Fourier Transform Infrared Evidence for Schiff Base Alteration in the First Step of the Bacteriorhodopsin Photocycle†

Kenneth J. Rothschild,* Paul Roepe, Johan Lugtenburg, and Johannes A. Pardoen

ABSTRACT: The first step of the bacteriorhodopsin (bR) photocycle involves the formation of a red-shifted product, K. Fourier transform infrared difference spectra of the bR570 to K630 transition at 81 K has been measured for bR containing different isotopic substitutions at the retinal Schiff base. In the case of bacteriorhodopsin containing a deuterium substitution at the Schiff base nitrogen, carbon 15, or both, we find spectral changes in the 1600–1610- and 1570–1580-cm^{-1} region consistent with the hypothesis that the K630 C=N stretching mode of a protonated Schiff base is located near 1609 cm^{-1}. A similar set of Schiff base deuterium substitutions for retinal containing a ^{13}C at the carbon 10 position strongly supports this conclusion. This assignment of the K630 C=N stretching vibration provides evidence that the bR Schiff base proton undergoes a substantial environmental change most likely due to separation from a counterion. In addition, a correlation is found between the C=N stretching frequency and the maximum wavelength of visible absorption, suggesting that movement of a counterion relative to the Schiff base proton is the main source of absorption changes in the early stages of the photocycle. Such a movement is a key prediction of several models of proton transport and energy transduction. Evidence is also presented that one or more COOH groups are involved in the formation of the K intermediate.

The mechanism by which bacteriorhodopsin (bR),[1] a protein in the purple membrane of *Halobacteria halobium*, acts as a light-driven proton pump is an area of extensive investigation (Stoeckenius et al., 1979; Stoeckenius & Bogomolni, 1982). The first light-driven step of the bR photocycle produces a red-shifted intermediate, K, which is formed from bR in less than 10 ps (Applebury et al., 1978). Since subsequent steps of the photocycle occur without light, the absorbed photon energy must be somehow stored in the K intermediate. Two important questions are how this energy is stored and how it is transduced into the movement of a proton across the membrane. A related problem which has received much attention is the molecular origin of absorption shifts which occur at each step in the bR photocycle (Birge, 1981).

Two types of molecular processes have thus far been implicated in the bR570 to K transition. On the basis of picosecond visible absorption studies, it was proposed that a proton movement or tunneling occurs (Applebury et al., 1978). Since this event appears to involve an exchangeable proton, the Schiff base proton was considered a likely candidate, although protein groups might also be involved (Dinur et al., 1981). An all-trans to 13-cis isomerization of the chromophore has also been deduced on the basis of low-temperature (Braiman & Mathies, 1982) and room temperature resonance Raman measurements (Smith et al., 1983a,b) as well. While it has been concluded from these studies and others (Terner et al., 1979; Pande et al., 1981) that the Schiff base of K is still protonated, it is not clear if the Schiff base proton becomes altered relative to bR570. As mentioned above, such a change might be expected on the basis of picosecond visible absorption spectroscopy. A change in the environment of the Schiff base is also a key feature in many models of wavelength regulation and proton transport (Honig et al., 1976, 1979b; Blatz et al., 1972; Nagle & Mille, 1981; Warshel & Barboy, 1982; Schulten & Tavan, 1978; Schulten, 1978; Stoeckenius, 1978).

In this paper, we present evidence that the Schiff base proton environment is altered in the first step of the bR photocycle. By comparing the FTIR difference spectra for bR isotopically labeled at the Schiff base, we conclude that the C=N stretching frequency undergoes a large downshift during the bR570 to K transition. On the basis of these (and other) data, a possible linear correlation between the maximum wavelength of absorption and the C=N stretching frequency ($\nu_{C=N}$) for several states of the photocycle is discussed. Variation in the charge separation between the protonated Schiff base and its negative counterion(s) is considered as a mechanism consistent with these findings.

