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Fourier transform infrared difference spectroscopy of bacteriorhodopsin and its photoproducts*

(purple membrane/Schiff base/conformational changes/low temperature/hydrated films)

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ABSTRACT **Fourier transform infrared difference spectroscopy has been used to obtain the vibrational modes in the chromophore and apoprotein that change in intensity or position between light-adapted bacteriorhodopsin and the K and M intermediates in its photocycle and between dark-adapted and light-adapted bacteriorhodopsin. Our infrared measurements provide independent verification of resonance Raman results that in light-adapted bacteriorhodopsin the protein–chromophore linkage is a protonated Schiff base and in the M state the Schiff base is unprotonated. Although we cannot unambiguously identify the Schiff base stretching frequency in the K state, the most likely interpretation of deuterium shifts of the chromophore hydrogen out-of-plane vibrations is that the Schiff base in K is protonated. The intensity of the hydrogen out-of-plane vibrations in the K state compared with the intensities of those in light-adapted and dark-adapted bacteriorhodopsin shows that the conformation of the chromophore in K is considerably distorted. In addition, we find evidence that the conformation of the protein changes during the photocycle.**

Bacteriorhodopsin (bR) is the light-energy transducing protein found in the purple membrane (PM) of the extreme halophile *Halobacterium halobium* (1–4). The chromophore in bacteriorhodopsin is a single molecule of retinal, covalently bound to the ε-amino group of a lysine (Lys-216) via a Schiff base linkage (Fig. 1). Upon absorption of light, the light-adapted form of bR (bR^{LA}) undergoes a photocycle, $bR^{LA} \leftrightarrow K \rightarrow L \rightarrow M \rightarrow O \rightarrow bR^{LA}$, during which protons are pumped from the inside of the cell to the extracellular medium. The resulting proton gradient is used by the cell to generate chemical energy in the form of ATP and drive other energy-requiring processes. In the dark, bR^{LA} thermally converts to the dark-adapted form of bR (bR^{DA}).

The mechanism of this light-driven proton pump has been studied by using visible and ultraviolet, resonance Raman (5), and infrared (IR) (6–8) spectroscopies and chemical extraction techniques. These investigations strongly suggest that during the photocycle changes occur in both the isomeric state of the chromophore and the state of protonation of the Schiff base. In particular, chemical extraction experiments have provided evidence that the chromophore in bR^{LA} is in an *all-trans* configuration, that in the L and M states it is in a 13-*cis* configuration, and that in bR^{DA} the chromophore exists in two isomeric forms, *all-trans* and 13-*cis*, in a ratio of approximately 1:1 (9–11).

Evidence for the conformation of the chromophore *in situ* comes primarily from comparisons between the resonance Raman vibrational spectra in both ^1H_2O and ^2H_2O of native bR, bR in which analogs of retinal have been incorporated, and retinal Schiff bases. Analysis of the results from such work is difficult owing in part to the large perturbation of the chromophore by the protein environment (12). However, it is widely accepted that these studies have shown that in bR^{LA} *all-trans* retinal is bound to the protein via a protonated Schiff base linkage and that in M the chromophore is in the 13-*cis* conformation and the Schiff base linkage is unprotonated (13–19). For other intermediates in the photocycle there is not such a consensus on the interpretation of resonance Raman results, nor is there complete agreement among data obtained by different groups. Significant discrepancies between various resonance Raman data occur for the K intermediate, especially in the Schiff base region (16, 19, 20). These discrepancies must be resolved in order to understand the mechanism of the primary photochemical event ($bR \rightsquigarrow K$).

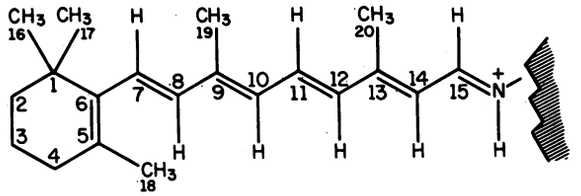

FIG. 1. *All-trans*-retinal bound to apoprotein via a protonated Schiff base linkage. In 15-deuterioretinal the hydrogen at carbon 15 is replaced by deuterium.

