Thr89 detected by low-temperature FTIR spectroscopy (*20*). One of the concerns regarding the diffraction studies is whether the non-native lipid and crystal packing may affect protein function. In addition, X-ray diffraction is limited in its ability to locate hydrogen atoms. Since active transport depends on the details of proton transfer, information about the structure of hydrogen bonds in the various photocycle intermediates is required for a better understanding of the pump mechanism.

Vibrational analysis is a powerful tool for investigation of hydrogen bonds. In particular, the O−H and N−H stretching modes are good probes. Polarized measurements of oriented BR have the advantage that they can characterize not only the frequencies of vibration, but also their dipolar orientations. In the past, we have applied low-temperature polarized FTIR spectroscopy to oriented BR, and successfully obtained K minus BR difference spectra throughout the whole midinfrared region (4000−700 cm^{-1}) (*21*). Furthermore, studies of isotope-labeled and mutant BR led to identification of specific vibrational bands. In particular, by using [3-^{18}O]threonine-labeled BR and mutating individual threonine residues, we detected changes in threonine hydrogen bonds which included Thr89 in the Schiff base region (Figure 1) (*14*). Further analysis of the L and M intermediates indicated continuing interaction between Thr89 and Asp85, suggesting that the Thr89−Asp85 region is not involved in the switch that enforces unidirectional proton transport (*20*). Although the observation of water stretching vibrations was initially limited to weak hydrogen bonds (*5*), recent accurate spectral comparison between samples in D_2O and $D_2{}^{18}O$ has extended observations to water molecules with strong hydrogen bonds (*22*). In these studies, changes in the dipolar orientation suggested rotational motion of a water molecule associated with a negative charge (*22*).

Among the various hydrogen bonds, one of the most interesting is the one between the Schiff base and water402 (Figure 1), which is presumably broken or distorted when the chromophore isomerizes. Therefore, information about the hydrogen bonding of the Schiff base is important. So far, the hydrogen bonding strength of the Schiff base has been estimated from its C=N stretching mode in vibrational analysis. In BR, the C=N stretch of the Schiff base has been assigned to 1641 cm^{-1} in H_2O and to 1628 cm^{-1} in D_2O (*16*). Because the upshift in H_2O is caused by coupling of the N−H bending vibration of the Schiff base, the difference in frequency between H_2O and D_2O has been regarded as the measure of the hydrogen bonding strength of the protonated Schiff base (*16*). The relatively small difference (13 cm^{-1}) in BR has been taken as an indication of weak hydrogen bonding of the Schiff base, which is consistent with solid-state NMR data (*23*). In the K intermediate, the C=N stretch of the Schiff base has been assigned to 1609 cm^{-1} in H_2O and to 1605 cm^{-1} in D_2O, and this very small difference has been taken as an indication of cleavage of the hydrogen bond upon photoisomerization (*16*).

In the study presented here, we attempted to assign the N−H (N−D) stretching vibration of the protonated Schiff base in BR and the K intermediate. To this end, low-temperature polarized FTIR spectroscopy was applied to highly oriented hydrated films of [ζ-^{15}N]lysine-labeled and unlabeled BR in D_2O. Highly accurate spectral comparison eventually led to identification of the frequency and dipolar orientation of the N−D stretching vibrations of the Schiff base. Structure and structural changes of the local environment of the Schiff base are discussed in light of these results.

MATERIALS AND METHODS

[ζ-^{15}N]Lys-labeled BR was prepared as described previously (*24*). A 120 μL aliquot of the sample in 2 mM phosphate buffer (pH 7) was dried on a BaF_2 window with a diameter of 18 mm. After hydration by 1 μL of D_2O, the sample was placed in a cell and then mounted in an Oxford DN-1704 cryostat, oriented at various tilt angles (0°, 17.8°, 35.7°, and 53.5°) in an FTIR spectrometer (Bio-Rad; FTS-40). The film was illuminated with >500 nm light for 1 min at 273 K to obtain the light-adapted state of BR.

