Polarized FTIR Spectroscopy Distinguishes Peptide Backbone Changes in the M and N Photointermediates of Bacteriorhodopsin

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Diffraction studies have detected significant conformation changes in the M and N photointermediates of bacteriorhodopsin. Fourier transform infrared (FTIR) difference spectra of the N state contain negative bands at 1669 and 1693 cm^{-1} and a positive band at 1649 cm^{-1} in the amide-I (C═O stretch vibration of the peptide backbone) region. With polarized FTIR spectroscopy we determined the angle of the dipoles of these bands to the membrane normal to be 55° at 1669 cm^{-1}, 30° at 1649 cm^{-1}, and 30° at 1693 cm^{-1}. In addition, two prominent peaks are newly observed, at 1671 (−) and 1663 cm^{-1} (+), whose dipole moments are nearly parallel to the membrane normal (<10°). In contrast, the spectral changes in the amide-I region are much less in M. The structural changes in peptide backbone thus presumably correspond to the larger displacement of the helices in N reported from diffraction studies.

Bacteriorhodopsin (BR) is a light-driven proton pump in the purple membrane of *Halobacterium salinarium*.[1] Illumination causes the isomerization of the retinal chromophore, which is followed by a series of thermal steps that result in intermediate states (J, K, KL, L, M, N, and O).[2] Diffraction studies have revealed global conformation changes at the cytoplasmic region in the M and N states, although greater change in N has been suggested recently.[3−6] The previous FTIR spectroscopy reported that two prominent peaks appear at 1669 (−) and 1649 (+) cm^{-1} in N, not in M.[7−10] Part of the negative band is due to the carbonyl of Tyr185.[11] Since IR spectra monitor local structure in the protein, however, the IR spectral changes do not necessarily correspond to the structural change in diffraction study. In particular, the orientation of the BR molecules was not sufficiently taken into account in previous FTIR studies.

It is well-known that polarized FTIR spectroscopy of the oriented BR film can determine particular angles of the dipole moment of the vibration in question, in addition to its frequency.[12−14] In fact, previous studies determined the angle of dipole moments of vibrations in retinal,[12−14] carboxylic C═O stretch,[12−14] and water O−H stretch[14] in K, L, and M, but not the amide-I band. In the present study, we applied polarized FTIR spectroscopy to highly oriented BR films to investigate the change in peptide backbone in M and N.

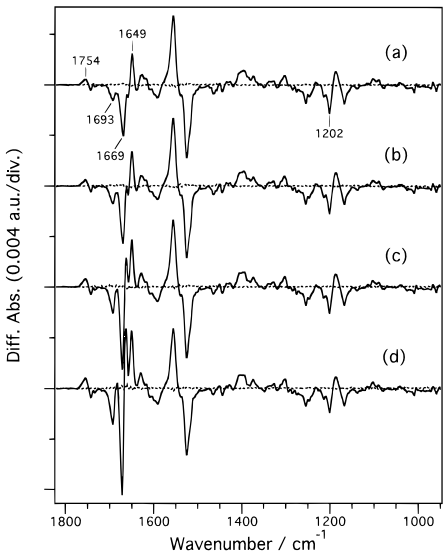

Figure 1. IR difference spectra after and before illumination of bacteriorhodopsin at 273 K (solid lines) with tilting of the sample window by 0° (a), 17.8° (b), 35.7° (c) and 53.5° (d). The photoproduct comprises 85% N and 15% M, as judged from the C═O stretch of Asp85 at 1754 cm^{-1} in N and 1762 cm^{-1} in M. Dotted lines represent the baseline, where the same measurement as the solid line was conducted without illumination.