It has been hypothesized that the resonance Raman process itself disturbs the ground state of the system being investigated and therefore the vibrational modes measured by resonance Raman are not the same as would be detected by IR absorption spectroscopy. In particular, Sándorfy and co-workers (21) have proposed that the Schiff base nitrogen is not fully protonated, but rather is only hydrogen bonded, and that the proton is partially transferred to the Schiff base during the scattering process, giving rise to the apparent protonation.

Conventional IR absorption spectroscopy cannot be used to study the conformation of the chromophore because the vibrational modes of the chromophore are superimposed on the much stronger infrared absorption bands of H_2O and the protein backbone. To overcome this problem infrared difference spectroscopy is used, because only modes that change intensity or position during the photocycle will appear in the difference spectrum. Kinetic IR difference spectroscopy (8) has been ap-

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Abbreviations: bR, bacteriorhodopsin; bR^{LA}, light-adapted form of bR; bR^{DA}, dark-adapted form of bR; $bR^{13\text{-}cis}$, 13-*cis* component of bR^{DA}; FTIR, Fourier transform infrared; PM, purple membrane.

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plied to bacteriorhodopsin. The results in the Schiff base region could not be correlated with those obtained in resonance Raman experiments.

To help resolve some of these conflicts and to provide additional information on the changes in conformation of the protein as well as the chromophore that occur during the photocycle, we have applied Fourier transform IR (FTIR) difference spectroscopy to bR and some of the intermediates in its photocycle. After completing these studies, we became aware of studies carried out by Rothschild and co-workers using similar techniques (22, 23).

MATERIALS AND METHODS

The sample is prepared by drying PM on an IR and visible light transmitting window (IRTRAN 2; Kodak) and adding enough water to bring the films to full hydration. The photocycle in these wet films is similar to that of aqueous suspensions (24). Deuterated films were prepared by repeated drying and rehydration of the films with ^2H_2O. The absorbance of the films was approximately 0.6 at 570 nm. Measurements were made on hydrated or deuterated films of native bR and bR regenerated with 15-deuterioretinal. The regeneration was done by following standard procedures (12).

The films were vacuum sealed and mounted in a helium refrigerator (NaCl windows) that was placed in a Nicolet 7199 FTIR spectrometer, equipped with an HgCdTe detector. After being light-adapted at 300 K, the sample was cooled to a temperature at which the intermediate to be investigated is stable. An IR transmission spectrum of bR^{LA}, at 2-cm^{-1} resolution in the region 4,900–700 cm^{-1} was collected and stored (2,048 scans, collection time ≈30 min). Light from a projector with a narrow bandpass interference filter ($\Delta\lambda$ = 10 nm) was used to create a photostationary state containing bR^{LA} and a large amount of the relevant intermediate, and the IR transmission spectrum of this photostationary state was collected. The logarithmic difference of the two spectra was calculated. If the absorbance of the intermediate at a particular wavenumber is greater than that of bR^{LA}, the difference spectrum has a positive peak at that frequency. If the absorbance of bR^{LA} is greater, the difference spectrum has a negative peak. For the dark-adapted minus light-adapted experiment the sample was dark-adapted at 300 K and cooled to 250 K to reduce the water vapor pressure, and a spectrum was taken. The sample was then light-adapted and another spectrum was taken. Because the chromophore in bR^{DA} exists in two isomeric forms, *all-trans* and 13-*cis*, and bR^{LA} is *all-trans* (see above), the bR^{DA} − bR^{LA} difference spectrum gives the vibrational modes of bR containing the 13-*cis* isomer of the chromophore, $bR^{13\text{-}cis}$. As a control all experiments were repeated with the refrigerator in a Cary 14 visible spectrophotometer.

The protein bands above 2,000 cm^{-1} were obscured by ^1H_2O or ^2H_2O, therefore only data below 2,000 cm^{-1} will be presented in this paper. The IR vibrational frequencies given in this paper are those that occur in the difference spectra. Except where specifically mentioned, no corrections have been made for the distortion in the shape and location of peaks that occur when difference spectra are calculated, nor were the spectra smoothed.