Illumination with 501 nm light [using a Toshiba interference filter with a full width at half-maximum of 4 nm (*25*)] at 77 K for 2 min converted BR to the K intermediate. Since the K intermediate completely reverted to BR upon illumination with >660 nm light for 1 min, as evidenced by the same but inverted spectral shape, the cycles of alternative illuminations with a 501 nm light and a >660 nm light were repeated a number of times. The difference spectrum was calculated from the spectra constructed with 128 interferograms before and after the illumination. Twenty-four spectra obtained in this way were averaged for each K minus BR spectrum under various conditions. The details of the polarized FTIR

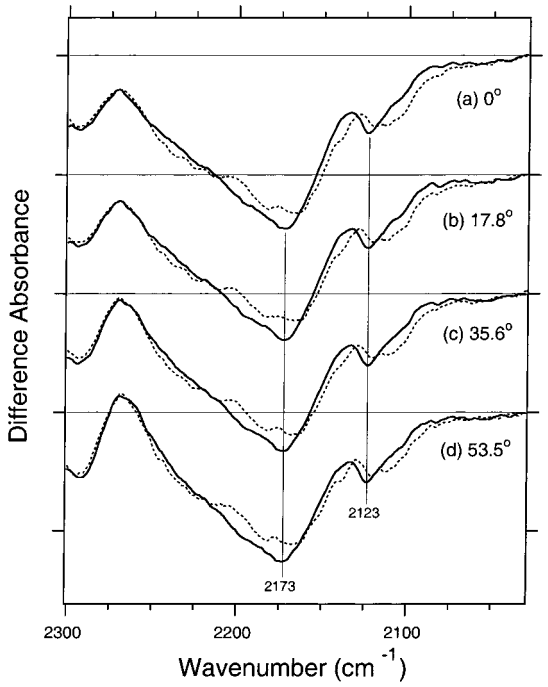

FIGURE 2: K minus BR difference infrared spectra of unlabeled (—) and [ζ-^{15}N]Lys-labeled (•••) BR in the 2300−2030 cm^{-1} region. The sample was hydrated with D_2O, and spectra were measured at 77 K. The window tilting angles are 0° (a), 17.8° (b), 35.7° (c), and 53.5° (d). One division of the *Y*-axis corresponds to 0.001 absorbance unit, and the horizontal straight lines represent zero lines.

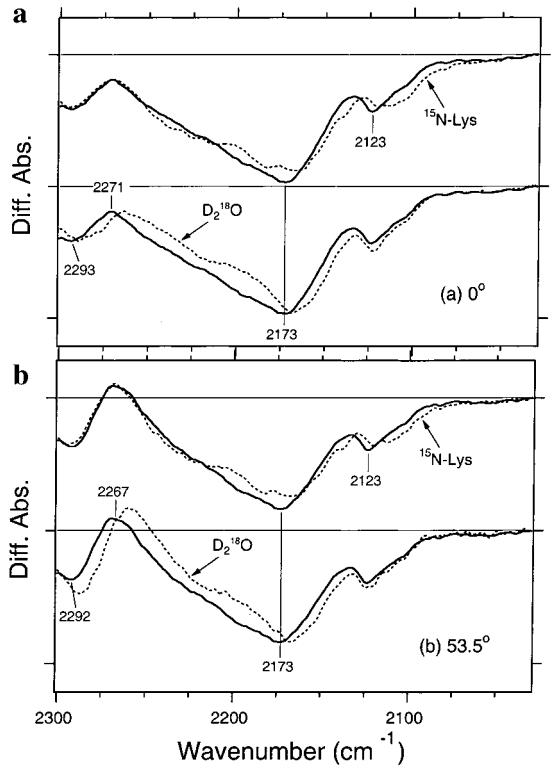

FIGURE 3: K minus BR difference infrared spectra in the 2300−2030 cm^{-1} region with window tilting angles of 0° (a) and 53.5° (b). The sample was hydrated with D_2O, and the spectra were measured at 77 K. The solid traces represent the spectra of unlabeled BR in D_2O. The top dotted traces are the spectra of [ζ-^{15}N]Lys-labeled BR in D_2O, while the bottom dotted traces are those of unlabeled BR in $D_2{}^{18}O$ reproduced from ref *19*. One division of the *Y*-axis corresponds to 0.0015 absorbance unit, and the horizontal straight lines represent zero lines.