Polarized FTIR spectroscopy was performed on a hydrated BR film at pH 10 as described previously.[14] Figure 1 shows difference spectra after and before illumination with >500 nm light at 273 K. The spectrum at 0° (Figure 1a) looks similar to the reported N minus BR spectrum, exhibiting sharp peaks at 1669 (−) and 1649 (+) cm^{-1}. From the C═O stretching frequency of Asp85, the products are estimated to be 85% N and 15% M. When the BR film is tilted prominent spectral changes appear in the amide-I region. Two negative bands increase their intensities and a new positive band appears. These bands did not show shift of frequency upon hydration with D_2O, thus being indicative of C═O stretching vibrations of the peptide backbone (amide-I).[15] These spectral features in the amide-I region are greatly different from the M minus BR spectrum measured at 230 K (Figure 2).

To numerically compare the spectra of M and N, we subtracted the M minus BR spectra from those in Figure 1, and obtained the N minus BR spectra at various tilting angles. Figure 3 compares the M minus BR (a) and the N minus BR (b) spectra in the 1780−1590 cm^{-1} region. The M minus BR spectra were multiplied by 0.152 to normalize the negative 1202 cm^{-1} band, so that the same amount of BR molecule is converted to M (a) and N (b) in Figure 3.

The positive bands at 1762 and 1754 cm^{-1} are the C═O stretch vibrations of Asp85 in M and N, respectively, while the negative band at 1742 cm^{-1} is the C═O stretch of Asp96. Since the

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(15) Other vibrations such as the C═N stretch of arginine also could be present.

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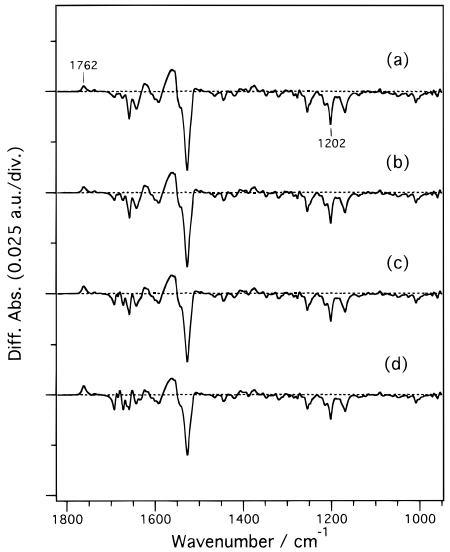

Figure 2. IR difference spectra after and before illumination of bacteriorhodopsin at 230 K (solid lines) with the tilting of the sample window by 0° (a), 17.8° (b), 35.7° (c), and 53.5° (d), which correspond to the M minus BR spectra. Dotted lines represent the baseline, where the same measurement as the solid line was conducted without illumination.

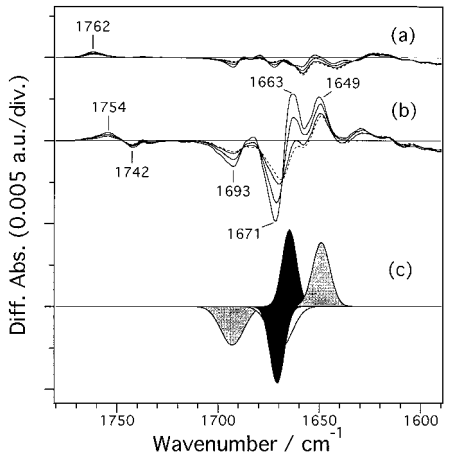

Figure 3. Spectral comparison of the M minus BR spectra (a) and the N minus BR spectra (b), which are normalized at 1202 cm^{-1}. Five prominent peaks in the amide-I region of (b) are fitted by five Gaussian curves (peaks: 1693 (−), 1671 (−), 1669 (−), 1663 (+), and 1649 (+) cm^{-1}) and shown as the tilting angle is 90° (c).

amplitude of bands increases with tilting, the dipole moments of these C═O stretches lie parallel to the membrane normal. By assuming the parameters in previous studies (refractive index of 1.7, and the degree of orientation of the membrane of 0.95),[14] we obtained the angle of C═O groups of Asp85 in M, Asp85 in N, and Asp96 in BR to be 36 ± 1.0°, 32 ± 2.0°, and 34 ± 2.5° with respect to the membrane normal, respectively. On the other hand, it appears that the C═O group of Asp115 is oriented by 50−60° in both BR and N. The C═O stretch angles of aspartic acids that participate in proton translocation are therefore 32−36°, which might correlate with proton transfer in the hydrogen-bonding network of the protein. The constancy of the angle of the C═O of Asp85 implies that there are no significant changes in its position in the M-to-N transition.