RESULTS AND DISCUSSION

FTIR difference spectra between K and bR^{LA} are given in Fig. 2, between M and bR^{LA} in Fig. 3, and between bR^{LA} and bR^{DA} in Fig. 4. The chromophore vibrational modes that one would expect to appear in the infrared difference spectra are those due to the terminal group (C=N, $C{=}NH^+$ Schiff base) above 1,600

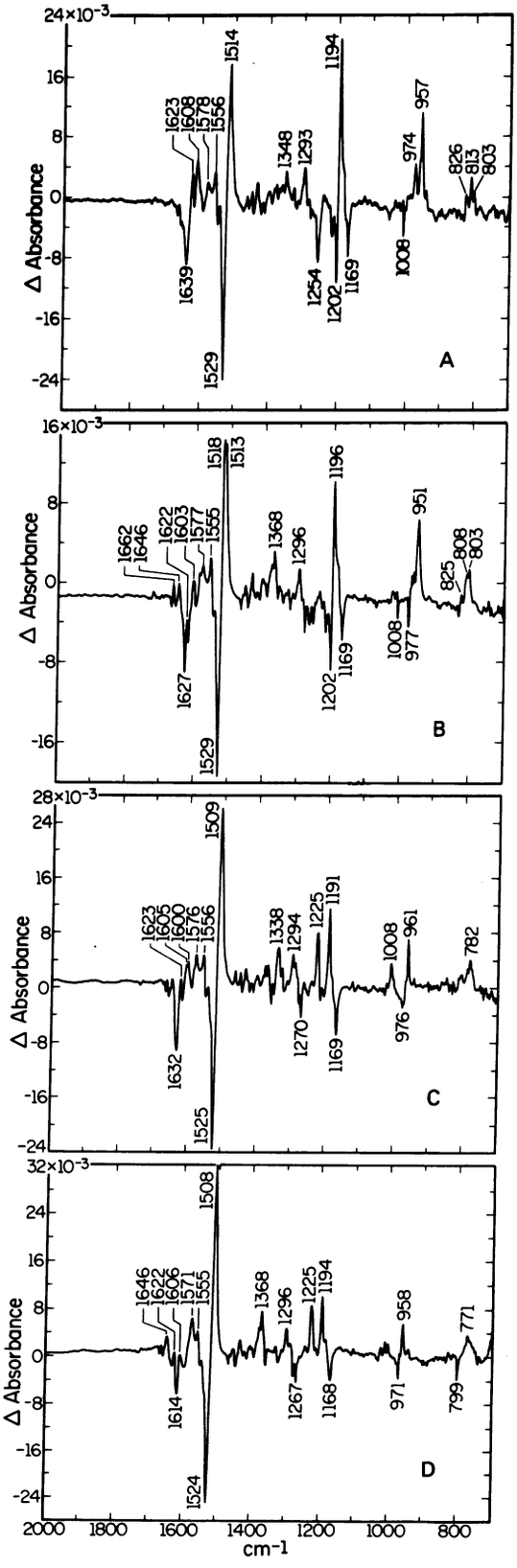

FIG. 2. IR difference spectra between K and bR^{LA}, K − bR^{LA}, at 70 K. (*A*) Hydrated film of native bR; (*B*) deuterated film of native bR; (*C*) hydrated film of bR regenerated with 15-deuterioretinal; (*D*) deuterated film of bR regenerated with 15-deuterioretinal. Light at 500 nm was used to create the photostationary state. Identical K − bR^{LA} difference spectra have also been obtained by using light at 640 nm to drive the photostationary state back to bR^{LA}. Resolution is 2 cm^{-1}.