spectroscopy are described elsewhere (*21*, *26*), including the derivation of the orientation of the vibrational dipole moment from the dependence of the measured dichroic ratio on the window tilt angle (*21*).

RESULTS

Assignment and Orientation of the N−D Stretching Vibration of the Schiff Base in BR. Since the Schiff base proton is deuterated in D_2O, the N−D stretching vibration is expected to appear in the 2700−2000 cm^{-1} region (*21*). In this range, an isotope-induced spectral shift of BR (the negative side of the difference spectra) was only observed between 2200 and 2100 cm^{-1}. With a focus on the 2300−2030 cm^{-1} region, Figure 2 shows the K minus BR difference infrared spectra of unlabeled and [ζ-^{15}N]Lys-labeled BR in D_2O. The negative band at 2123 cm^{-1} is downshifted to 2114 cm^{-1} in [ζ-^{15}N]-Lys-labeled BR, indicating that the band originates from the N−D stretching vibration of the Schiff base in BR. The 9 cm^{-1} shift is consistent with the expected effect of ^{15}N. The intensity of the negative 2123 cm^{-1} (—) and 2114 cm^{-1} (•••) bands is slightly reduced upon tilting the window, indicating that the angle between the dipole moment of the N−D stretching vibration and the membrane normal is greater than the magic angle (54.7°). We estimate the angle of the 2123 cm^{-1} band to be 60°.

In addition to the negative 2123 cm^{-1} band, there is also a reproducible spectral difference for the broad negative feature whose peak frequency is 2173 cm^{-1} (Figure 2). Part of this feature is attenuated and appears to be shifted to a lower frequency. Thus, it is suggested that the N−D stretch of the Schiff base in BR is also contained in the negative 2173 m^{-1} band. Previous comparison between spectra in D_2O and $D_2{}^{18}O$ indicated that the negative 2173 cm^{-1} band contains a water O−D stretching vibration, whereas the negative 2123 cm^{-1} band does not originate from water (*22*). The very low frequency (2173 cm^{-1}) relative to that of fully hydrated tetrahedral water indicates that the water is associated with a negative charge. It is noted that the water band at 2173 cm^{-1} may be shifted by introduction of ^{15}N at the Schiff base position, because the Schiff base interacts with water402, which in turn interacts with the negatively charged Asp85 and Asp212 (Figure 1). Such an isotope effect through a hydrogen bond has previously been observed between Thr89 and Asp85 (*20*). Thus, we next compared the isotope-induced spectral shift for the Schiff base and waters.

Figure 3 compares the K minus BR difference spectra of the unlabeled BR in D_2O with [ζ-^{15}N]Lys-labeled BR in D_2O (top trace) and with the unlabeled BR in $D_2{}^{18}O$ (bottom trace). In $D_2{}^{18}O$, most of the difference spectrum in the 2300−2030 cm^{-1} region is shifted except for the 2123 cm^{-1} band. Thus, we previously concluded that the peaks at 2292 (−), 2267 (+), and 2173 (−) cm^{-1} originate from O−D stretches of water molecules (*22*). It is noteworthy that the spectral feature at 2200−2150 cm^{-1} for [ζ-^{15}N]Lys-labeled BR in D_2O (top dotted lines) is different from that for unlabeled BR in $D_2{}^{18}O$ (bottom dotted lines). This indicates that the negative 2173 cm^{-1} band includes the N−D stretch of the Schiff base in addition to water vibrations. Since the spectral components are highly mixed, it is not easy to estimate the dipolar orientation of the N−D stretch at ∼2173 cm^{-1}. However, because the intensities of the shifted and

