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Infrared evidence that the Schiff base of bacteriorhodopsin is protonated: bR570 and K intermediates

(purple membrane/resonance Raman spectroscopy/proton pump/energy transduction/retinal)

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ABSTRACT **It is possible, by using Fourier-transform infrared (FTIR) difference spectroscopy, to detect the conformational changes occurring in both the protein and the chromophore of bacteriorhodopsin during the photocycle. In contrast to Raman spectroscopy, a laser is unnecessary and hence the problem of a perturbing probe beam is eliminated. Furthermore, the relatively high signal-to-noise ratio obtainable with FTIR enables measurements to be made in minutes over a large spectral range. In the study reported in this paper, we used this method to examine the state of protonation of the retinylidene Schiff base in light-adapted bR570 and in K, the first intermediate in the photocycle. Resonance Raman spectroscopy provides evidence that bR570 is protonated, but these results have been questioned on the basis of theoretical and experimental grounds. FTIR difference spectral changes in the bR570-to-K transition clearly indicate that bR570 contains a protonated Schiff base. In contrast, the K intermediate displays a Schiff base that is altered but still is associated to some degree with a proton. Because the low-temperature FTIR difference spectrum of bR570 and K is similar to the recently reported low-temperature resonance Raman spectra of bR570 and K [Braiman, M. & Mathies, R. (1982) *Proc. Natl. Acad. Sci. USA* 79, 403–407], we can assign most, but not all, vibrational changes in the bR570-to-K transition to the chromophore. These results are consistent with a simple model of the first step in the photocycle which involves a movement of the Schiff base proton away from a counterion.**

Understanding how ions move across biological membranes remains a key goal in biology. The membrane protein bacteriorhodopsin (bR) from the purple membrane of *Halobacterium halobium* (1) offers a unique system for studying light-driven active transport because both the amino acid sequence (2) and the three-dimensional structure to <7 Å is known (3). In addition, the retinal chromophore of bR offers a means to detect distinct steps in the proton transport by measuring changes in absorption of visible light. In order to elucidate the bR proton pump mechanism fully it will be necessary to understand the molecular changes occurring during each step of the photocycle.

One part of bR that has been accessible to study is the C=N Schiff base which links the retinal chromophore to a lysine residue of the protein (4–6). Resonance Raman spectroscopy, which selectively probes the vibrations of the chromophore, indicates that a deprotonation of the Schiff base occurs by the M412 intermediates (7, 8). Because proton release from the membrane lags slightly behind production of M412 (9), movement of the Schiff base proton is likely to play a key role in proton transport. However, the existence of a protonated Schiff base has been questioned on the basis of theoretical and experimental work (10–13). For example, the possibility has been raised that the exciting light used in resonance Raman studies produces a spectrum that does not reflect the ground state configuration of the chromophore (10). Furthermore, an NMR study (13) on the closely related protein rhodopsin suggests that the Schiff base is deprotonated, in contrast to resonance Raman results. Hence, it is important to develop a new approach to address this question as well as a general technique to probe specific changes occurring in bR during the photocycle.

It recently has been demonstrated that chromophore and protein vibrational changes can be detected by Fourier-transform infrared (FTIR) difference spectroscopy (14–16). There are several advantages to this approach. (*i*) In contrast to resonance Raman spectroscopy, infrared absorption measurements can be made on a sample that is not appreciably driven through the photocycle. This eliminates the necessity of using rapid flow and double-beam methods (17) which cope with the effects of the probe beam on the photocycle of bR. Furthermore, the absence of an intense probe beam eliminates the possibility of altering ground states or producing photoproducts. In addition, fluorescence which can hamper Raman measurements, particularly of K (18), does not affect infrared absorption measurements. (*ii*) Because infrared probes all the modes of purple membrane (19), not just the chromophore vibrations as in resonance Raman spectroscopy, it is possible to examine the conformational changes of the protein during the photocycle. (*iii*) The intrinsic high signal-to-noise ratio of FTIR spectroscopy allows spectra to be recorded in minutes, compared to hours in conventional Raman measurements. (*iv*) The direction of bonds can be determined with polarized IR radiation—for example, as demonstrated for the peptide groups in the α-helices of bR (19).