It has recently been demonstrated that Fourier transform infrared (FTIR) difference spectroscopy can be used to detect and characterize conformational changes occurring during the bR photocycle (Rothschild et al. 1981, 1982; Bagley et al., 1982; Siebert & Maentele, 1983) and rhodopsin bleaching (Rothschild et al., 1983). In contrast to resonance Raman spectroscopy, this method is sensitive to all vibrational changes occurring in bacteriorhodopsin, not just those limited to the chromophore (Rothschild et al., 1982). Thus, it is possible to simultaneously detect conformational changes of both the chromophore and the protein. The necessity of using double beam or rapid flow techniques (Oseroff & Callender, 1974) is also eliminated with this method since the wavelength of the IR probe beam does not appreciably drive the bR photocycle.

Our initial study of the bR570 to K transition (Rothschild & Marerro, 1982) was performed at 77 K, a temperature that blocks the transition[2] of K630 to L550 (Hurley & Ebrey,

† From the Departments of Physics and Physiology, Boston University, Boston, Massachusetts 02215 (K.J.R. and P.R.), and the Department of Chemistry, University of Leiden, Leiden, The Netherlands (J.L. and J.A.P.). *Received April 2, 1984.* This work was supported by National Institutes of Health Grants EY02142 and EY01996, National Science Foundation (80-11509), and an Established Investigatorship Award from the American Heart Association to K.J.R. Research in Leiden was supported by grants from SON, ZWO. A preliminary account of this work was presented at the 28th Annual Meeting of the Biophysical Society, San Antonio, TX, Feb 1984.

* Address correspondence to this author at the Department of Physics, Boston University.

[1] Abbreviations: bR, bacteriorhodopsin; PM, purple membrane; FTIR, Fourier transform infrared spectroscopy; SB, Schiff base.

[2] Since K has a visible absorption maximum at 77K of 630 nm, it is referred to throughout the rest of this paper as K630 in order to distinguish it from the room temperature intermediate K610 which absorbs near 610 nm.

0006-2960/84/0423-6103$01.50/0

Table I: Isotopic Substitutions at the Schiff Base for Bacteriorhodopsin Regenerated with *all-trans*-Retinal

	C=N stretch (cm^{-1})	
	BR570	K630
H –C=N– H$^+$	1639	1609
D –C=N– H$^+$	1632	1600 or 1578
H –C=N– D$^+$	~1625	1575
D –C=N– D$^+$	1615	1575[a]

[a] Although this peak has a maximum frequency at 1575 cm^{-1}, it has a significant increase in integrated intensity below this frequency relative to the case of ND listed above.

1978). The FTIR difference spectrum of bR570 to K630 closely resembles the difference spectrum computed from the resonance Raman of bR570 and K630 (Rothschild et al., 1984), indicating that bacteriorhodopsin structural changes at 77 K involve mainly the chromophore. In particular, a negative peak at 1640 cm^{-1} appears, which is assigned to the C=N stretching vibration in agreement with resonance Raman studies (Lewis et al., 1974; Stockburger et al., 1979). The appearance of this peak in the FTIR difference spectrum raises the possibility that the Schiff base undergoes a change during the bR570 to K transition. A *single* negative peak could arise from a simple reorientation of the C=N group which would cause intensity changes due to polarization effects. On the other hand, a negative and a positive peak should appear if the C=N vibration undergoes a frequency shift. The assignment of a positive peak to the C=N vibration in K630 would clearly indicate such a shift. Two positive peaks at 1623 and 1609 cm^{-1} were in fact considered candidates for the K630 C=N stretch (Rothschild & Marerro, 1982). Such a large frequency shift, however, is unexpected since protonated retinylidene Schiff bases normally exhibit $\nu_{C=N}$ in the range 1640–1660 cm^{-1} (Lewis, 1982 and references cited therein). Furthermore, the 1623- and 1609-cm^{-1} peaks could originate from C=C stretching modes which can fall within this region (Curry et al., 1982).