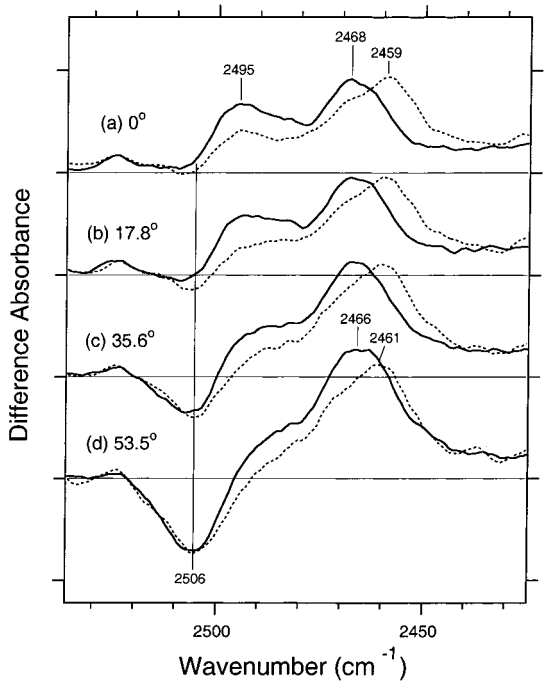

FIGURE 4: K minus BR difference infrared spectra of unlabeled (—) and [ζ-^{15}N]Lys-labeled (•••) BR in the 2535−2425 cm^{-1} region. The sample was hydrated with D_2O, and the spectra were measured at 77 K. The window tilting angles are 0° (a), 17.8° (b), 35.7° (c), and 53.5° (d). One division of the *Y*-axis corresponds to 0.001 absorbance unit, and the horizontal straight lines represent zero lines.

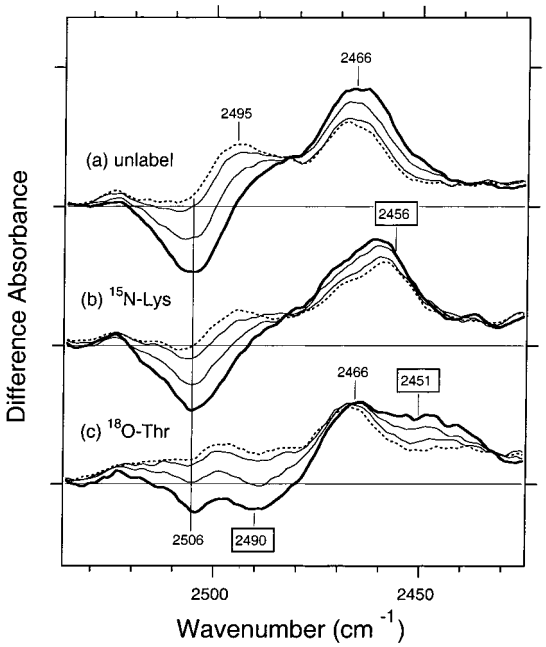

FIGURE 5: K minus BR difference infrared spectra of unlabeled (a), [ζ-^{15}N]Lys-labeled (b), and [3-^{18}O]Thr-labeled (c) BR in the 2535−2425 cm^{-1} region. The sample was hydrated with D_2O, and the spectra were measured at 77 K. The window tilting angles are 0° (dotted traces), 17.8° (thin solid traces close to the dotted traces), 35.7° (thin solid traces close to the thick solid traces), and 53.5° (thick solid traces). The spectra in part c are reproduced from ref *14*. One division of the *Y*-axis corresponds to 0.0015 absorbance unit, and the horizontal straight lines represent zero lines. Frequencies in the boxes are those expected according to the isotope shifts.

unshifted bands vary similarly upon tilting of the sample window, we infer that the orientations of the dipole moments of the Schiff base N−D and water O−D stretches at ∼2173 cm^{-1} relative to the membrane normal are both close to 65°.