In this paper, we report on FTIR difference measurements made at 77 K, a temperature at which the decay of the K intermediate of bR is blocked (20). This intermediate is the first photoproduct of bR with a rise time of <11 psec at room temperature (21). Its absorption is red-shifted to near 630 nm and is related in this respect to the bathorhodopsin primary photoproduct of the visual pigment rhodopsin which is also red-shifted (22). There is considerable interest in elucidating the molecular changes that occur during the bR570-to-K transition, the only light-driven step in the photocycle. It is likely that key events occur at this step in both the transduction of the photon energy and the movement of a proton through the membrane. Resonance Raman studies on K (23–25) indicate that the retinal conformation is different from that of bR570. However, the reported spectra all differ in several important features including the C=C and C=N stretching modes. Our FTIR difference data help to resolve this controversy and offer confirming evidence that bR570 contains a protonated Schiff base. Of equal importance, we find that the Schiff base in K undergoes a major

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Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier-transform infrared.

alteration which is consistent with an energy storage and proton "switch" mechanism. We also detect vibrational changes which may be associated with peptide groups in the protein.

MATERIALS AND METHODS

Sample Preparation. Purple membrane was purified from strain S9 (26). Thin films with OD 0.3–0.5 at 570 nm were deposited on 1-cm-diameter AgCl windows (Fisher) by the method of isopotential spin-drying (27, 28). On the basis of freeze-fracture electron microscopy and linear dichroism measurements (27), these films have been shown to contain a multilamellar array of membranes that are highly ordered relative to the film plane. Polarized FTIR measurements on these films reveal clearly the transverse orientation of the bR α-helices (19).

FTIR Measurements. Films were sealed in a humidified cell formed by an "O" ring and a second AgCl window. Humidification was accomplished by exposing the film to H_2O or ^2H_2O vapor at 100% relative humidity for 1 min. The sealed cell was mounted on the copper tail of a low-temperature cryostat (Janis Research, Stoneham, MA) equipped with a KBr and a Ge window (to filter out the spectrometer laser beam). The presence of H_2O or ^2H_2O could be monitored during the experiment by the presence of a strong water vibration at 3,400 or 2,600 cm^{-1}, respectively. Prior to cooling, the sample was light adapted for 10 min by exposure to a 600-W incandescent light filtered through a yellow Wratten filter and two glass IR filters. A fiber optic cable was used to guide the light into the spectrometer compartment. At 273 K the adapting light was shut off and the sample was cooled to 77 K in the dark.

FTIR measurements were made with a Nicolet MX-1 spectrometer. Each interferogram consisted of the average of 196 scans; averaging took a total of 3 min of recording time. The Fourier transform was preformed with triangular apodization. The spectra were recorded with 2-cm^{-1} resolution and the difference spectra were smoothed by using a 17-point fit which resulted in an effective resolution of 8 cm^{-1}. However, the frequency of peaks varied little for each measurement, resulting in an overall variability of <2 cm^{-1} for peak frequencies. All measurements were repeated at least three times. Measurements of absorption of visible light were made with a Cary 219 spectrometer interfaced to an Apple computer.

Difference spectra of bR570 vs. K were made by recording a spectrum before and during illumination. In order to drive the sample into the K intermediate, 3-min exposure to light of frequency peaked at 500 nm (Wratten filter 65) was used. Light of wavelengths >690 nm (Wratten filter 70) was used for reversal of the sample back to bR570.