One method of identifying the C=N stretch peak of K630 is to examine the FTIR difference spectra of bR containing isotopic substitutions at the Schiff base. A variety of isotopic substitutions are possible, some of which are listed in Table I. Resonance Raman spectra of K630 recorded under similar conditions to our measurements have already been reported for one of these labels [C(15)-D] (Braiman & Mathies, 1982). However, peaks in the C=N region were not detected above the noise, and in the case of unlabeled PM two weak peaks at 1623 and 1608 cm^{-1} were observed only after extensive signal averaging.[3] The relatively stronger intensity of these peaks in the FTIR-difference spectrum facilitates a detailed study of the spectral changes in this region due to isotope substitution. Previous FTIR-difference measurements have been reported by Rothschild & Marrero (1982) and by Bagley et al. (1982) and Siebert & Maentele (1983), who both also studied PM regenerated with C(15)-D.

[3] A recent resonance Raman measurement of room temperature K by Braiman & Mathies (1983) does not reveal a C=N peak above the noise level.

Materials and Methods

Fourier transform infrared difference measurements were made on films of purple membrane formed by the isopotential spin-dry method (Clark et al., 1980) as previously reported (Rothschild & Marerro, 1982). Briefly, films deposited on AgCl are humidified by exposing them to H_2O or D_2O vapor while cooling the backside of the window with a stream of cold N_2 gas or by direct exposure to H_2O or D_2O for 3–5 min. The films are then sealed in a chamber made by adding a second AgCl window and attached to the cold finger of a low-temperature cryostat (Helitran, Air Products, Allentown, PA). The films are irradiated for 15 min with yellow light in order to light-adapt them while cooling slowly to 0 °C. The adapting light is then turned off and the sample rapidly cooled to 81 K. The light-adapting procedure is essential, since results obtained on dark-adapted films differ significantly from light-adapted films.

Illumination is produced by Dolan-Jenner 180 Fiber-lite with a 150-W bulb, which passes through two heat filters and an interference filter. The light is focused onto a fiber optic cable and guided into the spectrometer cabinet where it is focused onto the sample using an annular fiber optic illuminator. Under these conditions the sample reaches a steady-state composition in only a few minutes which results from a 30% interconversion of bR570 to K630 (Hurley & Ebrey, 1978).

Measurements are made with an MX-1 spectrometer interfaced to a 1200S data analysis system (Nicolet Instrument, Madison, WI). The FTIR-difference spectrum is obtained by recording the spectrum of the bR film while it is irradiated for 15 min with green light (Ditric Optics, Hudson, MA, 500-nm broad band three-cavity filter) and then fully photoreversing the K intermediate produced with the green light by irradiation for 15 min with red light (Ditric Optics, 640-nm interference filter), again recording a second spectrum. This procedure is repeated under computer control at least 10 times resulting in a corresponding number of FTIR-difference spectra which are averaged together. This averaged difference spectrum exhibits extremely low signal/noise, allowing us to reliably detect peaks with an OD as small as 2×10^{-4}. Furthermore, a base-line check performed by averaging the differences of successive spectra recorded under similar conditions (i.e., with either green or red illumination) displays no artifacts as reported in the amide I and II region by Siebert & Maentele (1983). The resolution of all spectra shown, which are unsmoothed, is better than 2 cm^{-1}.

White membrane from a retinal-deficient strain of *Halobacteria halobium* (JW5) was isolated by using a method based on sucrose density centrifugation (Becher & Cassim, 1975). Regeneration with *all-trans*-retinal was done at room temperature under dim red light and monitored with a Cary 219 UV–visible spectrometer. The synthesis of $^{13}C(10)$ retinal with 93% ^{13}C incorporation has been published elsewhere (Pardoen et al., 1983). $^{13}C(10)/C(15)$-D retinal with 93% ^{13}C incorporation and 99% deuterium incorporation was prepared from $^{13}C(10)$ retinal as described in Pardoen et al. (1984).