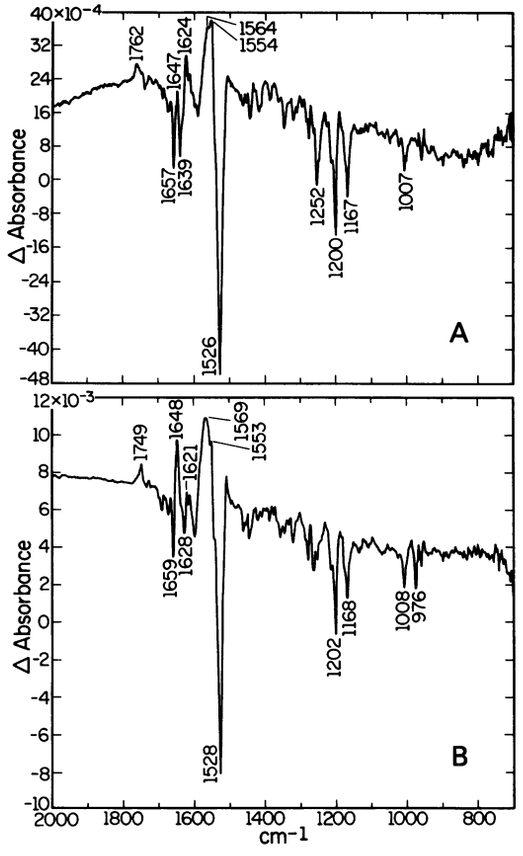

FIG. 3. IR difference spectra between M and bR^{LA}, M − bR^{LA}, at 210 K. (*A*) Hydrated film of native bR; (*B*) deuterated film of native bR. Light at 500 nm was used to create the photostationary state.

cm^{-1}, C═C stretching vibrations (ethylenic stretch) between 1,500 and 1,620 cm^{-1}, C—C stretches and C—C—H bends between 1,100 and 1,400 cm^{-1} (fingerprint region), methyl rocks around 1,000 cm^{-1}, and hydrogen out-of-plane vibrations between 750 and 1,050 cm^{-1} (18, 19, 25). The major protein vibrational modes that may appear in the infrared difference spectra are amide I (C═O ≈ 1,660 cm^{-1}) and amide II (C═N, N—H ≈ 1,550 cm^{-1}).

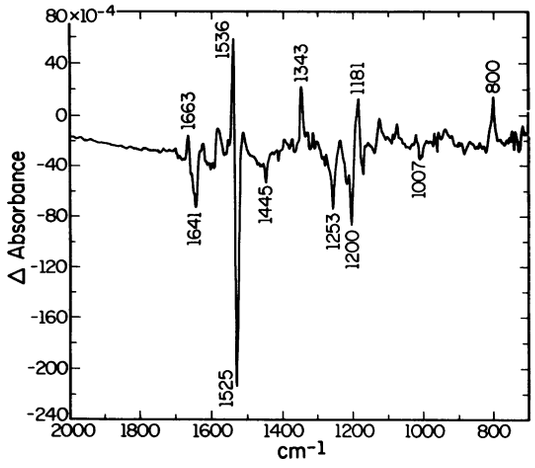

FIG. 4. IR difference spectrum between bR^{DA} and bR^{LA}, bR^{DA} − bR^{LA}, at 250 K in a hydrated film. The difference spectrum is equivalent to $bR^{13\text{-}cis}$ − $bR^{all\text{-}trans}$. Light at 560 nm was used to light adapt the sample.

Agreement Between Chromophore Vibrational Modes Obtained by IR and Resonance Raman. The IR difference spectra show that bR^{LA} and $bR^{13\text{-}cis}$, below 1,650 cm^{-1}, have absorption peaks at the same positions as observed in resonance Raman experiments (5, 13–20, 26–29). For K, the peaks below 1,550 cm^{-1} agree with those found by resonance Raman (16, 19, 20, 30); in particular the overall data for K presented in this communication are in good agreement with those obtained by Braiman and Mathies (20). For M, all peaks except those at 1,762, ≈1,650, and 1,555 cm^{-1} are observed in resonance Raman (13–15, 17–18). In addition, the major shifts in these lines on deuteration of the films (Fig. 2*A* vs. Fig. 2*B*, Fig. 3*A* vs. Fig. 3*B*) and when bR is reconstituted with 15-deuterioretinal (Fig. 2*A* vs. Fig. 2*C*) are consistent with those obtained in resonance Raman experiments. Because the two processes are governed by different selection rules and because resonance Raman only measures the chromophore vibrations and not those due to the apoprotein moiety, the two methods yield complementary data.