RESULTS AND DISCUSSION

Fig. 1 presents the FTIR difference spectrum (curve A) of purple membrane obtained by subtracting a reference spectrum recorded in the dark from a spectrum of the same film recorded immediately afterward under constant 500-nm illumination. Fig. 2 presents similar data for a ^2H_2O-humidified film. Visible absorption measurements showed that >20% of bR570 is converted to K, in agreement with previous reports (20). Hence, the negative peaks in the FTIR difference spectrum reflect a loss in absorption due to the depletion of bR570 and the positive peaks reflect an increase in absorption and the production of K. Reversal of the K intermediate back to bR570 was performed by illumination of the sample for 3 min with light of wavelength >690 nm. Curve B in Fig. 1 shows the FTIR difference spectrum obtained when the absorbance axes have been reversed. The close similarity between this spectrum (K to bR570) and curve A (bR570 to K) indicates the almost complete reversability of this transition.

Interpretation of the FTIR difference spectra depends on the assignment of peaks to specific protein and chromophore groups. This task can be aided by the comparison with resonance Raman data which reflect only chromophore vibrations. This approach was used by us previously in conjunction with isotope labeling to verify that the largest vibrational changes observed in the bR570-to-M412 transition can be attributed to the ethylenic modes of the chromophore (14). For the present study, resonance Raman data were available for comparison for both bR570 and K at 77 K (23).

bR570. Table 1 compares the major negative peaks in the FTIR spectrum (Fig. 1) with the frequencies of the prominent resonance Raman peaks of bR570 measured at 77 K in a spinning cell by Braiman and Mathies (23). In addition to the appearance

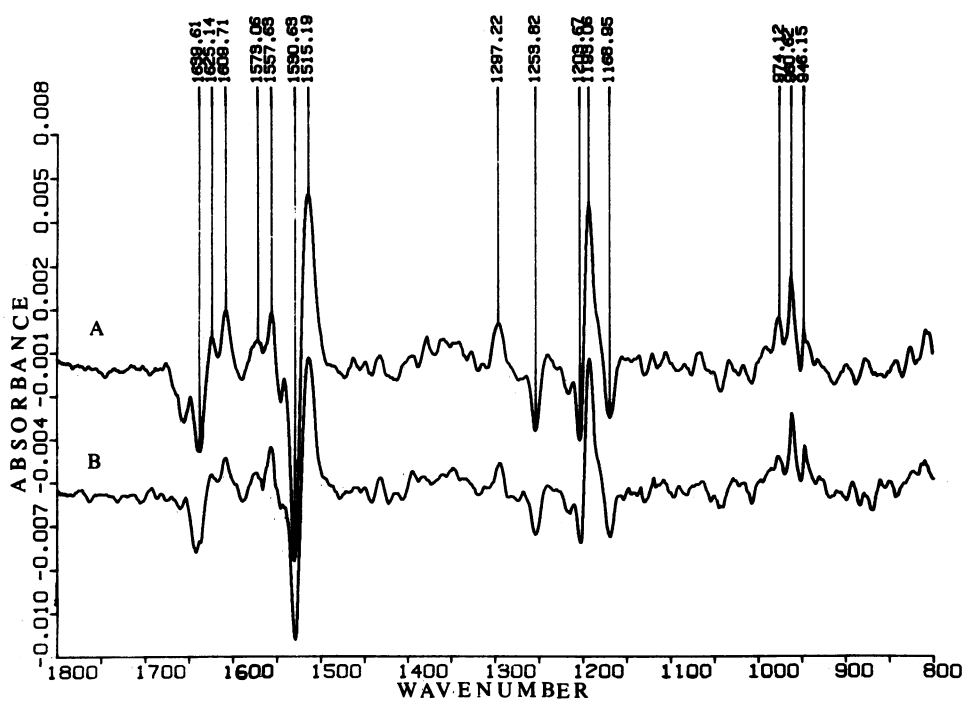


FIG. 1. FTIR difference spectra of purple membrane film deposited on AgCl at 77 K and fully humidified. Curve A: difference spectrum obtained by subtracting a reference spectrum recorded in the dark from the same sample recorded during illumination for 3-min with 500-nm light. Curve B: difference spectrum obtained by subtracting a reference spectrum in the dark obtained after 500-nm illumination from a spectrum recorded during 3 min of illumination with light of wavelengths >690 nm. For additional details see text.