Assignment and Orientation of the N−D Stretching Vibration of the Schiff Base in the K Photointermediate. For the K intermediate (positive side of the difference spectra), isotope-induced spectral shifts were only observed between 2500 and 2450 cm^{-1}. With a focus on the 2535−2425 cm^{-1} region, Figure 4 shows the K minus BR difference infrared spectra of unlabeled and [ζ-^{15}N]Lys-labeled BR in D_2O. The positive band at 2468 cm^{-1} for the untilted sample (Figure 4a) exhibits a clear shift to 2459 cm^{-1} for [ζ-^{15}N]Lys-labeled BR, indicating that the band originates from the N−D stretching vibration of the Schiff base in the K intermediate. The 9 cm^{-1} shift is consistent with the expected isotope shift (10 cm^{-1}) due to ^{15}N. When the window is tilted, the intensity of the positive 2468 cm^{-1} band significantly increases, and the isotope shift becomes less clear. In fact, at 53.5° (Figure 4d), the peaks are at 2466 cm^{-1} for the unlabeled BR (—) and at 2461 cm^{-1} for [ζ-^{15}N]Lys-labeled BR (•••). In previous work, we identified the O−D stretch of Thr89 at 2466 cm^{-1} in the K intermediate, while the corresponding vibration in BR was located at 2506 cm^{-1} (*14*). No isotope shift was observed for the 2506 cm^{-1} band in [ζ-^{15}N]Lys-labeled BR (Figure 4). However, we conclude that the positive 2466 cm^{-1} band contains the N−D stretch of the Schiff base in addition to the O−D stretch of Thr89.

Figure 4 also shows a reproducible spectral difference between unlabeled and [ζ-^{15}N]Lys-labeled BR in the 2500−2480 cm^{-1} region. However, a clear isotope shift was not observed for the 2495 cm^{-1} band (Figure 4a). When the window is tilted, the positive peak at 2495 cm^{-1} disappears because of the intense negative band at 2506 cm^{-1} due to the O−D stretch of Thr89. Since there are spectral differences due to the isotope effect in the 2500−2480 cm^{-1} region at all tilting angles in Figure 4, the positive 2495 cm^{-1} band is likely to contain the N−D stretch of the Schiff base.

Figure 5 compares the K minus BR spectra of unlabeled (a), [ζ-^{15}N]Lys-labeled (b), and [3-^{18}O]Thr-labeled (c) BR. No spectral alterations were observed for the negative 2506 cm^{-1} band between unlabeled (a) and [ζ-^{15}N]Lys-labeled (b) BR, nor was a clear isotope shift visible for the positive 2495 cm^{-1} band (Figure 4). However, a clear isotope shift due to ^{15}N occurs for the positive 2466 cm^{-1} band (Figure 5a,b), somewhat masked by the dichroic O−D stretch of Thr89. In fact, the dichroic 2466 cm^{-1} (+) band of the K intermediate (Figure 5a) exhibits the expected isotope shift by 15 cm^{-1} in [3-^{18}O]Thr-labeled BR (Figure 5c). The highly dichroic 2506 cm^{-1} (−) band of BR (Figure 5a) also exhibits the expected isotope shift of 16 cm^{-1} in [3-^{18}O]Thr-labeled BR (Figure 5c), with residual unshifted intensity due to incomplete labeling of the threonine residues (*14*).

It is noted that the amplitude of the O−D stretch of Thr89 is much greater than that of the N−D stretch of the Schiff base, though they each represent a single vibration. This can be explained in terms of hydrogen bonding strength. In general, the IR signal is intensified (and lowered in frequency) for stronger hydrogen bonds. The O−D stretch of Thr89 at 2466 cm^{-1} is lower than that for a neat secondary alcohol, indicating an "unusually" strong hydrogen bond (*14*). In contrast, the frequency of the N−D stretch of the Schiff base in K is relatively high, consistent with even weaker

hydrogen-bonding than in BR. This accounts for the relatively weak intensity of the band.