Figure 3 shows large differences in the 1710−1640 cm^{-1} region between M and N. The spectral changes are 2.2- or 5.2-times greater in N than in M at 0° or 53.5°, respectively, in the 1710−1610 cm^{-1} region, as calculated from the areas. In the N minus BR spectrum, there are three peaks at 0° (dotted line): 1693 cm^{-1} (−), 1669 cm^{-1} (−), and 1649 cm^{-1} (+). When the BR film is tilted, the negative 1693 cm^{-1} and the positive 1649 cm^{-1} bands increase their intensities. In addition, a pair of sharp peaks appears in the 1670−1660 cm^{-1} region. Since four spectra in Figure 3b exhibit an isosbestic point at 1688 cm^{-1}, it is likely that the negative 1669 cm^{-1} band is insensitive to the tilting and the negative 1671-cm^{-1} band and the positive 1663 cm^{-1} band newly appear with tilting. The difference between 53.5° and 0° shows the two peak positions at 1672 and 1665 cm^{-1}.

These bands probably comprise multiple C═O stretches in the amide-I region, and the angles of the dipole moment of each C═O group cannot be determined uniquely. However, it would be meaningful to estimate the angles to the membrane normal. We estimate the angle of the 1693 (−) and 1649 cm^{-1} (+) bands to be about 30°, and that of the 1669 cm^{-1} band is close to the magic angle (54.7°). On the other hand, the newly visible peaks at 1671 (−) and 1663 cm^{-1} (+) possess angles of <10°.

Absorption change in the visible upon M and N formation suggested that 12% of BR is converted to N under the illumination conditions used. Thus, comparison of the integrated band intensity in the difference spectra with that of the absolute spectra at various tilting angles can lead to an estimation of the number of amino acids contributing to the spectral change. Taking into account the total number of amino acids of BR (248), we obtained the average number of residues that participate in the 1669 cm^{-1} band at the magic angle to be 4.2. Similarly, the 1693 and 1649 cm^{-1} bands correspond to 1.8 and 2.4 amino acids, respectively. The bilobe at 1671 (−) and 1663 cm^{-1} (+) that is parallel to the membrane normal corresponds to 2−3 amino acids, respectively. Thus, about 10 amino acids appear to display significant frequency change upon N formation.

The structure of BR[16−18] shows that most of the membrane embedded amino acids orient their peptide carbonyls parallel to the membrane normal. In fact, only three residues (Thr46, Leu87, Trp182) have their C═O groups at angles with the membrane normal greater than 45° in the membrane spanning region.[16] This may suggest that the amide-I bands of these residues are the ones at 1669 cm^{-1} and change their frequencies upon N formation. For this conclusion, their accurate identification will be necessary. Since the angle between the C═O group of Tyr185 and the membrane normal is ∼14°,[16] the signal identified by site-directed isotope labeling is attributable for the negative band at 1671 cm^{-1}, not at 1669 cm^{-1}.[11]

The polarized FTIR spectroscopy clearly shows that the spectral changes in amide-I are much greater in N than in M, and several C═O groups parallel to the membrane normal show shifts to lower frequencies in N (Figure 3), indicating stronger hydrogen bonding formation. Such local structural changes of the peptide backbone presumably allow the global movement of helices at the cytoplasmic side in N, as reported previously.[19] It should be noted that the correlation between negative and positive bands is at this time not unique. Conclusive identification of these peptide backbone changes will lead to better understanding of the conformation change of BR in the process of its proton pumping, which is our future focus.

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