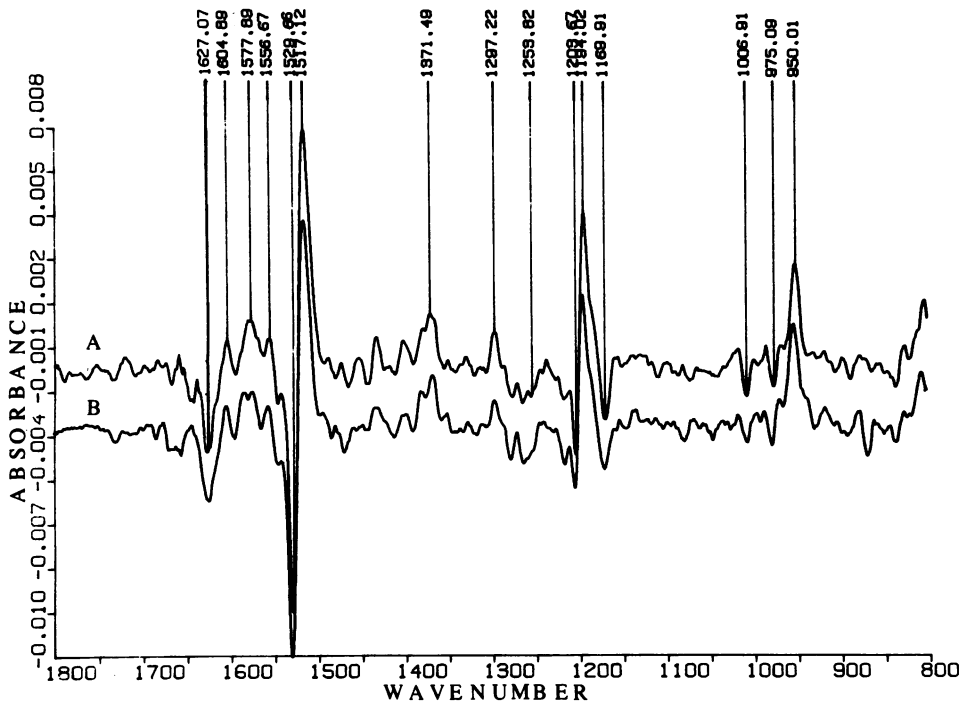


FIG. 2. FTIR difference spectra of purple membrane film at 77 K and fully humidified with ^2H_2O. Curves A and B were recorded under the same conditions as curves A and B in Fig. 1.

of a large negative peak at 1,530 cm^{-1} which is assigned in the resonance Raman spectrum to the ethylenic stretching mode, there is excellent agreement with most of the other major peaks. The exceptions are peaks observed in the resonance Raman from bR570 and K that are close or completely overlapping frequencies such as 1,009, 1,274, 1,323, and 1,347 cm^{-1}. This is expected because an overlapping positive and negative peak would cause either cancellation or splitting in the FTIR difference spectrum.

Table 1. Comparison of FTIR difference and resonance Raman spectra

FTIR (−)	Raman (bR570)	FTIR (+)	Raman (K)
1,640 (absent)	1,641 (absent)		
(1,627)	(1,624)		
		1,625	1,623
		1,610 (1,605)	1,610
	1,584*		1,591
		1,573 (1,578)	
		1,557 (absent)	1,561
1,530	1,529		
		1,515 (1,517)	1,516
	1,347		1,347*
	1,323		1,327
		1,297	1,294
	1,274		1,278
1,254 (absent)	1,250 (absent)		
	1,217		1,217*
		1,193	1,194
1,169	1,171		
	1,127*		1,127
	1,009		1,012
(975)	(974)	974 (absent)	974
		961 (950)	957
		946	942

Columns 1 and 3 list the prominent positive (+) and negative (−) peaks from Figs. 1 and 2. Columns 2 and 4 list resonance Raman data from refs. 23 and 29. Parentheses denote new peaks or disappearance of peaks induced by deuteration in the resonance Raman spectrum (see ref. 29).