From the residual unshifted positive band at ~2466 cm^{-1} in Figure 5c, we are able to estimate the dipolar orientation of the N−D stretch of the Schiff base in the K intermediate if we take into account that there is still a contribution from the O−D stretch of Thr89 due to incomplete ^{18}O labeling. Overall, there is little change in the intensity of the 2466 cm^{-1} band in Figure 5c upon tilting of the window. However, since the O−D contribution increases with tilting (as can be seen from the shifted band at 2451 cm^{-1}), the N−D contribution must be decreasing with tilting. Thus, the dipolar orientation of the 2466 cm^{-1} N−D stretch is greater than the magic angle (54.7°). The angle was estimated to be close to 90°. The decline of the intensity of the 2495 cm^{-1} band with tilting (see also Figure 4) indicates that it too has a dipolar orientation greater than the magic angle. Thus, we conclude that that N−D stretches of the Schiff base in the K intermediate are located at 2468 and 2495 cm^{-1} and that, in both cases, the dipolar orientation is ~90° to the membrane normal.

DISCUSSION

The present polarized FTIR spectroscopy identified the frequencies and dipolar orientations of the N−D stretching vibrations of the Schiff base in BR and the K intermediate at 77 K (Table 1). The frequencies were observed to be 2123 and 2173 cm^{-1} for BR and 2468 and 2495 cm^{-1} for the K intermediate. Observation of two frequencies in each state may originate from structural heterogeneity or vibrational coupling. The corresponding N−H stretches are estimated to be ~2800 cm^{-1} for BR and ~3350−3310 cm^{-1} for the K intermediate on the basis of the previous spectral comparison (*21*). Dipolar orientations of the N−D stretches were approximately parallel to the membrane for both BR (60−65° to the membrane normal) and the K intermediate (~90° to the membrane normal).

Frequencies in stretching vibrations are generally lowered when hydrogen bonds are strengthened. Therefore, the shift to a higher frequency upon K formation by ~350 cm^{-1} as the N−D stretch (~500 cm^{-1} as the N−H stretch) indicates a weakened hydrogen bond accompanying photoisomerization. This observation is consistent with the previous vibrational analysis for the C=N stretching mode, where the difference in frequency between observations in H_2O and D_2O has been regarded as the marker of the hydrogen bonding strength of the Schiff base (*16*). The difference in the C=N frequency is 13 cm^{-1} for BR and ~4 cm^{-1} for the K intermediate (*16*). The latter has been taken as an indication of cleavage of the hydrogen bond upon photoisomerization. This FTIR analysis provides the same conclusion by use of the more direct vibrational mode, the N−H (N−D) stretch.

Local Environment of the Protonated Schiff Base in BR. This FTIR study determined the dipolar orientation of the N−D stretch as 60−65° to the membrane normal. This observation seems inconsistent with the X-ray structure. Figure 1 shows a distance of 2.9 Å between the Schiff base nitrogen and the oxygen of water402, with an angle between the N−O vector and the membrane normal of 22° (*8*). According to the structure determined by Belrhali et al. (*7*), the angle is 24°. We infer that the much larger dipolar angle observed for the N−D stretch reflects coupling through hydrogen bonds.

Table 1: Frequencies and Dipolar Orientations of the X−D Vibrations in the Schiff Base Region of Bacteriorhodopsin

	BR		K	
	frequency (cm^{-1})	angle (deg)	frequency (cm^{-1})	angle (deg)
N−D stretch of the Schiff base[a]	2123	~60	2468	~90
	2173	~65	2495	~90
O−D stretch of water[b]	2173	~65	2267	0−20
(strongly H-bonded)	2292	~55	2359	~50
O−D stretch of Thr89[c]	2506	21	2466	29

[a] From this work. [b] From ref *22*. [c] From ref *14*.