Results

C=N Stretching Mode in bR570 and K630. Figure 1 compares the region 1540–1750 cm^{-1} for purple membrane regenerated with *all-trans*-retinal and *all-trans*-15-deuterioretinal in both H_2O and D_2O (cf. Table I). As previously described (Rothschild & Marrero, 1982; Bagley et al., 1982), the negative peak at 1639 cm^{-1} in normal purple membrane originates from the C=N stretching vibration of the Schiff

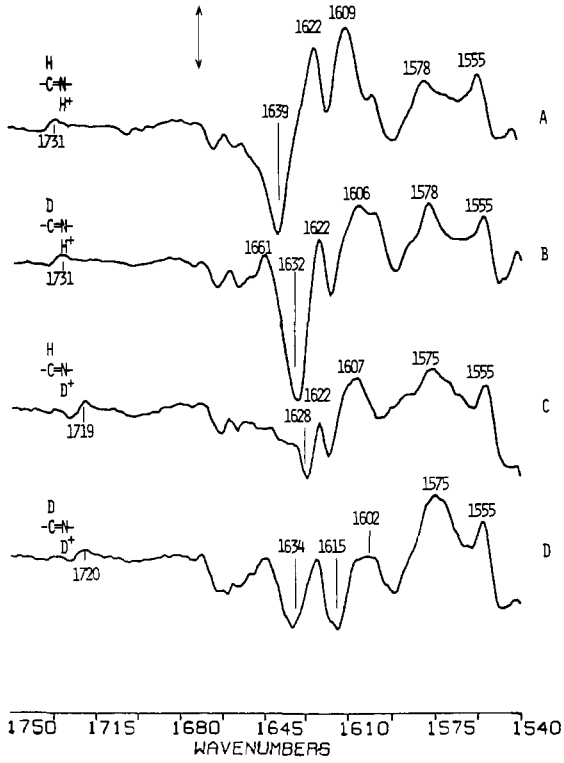

FIGURE 1: Comparison of 1540–1750-cm^{-1} region of FTIR difference spectra at 81 K for white membrane regenerated with *all-trans*-retinal or *all*-trans-15-deuterioretinal in the presence of either H_2O or D_2O. Films were exposed to a drop of H_2O or D_2O for 3–5 min and then partially dried with N_2. The amount of humidification was monitored by using the peak at 3400 (H_2O) or 2400 cm^{-1} (D_2O). Each spectrum is the average of at least 12 difference spectra each consisting of 15 min of green illumination and 15 min of red illumination. The arrow corresponds to an absolute OD as follows: (A) 0.0025, (B) 0.0028, (C) 0.0011, and (D) 0.0038. The spectra shown are all unsmoothed with a minimum resolution of 2 cm^{-1}. The Fourier transform was computed by using triangular apodization.

base in bR570. This assignment is strongly supported by both the isotope downshifts observed (cf. Table I) and the close agreement with resonance Raman results (Alshuth & Stockburger, 1981: Argade et al., 1981). It is noted that the downshift increases in the order

$$-\overset{D}{\underset{H}{C}}=N- \;<\; -\overset{H}{\underset{D}{C}}=N- \;<\; -\overset{D}{\underset{D}{C}}=N-$$

The assignment of the K630 C=N stretching mode is not as straightforward. Two positive peaks appear at 1622 and 1609 cm^{-1}. We can exclude the positive 1622-cm^{-1} peak since its frequency is constant for all of the isotopic substitutions examined (cf. Figure 1). The reduction in intensity of this peak can be accounted for by the downshift of the negative 1639-cm^{-1} peak. For example in the case of N-D, the bR570 C=N stretch is near 1625 cm^{-1}, cancelling much of the intensity at 1622 cm^{-1}.