* Peaks not explicitly listed but appear in spectra in ref. 23.

Additional evidence that many of the negative peaks in the FTIR difference spectrum can be assigned to the vibrations of the chromophore can be found from the effects of hydrogen/deuterium exchange. Several changes in the resonance Raman spectrum are observed upon deuteration of purple membrane at room temperature (29). The 1,250 cm^{-1} vibration disappears, a peak at 974 cm^{-1} appears, and the 1,641 cm^{-1} peak shifts to 1,624 cm^{-1} (Table 1). Each of these changes can also be detected in the FTIR difference spectra (Fig. 2). For example, a negative peak appears near 975 cm^{-1} (where previously there was a positive peak) whereas the negative peak at 1,254 cm^{-1} is absent. Finally, a peak shift from 1,640 cm^{-1} to 1,627 cm^{-1} is observed. All these effects are due to deuterium/hydrogen exchange of the Schiff base proton (29) and are discussed in greater detail below. Therefore, we conclude that the negative peaks of the FTIR difference spectrum consist mainly of contributions from the retinal chromophore of bR570.

K Intermediate. Table 1 compares the positive peaks found in the FTIR difference spectrum (Fig. 1) to the peaks reported recently for the resonance Raman spectrum of K (23). Again, almost complete agreement is found for all the major vibrations including those in the Schiff base region. Major differences, however, are found with the room temperature K spectrum reported by Terner *et al.* (24) and the low-temperature double-beam measurements by Pande *et al.* (25). Both of these studies found a large vibration near 1,524 cm^{-1} which was assigned to the ethylenic mode of the K intermediate. However, because the probe wavelength used was <530 nm, whereas the K absorption maximum is at 630 nm, it is possible that there was strong enhancement of a photoproduct that absorbs at <600 nm.

The above comparisons indicate that the bR570-to-K transition involves mainly chromophore alterations (the evidence for nonchromophoric changes is discussed below) which possibly involve a *trans* to *13-cis* conformational change (23). We discuss below further evidence which indicates that a major alteration also occurs in the protonated Schiff base.

bR570 Schiff base is protonated

The FTIR difference spectra provide direct evidence that the Schiff base of bR570 is protonated. In particular, a deuteration-induced shift of 1,640 cm^{-1} to 1,627 cm^{-1} was found (Figs. 1 and 2) which agrees quantitatively with the results of resonance Raman spectroscopy. Such a shift is predicted (30) and is not expected to occur for an unprotonated Schiff base. Because our data are based on IR absorption, it is not subject to the questions raised concerning the use of a probe laser which has been postulated to cause an alteration in the state of the chromophore prior to detection by the Raman scattering (10).

The above conclusion rests on the assumption that the 1,640 cm^{-1} peak (1,627 cm^{-1} peak in the deuterated case) arises in the IR spectrum from the Schiff base C=N stretch. Because there is excellent agreement between the FTIR and Raman spectra in other regions of the spectrum, it is unlikely that the peaks in this region would match by coincidence. In fact, comparison of resonance Raman and FTIR spectra of retinylidene model compounds (31) indicates that the C=N mode is enhanced in the IR and its magnitude is comparable to that of the C=C ethylenic stretch modes. Hence, we would expect the C=N stretch mode to appear in the spectrum along with the C=C stretching modes (1,529 cm^{-1}) provided that there is also a change in the C=N stretching vibration. The possibility that the 1,640 cm^{-1} peak arises from the amide I vibration of the protein, which is the most intense peak in this region in the IR spectrum (19), was also considered. However, specific labeling of bR with ^{15}N on the ϵ-nitrogen of lysines causes a 10 cm^{-1} downshift in both the FTIR difference (unpublished data) and resonance Raman spectra (6). In the resonance Raman spectrum this effect has been shown to be due to the specific incorporation of ^{15}N into the Schiff base. Because the labeled nitrogens are not incorporated into peptide groups, we can exclude amide I as a major contribution to the peak at 1,640 cm^{-1}.