In fact, recent ab initio quantum mechanical/molecular mechanical (QM/MM) calculation by Hayashi and Ohmine (*27*) showed that the orientation of the N−D stretch of the Schiff base is highly sensitive to the vibration−electron coupling in the pentagonal cluster structure (Figure 1), resulting in a significant deviation from the orientation of the N−D bond. Their calculated angle for the stretch orientation (52°) (*27*) is close to the angle observed here (60−65°). Thus, it is likely that strong intermolecular vibrational mixing due to electron−vibration interaction is present. This is consistent with our observation that the higher N−D stretch of the Schiff base in BR (2173 cm^{-1}) appears at the frequency of the water O−D stretch (Figure 3) and the lower N−D stretch of the Schiff base at 2123 cm^{-1} is nearby. Thus, the present vibrational analysis, as well as the theoretical calculation (*27*), indicates a highly coupled vibrational feature in the pentagonal cluster structure of the Schiff base region, which may be related to the specific architecture for the function of BR.

With regard to the N−H (or N−D) stretching vibrations of the Schiff base in rhodopsins, there have been no assignments so far. Nagata et al. observed a bilobe spectral feature at 3040−2880 (+) and 2880−2740 (−) cm^{-1} in the bathorhodopsin minus rhodopsin spectrum of the E113Q mutant protein of bovine rhodopsin (*28*). Though not yet assigned, the origin is most likely the N−H stretch of the Schiff base. In fact, the feature was downshifted to 2240−2200 (+) and 2200−2140 (−) cm^{-1} in D_2O. The latter is close to the N−D stretch of BR assigned in this study.

Local Environment of the Protonated Schiff Base in the K Photointermediate. This FTIR study determined the dipolar orientation of the N−D stretch of the K intermediate to be ~90° to the membrane normal. As for BR, we need to consider possible complications due to vibrational coupling. However, the higher-frequency shift of the N−D stretch by ~350 cm^{-1} suggests the lack of a hydrogen bond in the K state. The lack of the hydrogen bond is also supported by the small frequency difference between C=NH and C=ND stretches of the Schiff base (*16*). Thus, we infer that vibrational coupling through intermolecular modes in the K intermediate is much less extensive than in BR, and the dipolar orientation of the K intermediate (~90°) is closer to the actual bond than in BR.

Orientation of the N−D group of the Schiff base in the membrane plane requires local distortion of the chromophore. Vibrational analysis of the hydrogen out-of-plane (HOOP) modes of the retinal chromophore has revealed that the

chromophore is distorted around the Schiff base region in the K intermediate (*16*). In particular, a twist around the C15═N bond in the K state was suggested by the previous low-temperature FTIR spectroscopy (*17*). Thus, a considerable part of the light energy seems to be stored in chromophore distortion in the Schiff base region, as well as in hydrogen bonding alterations. Recent comparative IR studies of BR and *pharaonis* phoborhodopsin (*p*pR) indicate that the chromophore distortion around the Schiff base is unique for BR (*29*).