Protein–Chromophore Linkage in bR^{LA}. The IR difference spectra in Fig. 2 provide independent verification that in bR^{LA} the protein–chromophore linkage is a protonated Schiff base. Evidence that the 1,639-cm^{-1} line in bR^{LA} is associated with the Schiff base comes from the behavior of this line upon deuteration and substitution of the hydrogen at the carbon 15 position with deuterium. The shift from 1,639 cm^{-1} to 1,632 cm^{-1} in bR reconstituted with 15-deuterioretinal shows that this line is associated with the carbon atom at position 15. The fact that upon deuteration of the films this line shifts from 1,639 cm^{-1} to 1,627 cm^{-1} for native bR and from 1,632 cm^{-1} to 1,614 cm^{-1} for bR reconstituted with 15-deuterioretinal proves that this line is associated with exchangeable protons. The only known exchangeable proton in the chromophore is that associated with the Schiff base nitrogen. Similar shifts have been observed in resonance Raman experiments. The fact that the shifts that occur when the proton associated with the nitrogen is exchanged for a deuteron (12 cm^{-1} and 18 cm^{-1}) are much larger than the shifts that occur when the carbon at position 15 is deuterated (7 cm^{-1} and 13 cm^{-1}) shows that the Schiff base mode involves strong coupling of the C═N stretch with the C—N—H in-plane bend. This is in agreement with the calculations of Aton *et al.* (31) and Kakitani *et al.* (32). The agreement of our IR measurements in the Schiff base region with those of resonance Raman proves that the resonance Raman result is not an artifact of the scattering process.

Protein–Chromophore Linkage in K. There is controversy in the resonance Raman results in the Schiff base region for the K intermediate. Pande *et al.* (19), using a probe wavelength of 530.9 nm, report a C═N stretch at 1,637 cm^{-1} for K that shifts to 1,620 cm^{-1} when the sample is suspended in ^2H_2O. With a probe wavelength of 676 nm, Braiman and Mathies (20) do not observe any significant lines in the 1,620- to 1,660-cm^{-1} Schiff base region for K. However, when they use a probe wavelength of 647 nm and multiple scanning to obtain a high signal-to-noise ratio, a small line at 1,623 cm^{-1} is the highest observed K Raman frequency. Terner *et al.* (16), utilizing probe wavelengths of 552.3 and 568.2 nm, reported a line in the room-temperature K spectrum at 1,626 cm^{-1} that shifts to 1,616 cm^{-1} in ^2H_2O.

For K (Fig. 2*A*) we observe peaks at 1,608 and 1,623 cm^{-1} in the Schiff base region. Upon deuteration of the film (Fig. 2*B*) or reconstitution with 15-deuterioretinal (Fig. 2*C*) the 1,623-cm^{-1} line does not shift. The 1,608-cm^{-1} line does shift by small amounts—i.e., 1,608 → 1,603 cm^{-1}—upon deuteration (Fig. 2*B*), and 1,608 → 1,600, 1,605 cm^{-1} on reconstitution with 15-deuterioretinal (Fig. 2*C*). However, because the Schiff base line in bR^{LA} also shifts to lower wavenumbers, it is not at all clear that these small shifts are due to shifts in K lines. Nor can we

rule out the possibility that the Schiff base line in K is less intense than in bR^{LA} and is at the same position (1,639 cm^{-1}) (19).

Upon deuteration of the films we observe several changes in the K lines that strongly support the idea that in K the Schiff base is indeed protonated. In particular the ethylenic line at 1,514 cm^{-1} splits and the prominent 957-cm^{-1} line shifts to 951 cm^{-1}. Because these lines are associated with chromophore vibrations (C=C stretches and hydrogen out-of-plane vibrations, respectively), it is more likely that they will be influenced by exchangeable protons in the chromophore than exchangeable protons in other parts of the protein.