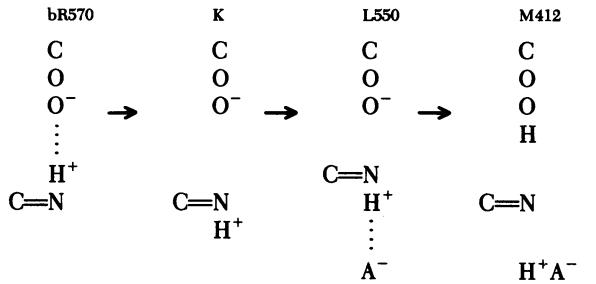
K Schiff base is perturbed

Two prominent positive peaks, at 1,625 and 1,610 cm^{-1}, in the FTIR difference spectrum are assigned to K. A weak shoulder at 1,623 and a peak at 1,610 cm^{-1} also were reported by Braiman and Mathies (23), making it likely that these vibrations are of chromophoric origin. This fact, together with the appearance of a negative peak at 1,640 cm^{-1}, indicates that a change occurs in the Schiff base of K from its original state in bR570. However, it is emphasized that this conclusion largely rests on the absence of a positive peak at a higher frequency in the resonance Raman data (23). In particular, a positive peak at such a frequency in the FTIR spectrum might be canceled by a stronger negative peak due to the Schiff base of bR570 and hence would not be observed. Furthermore, the reliable assignment of the origin of the 1,626 and 1,610 cm^{-1} bands can only be made by utilizing isotope replacement of the chromophore and protein.

Normally, a vibration near or lower than 1,620 cm^{-1} would be associated with an unprotonated Schiff base—for example, as found in the M412 intermediate (7, 8). However, changes in the local environment of a protonated Schiff base might also produce significant alterations in its frequency. The fact that there are shifts in this region due to deuteration (Fig. 2) supports this hypothesis. In particular, there is a reduction in the peak intensity at 1,610 cm^{-1} with a shift to 1,605 cm^{-1} and increased intensity near 1,580 cm^{-1}. One possible explanation is that the 1,610 cm^{-1} vibration has shifted to lower frequencies. However, a downshift of the negative 1,640 cm^{-1} peak to 1,627 cm^{-1} might also affect the positive peaks in this region. Thus, it is difficult to determine if the Schiff base of K is protonated on the basis of these data. However, deuteration also produces a positive peak shift from 961 cm^{-1} to 950 cm^{-1}, in agreement with a similar shift observed by resonance Raman measurements (R. Mathies, personal communication). This region is assigned to an out-of-plane hydrogen bending mode and has contributions from the 15—C—H (23, 32). FTIR difference measurements on bR regenerated with 15—C—^{2}H retinal (unpublished results) also confirm this conclusion. We would therefore expect changes to occur in this region upon hydrogen/deuterium exchange only if the Schiff base contained a protonated Schiff base coupled directly to the 15—C—H out-of-plane hydrogen bending mode. It should be noted that the NH out-of-plane bending mode might occur at a different frequency and deuteration of the nitrogen would still effect the 15—C—H out-of-plane bending motion through coupling effects. In the absence of a protonated Schiff base one would have to postulate more indirect interactions with deuterium/hydrogen exchangeable groups on the protein. We therefore tentatively conclude that the K intermediate still has a protonated Schiff base which is likely to be altered from its original state in bR570.