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REFERENCES

1. Haupts, U., Tittor, J., and Oesterhelt, D. (1999) *Annu. Rev. Biophys. Biomol. Struct. 28*, 367−399.
2. Lanyi, J. K. (2000) *J. Phys. Chem. B 104*, 11441−11448.
3. Krebs, M. P., and Khorana, H. G. (1993) *J. Bacteriol. 175*, 1555−1560.
4. Sasaki, J., Brown, L. S., Chon, Y.-S., Kandori, H., Maeda, A., Needleman, R., and Lanyi, J. K. (1995) *Science 269*, 73−75.
5. Kandori, H. (2000) *Biochim. Biophys. Acta 1460*, 176−190.
6. de Groot, H. J., Smith, S. O., Courtin, J., van den Berg, E., Winkel, C., Lugtenburg, J., Griffin, R. G., and Herzfeld, J. (1990) *Biochemistry 29*, 6873−6883.
7. Belrhali, H., Nollert, P., Royant, A., Menzel, C., Rosenbusch, J. P., Landau, E. M., and Pebay-Peyroula, E. (1999) *Struct. Folding Des. 15*, 909−917.
8. Luecke, H., Schobert, B., Richter, H.-T., Cartailler, J. P., and Lanyi, J. K. (1999) *J. Mol. Biol. 291*, 899−911.
9. Edman, K., Nollert, P., Royant, A., Belrhali, H., Pebay-Peyroula, E., Hajdu, J., Neutze, R., and Landau, E. M. (1999) *Nature 401*, 822−826.
10. Royant, A., Edman, K., Ursby, T., Pebay-Peyroula, E., Landau, E. M., and Neutze, R. (2000) *Nature 406*, 645−648.
11. Luecke, H., Schobert, B., Richter, H.-T., Cartailler, J. P., and Lanyi, J. K. (1999) *Science 286*, 255−261.
12. Luecke, H., Schobert, B., Cartailler, J.-P., Richter, H.-T., Rosengarth, A., Needleman, R., and Lanyi, J. K. (2000) *J. Mol. Biol. 300*, 1237−1255.
13. Sass, H. J., Büldt, G., Gessenich, R., Hehn, D., Neff, D., Schlesinger, R., Berendzen, J., and Ormos, P. (2000) *Nature 406*, 649−653.
14. Kandori, H., Kinoshita, N., Yamazaki, Y., Maeda, A., Shichida, Y., Needleman, R., Lanyi, J. K., Bizounok, M., Herzfeld, J., Raap, J., and Lugtenburg, J. (1999) *Biochemistry 38*, 9676−9683.
15. Kandori, H., Kinoshita, N., Yamazaki, Y., Maeda, A., Shichida, Y., Needleman, R., Lanyi, J. K., Bizounok, M., Herzfeld, J., Raap, J., and Lugtenburg, J. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 4643−4648.
16. Maeda, A. (1995) *Isr. J. Chem. 35*, 387−400.
17. Gat, Y., Grossjean, M., Pinevsky, I., Takei, H., Rothman, Z., Sigrist, H., Lewis, A., and Sheves, M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2434−2438.
18. Balashov, S. P., and Ebrey, T. G. (2001) *Photochem. Photobiol. 73*, 453−462.
19. Subramaniam, S., and Henderson, R. (2000) *Nature 406*, 653−657.
20. Kandori, H., Yamazaki, Y., Shichida, Y., Raap, J., Lugtenburg, J., Belenky, M., and Herzfeld, J. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 1571−1576.
21. Kandori, H., Kinoshita, N., Maeda, A., and Shichida, Y. (1998) *J. Phys. Chem. B 102*, 7899−7905.
22. Kandori, H., and Shichida, Y. (2000) *J. Am. Chem. Soc. 122*, 11745−11746.
23. de Groot, H. J. M., Harbison, G. S., Herzfeld, J., and Griffin, R. G. (1989) *Biochemistry 28*, 3346−3353.
24. Hu, J. G., Sun, B. Q., Bizounok, M., Hatcher, M. E., Lansing, J. C., Raap, J., Verdegem, P. J. E., Lugtenburg, J., Griffin, R. G., and Herzfeld, J. (1998) *Biochemistry 37*, 8088−8096.
25. Kandori, H., and Maeda, A. (1995) *Biochemistry 34*, 14220−14229.
26. Kandori, H. (1998) *J. Am. Chem. Soc. 120*, 4546−4547.
27. Hayashi, S., and Ohmine, I. (2000) *J. Phys. Chem. B 104*, 10678−10691.
28. Nagata, T., Terakita, A., Kandori, H., Kojima, D., Shichida, Y., and Maeda, A. (1997) *Biochemistry 36*, 6164−6170.
29. Kandori, H., Shimono, K., Sudo, Y., Iwamoto, M., Shichida, Y., and Kamo, N. (2001) *Biochemistry 40*, 9238−9246.

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