Protein–Chromophore Linkage in M. For M we observe a peak near 1,624 cm^{-1} in the Schiff base region in both hydrated and deuterated films, in agreement with resonance Raman results (13–15, 17, 18, 26). The facts that the shift in the frequency of the Schiff base mode between M and bR^{LA} is in the same direction as occurs between unprotonated and protonated Schiff bases of retinals (14) and that the frequency is not significantly changed when films are deuterated support the conclusion that in M the chromophore–protein linkage is an unprotonated Schiff base.

Ethylenic Stretch. The ethylenic stretching frequencies obtained from our IR measurements for bR^{LA} (1,529 cm^{-1}), K (1,514 cm^{-1}), M (1,569 cm^{-1}), and bR^{DA} (1,536 cm^{-1}) are in good agreement with those obtained in resonance Raman experiments and show the well-known correlation between ethylenic stretching frequency and wavelength of the absorption maximum in the visible (14, 33). The position of the ethylenic stretch in K (1,514 cm^{-1}) is closer to the 1,516-cm^{-1} value obtained by Braiman and Mathies (20) than it is to the 1,524-cm^{-1} value obtained by Pande *et al.* (19) or the 1,528-cm^{-1} value obtained by Terner *et al.* (16).‖ The shifts to lower frequencies in the major C=C band when bacteriorhodopsin is reconstituted with 15-deuterioretinal (Fig. 2*A* vs. Fig. 2*C*)—i.e., 1,514 → 1,509 cm^{-1} in K and 1,529 → 1,525 cm^{-1} in bR^{LA}—are in agreement with those obtained in resonance Raman experiments. We observe two peaks at 1,518 and 1,513 cm^{-1} in the ethylenic stretching region for K in deuterated films (Fig. 2*B*); the reason for this splitting is not clear at this stage, but the splitting may be due to the formation of a new blue-shifted K intermediate.

Fingerprint Region. Braiman and Mathies have shown that 15-deuterio-induced changes between 1,100 and 1,300 cm^{-1} can be used to distinguish between 13-*cis* and *all-trans* configurations of the chromophore even in the presence of large perturbations due to the protein environment (18). The pattern of changes observed in the infrared difference spectra (Fig. 2 *A* and *C*) for bR^{LA} (disappearance of the 1,254-cm^{-1} and 1,202-cm^{-1} lines and appearance of the 1,270-cm^{-1} line) and for K (a decrease in intensity of the 1,194-cm^{-1} line, appearance of the 1,225-cm^{-1} line) are in agreement with those obtained in resonance Raman experiments (18, 20), from which it was concluded that the conformation of the chromophore in bR^{LA} is closer to that of *all-trans* and in K it is closer to that of 13-*cis*. Although our IR measurements support these conclusions, verification awaits IR measurements on Schiff bases and protonated Schiff bases of various isomers of retinal.

Hydrogen Out-of-Plane Vibrations. The *trans*-disubstituted 7-ene and 11-ene bonds present in the retinal chromophore are characterized by strong out-of-plane hydrogen bending modes that appear near 965 cm^{-1} (25). It is known that this characteristic frequency is little affected by electronic, steric, and other factors and is thus diagnostic of the double bond substitution type (34). The hydrogen atom on a trialkyl-substituted double bond (9-ene and 13-ene for the retinal moiety) is associated with an 840- to 800-cm^{-1} band assigned to an out-of-plane wag vibration; however, this is not as characteristic as the 965 cm^{-1} band described above.