The question arises as to the nature of the Schiff base alteration that produces a shift in the C=N vibration to lower frequency by at least 14 cm^{-1} and as much as 30 cm^{-1}. One attractive explanation is based on the experiments and calculations of Blatz and Mohler (33) who demonstrated that a counterion or solvent interaction with the Schiff base proton can raise its frequency from what should be observed in the vacuum state. This effect is due to the localization of the positive charge by the counterion. Without this counterion, charge is more delocalized throughout the conjugated chain, promoting resonance structures that were originally discussed by Kropf and Hubbard (34) to explain the red-shift upon protonation of the Schiff base in model compounds. Hence, one would predict that an increase in the distance between the counterion and the Schiff base proton would lower the C=N frequency and also produce the observed red-shift in the K absorption. As pointed out by Honig *et al.* (35, 36), such a separation of the counterion from the proton is an effective means of storing part of the photon energy in the K state, which can be used to drive bR through the rest of the photocycle.

The movement of the Schiff base proton away from its counterion would also be a logical first step in the proton translocation. However, the Schiff base would become more basic in such an environment and prevent the complete removal of the proton. We would therefore expect the next step of the cycle to involve interaction with a proton acceptor, thereby making the Schiff base more acidic and again raising the Schiff base frequency. There is evidence, in fact, that the next intermediate, L550, is also protonated with a Schiff base frequency near 1,640 cm^{-1} (29, 37). Thus, one model consistent with our data is movement of the Schiff base proton from the counterion in K with movement toward a second counterion which serves as a proton acceptor by the L550 state:



These steps satisfy the requirements for a proton switch, a key

feature postulated for a proton pump (38–40). This switch acts as a molecular group "carrier" of the proton from a negative site that is accessible from the inside of the cell to another that is accessible from the outside.

Protein conformational changes

Evidence, from FTIR difference spectra of dehydrated purple membrane film, that a carboxylate group becomes protonated upon formation of M412 has been reported (14). This conclusion was based on the appearance of a peak near 1,760 cm^{-1} which correlated well with the frequency of a low pK_a COOH group such as could be present in aspartate. In the present work, no evidence was found for the formation of a protonated carboxylate group by the K intermediate. However, we do detect this peak at 233 K upon formation of M, again indicating that a carboxylate protonation does occur later in the photocycle (unpublished data). It is interesting to also note that the appearance of a COOH group in M412 could reflect a protonation of the Schiff base counterion. This group would act as a proton donor to recharge the Schiff base and complete the photocycle. The proton in this step would originate from the inside of the cell.

We also detect a positive peak at 1,557 cm^{-1} which may have nonchromophoric origins. First, this peak is much larger than a weak vibration detected near 1,560 cm^{-1} in the resonance Raman spectrum of K. This discrepancy, however, could be due to differences in the resonance Raman activity of this mode. Second, deuteration considerably lowers the intensity of the 1,557 cm^{-1} peak (Figs. 1 and 2). We also detect a negative peak at 1,660 cm^{-1} which is not fully reversed by red illumination and may be due to an artifact.

Conclusions

The present study clearly demonstrates the power of FTIR spectroscopy for probing conformational changes in bR. We have established that the bR570 state has a protonated Schiff base, thus removing questions raised concerning the resonance Raman results. Significantly, we also find evidence for the alteration of the Schiff base by the K intermediate. The effects of deuterium/hydrogen exchange suggest that this step does not involve deprotonation of the Schiff base. These conclusions and our previous finding of a carboxylate protonation at M412 (14) are consistent with a simple model of changes involving the Schiff base during the photocycle as diagrammed above. The key feature of this model is the movement of the Schiff base proton away from a counterion which could serve as a proton "switch" and energy storage mechanism as previously discussed (35, 38–40). We do not claim, however, that this is the only possible interpretation of our data. For example, further work will be necessary in order to establish firmly the movement of the C=N proton from one anionic site toward another during the bR-to-K transition.

Note Added in Proof. We have recently used FTIR difference spectroscopy to measure the rhodopsin-to-bR transition.

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