Our IR difference spectra show a large peak for K at 957 cm^{-1} and three smaller peaks at 826, 813, and 803 cm^{-1}. In order to determine whether the intense 957-cm^{-1} *trans*-disubstituted ethylenic band is unique to K or whether it is characteristic of all 13-*cis* chromophores in the bacteriorhodopsin system, we have examined the region below 1,000 cm^{-1} in the $bR^{DA} - bR^{LA}$ IR difference spectrum (Fig. 4) and the $M - bR^{LA}$ IR difference spectrum (Fig. 3). The fact that the only significant difference between bR^{DA} and bR^{LA} in this region (Fig. 4) occurs at 800 cm^{-1}, and not around 965 cm^{-1}, agrees with the idea that the major difference between $bR^{13\text{-}cis}$ and $bR^{all\text{-}trans}$ is the geometry of the trisubstituted 13–14 double bond. Thus the large intensity of the *trans*-disubstituted hydrogen out-of-plane band in K suggests that the change between bR^{LA} and K is more than just a *trans* to 13-*cis* isomerization of the chromophore. This conclusion has already been obtained from resonance Raman studies on the basis of the anomalously large 960-cm^{-1} band in the K state (20).

Protein Conformational Changes. The data presented in Figs. 2–4 show several lines that have not been observed in resonance Raman experiments. The $K - bR^{LA}$ difference spectra in deuterated films show weak K lines at 1,646 (Fig. 2 *B* and *D*) and 1,662 cm^{-1} (Fig. 2*B*); the $bR^{DA} - bR^{LA}$ difference spectrum shows a $bR^{13\text{-}cis}$ line at 1,663 cm^{-1} (Fig. 4); the $M - bR^{LA}$ difference spectrum shows positive peaks at 1,554, 1,647, and 1,762 cm^{-1} and a negative peak at 1,657 cm^{-1} (Fig. 3*A*). These lines probably reflect conformational changes in the protein; however, we cannot rule out the possibility that some are due to chromophore vibrational modes that have small Raman intensities.

Conformational changes in the protein at the stage of M have previously been observed (8, 23, 35, 36). In particular, the line in M at 1,762 cm^{-1}, which shifts to 1,749 cm^{-1} in deuterated films, has recently been interpreted by Rothschild *et al.* (23) to be due to the carbonyl stretching vibrations of COOH groups of aspartate or glutamate. For the dark-adapted to light-adapted transition, spectroscopic (37), enthalpy (38, 39), and pressure studies (40) have not found any evidence for protein conformational changes, whereas IR studies (41) have detected an increase in sorbed water on light adaptation.

It is interesting to speculate on the reason for the changes we observe in the amide I (≈1,660-cm^{-1}) and amide II (≈1,555-cm^{-1}) regions between the M and bR^{LA} states. Films of PM are oriented with the membrane normals predominantly along the film normal (6). Because the amide I and amide II transition dipoles lie roughly parallel and perpendicular to the α-helix axes, respectively, a protein conformational change, involving a rotation of the α-helices towards the membrane normal, would result in an increased absorption due to amide II and a decreased absorption due to amide I. Thus the changes observed in the amide I and amide II regions are consistent with a rotation of the α-helices towards the membrane normal in the bR-to-M transition. Using the relative absorption changes of the amide I and amide II bands, the amount of M present in the photostationary state (≈30%), and the nested-cone model developed in ref. 6, we calculate the angle of rotation to be less than 2°.

CONCLUSIONS

The generally excellent agreement found in the chromophoric vibrational modes as measured by FTIR difference and reso-

‖ For these bands we have deconvoluted the difference spectrum and find that the peak positions are shifted by less than 1 cm^{-1} from the values measured in the difference spectrum.

nance Raman studies and the appearance of apoprotein modes in the FTIR spectra show that the two techniques can be used in both a collaborative and complementary manner. Our studies confirm the resonance Raman results which show that the Schiff base linkage in bR is protonated whereas in M it is unprotonated. Although the Schiff base frequency of the K intermediate could not be unambiguously identified, the most likely interpretation of our results is that it is protonated. We also find that the conformation of the chromophore in the K state is significantly different from that of the 13-*cis* component of bR^{DA} and the *all-trans* isomer in bR^{LA} and furthermore that conformational changes in the protein have occurred by the M stage in the photocycle.

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