In contrast to the 1622-cm^{-1} band, the positive peak at 1609 cm^{-1} undergoes a change in intensity, frequency, and shape for all of the isotopic substitutions relative to the unlabeled sample. In order to compare the relative integrated intensity of this band for the different cases, we scaled all the spectra to specific peaks between 1400 and 1475 cm^{-1}, which appear to be unaffected by the isotope substitutions examined. The correctness of the scaling was also confirmed separately for samples in H_2O and D_2O by comparing the size of the bands either at 1731 (H_2O) or 1719 cm^{-1} (D_2O), respectively. As discussed below, these bands are due to COOH group vibrations of the protein and therefore should be unaffected by

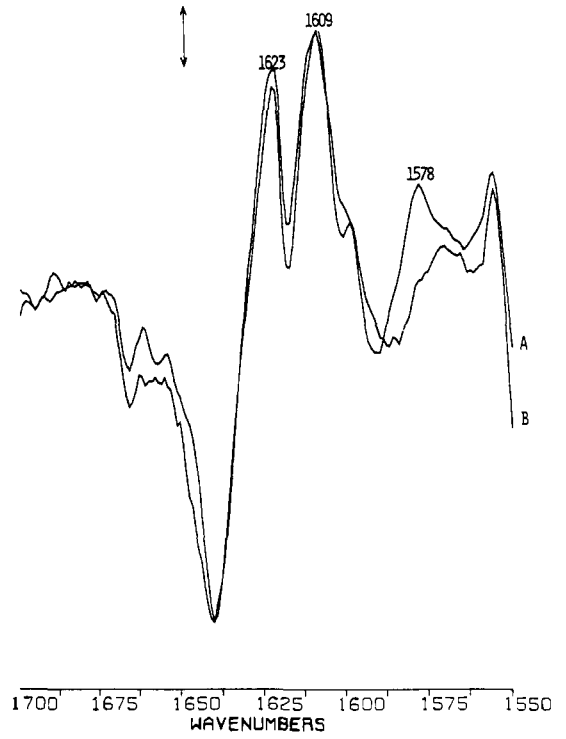

FIGURE 2: Comparison of FTIR difference spectra at 81 K in the region 1500–1700 cm^{-1} for white membrane regenerated with either (A) *all-trans*-retinal or (B) *all-trans*-[10-^{13}C]retinal. Both samples were recorded under the same conditions as described in Figure 1. The arrow corresponds to an absolute OD of (A) 0.0006 and (B) 0.0004.

isotope substitutions at the Schiff base. By use of this method of scaling, it is found that the integrated intensity between 1608 and 1612 cm^{-1} is reduced for all of the substitutions relative to the normal unsubstituted sample.

In addition to a reduction in intensity near 1610 cm^{-1}, an increase in integrated intensity is found at lower frequencies for each case, implying that the original band near 1610 cm^{-1} has downshifted. This downshift is evident by comparing the relative magnitude of the 1555-cm^{-1} band to those of the bands near 1578 cm^{-1}. The frequency of the increase in intensity appears to occur in the order C(15)-D, N-D and C(15)-D/N-D in agreement with the order found for the C=N stretch of bR570. For example, although the maximum peak frequency occurs at 1575 cm^{-1} for C(15)-D/N-D, it is clear that the increase in integrated intensity occurs at lower frequency relative to N-D. It is noted, however, that the assignment of the C=N stretch in C(15)-D is not completely certain since an increase in intensity near 1600 cm^{-1} is also found, which raises the possibility of a 10 cm^{-1} downshift from 1609 to 1600 cm^{-1}.

In order to further examine the effect of isotope labels at the Schiff base, we utilized purple membrane regenerated with *all-trans*-[10-^{13}C]retinal. As shown in Figure 2, the label does not appreciably affect the spectrum above 1600 cm^{-1} but eliminates the peak at 1578 cm^{-1}. Shifts into this region due to isotope labeling at or near the Schiff base are now more evident (cf. Figure 3). For example, new peaks are found at 1584, 1578, and 1570 cm^{-1} for the 15D, ND, and 15D/ND substitutions, respectively (cf. Figure 3). In addition, an increase in intensity near 1600 cm^{-1} is also detected in the case of C(15)-D. In all cases, the appearance of these new bands is accompanied by a reduction in the intensity near 1609 cm^{-1}. Significantly, these changes are consistent with the shifts observed with the set of substitution shown in Figure 1, strongly implying that a band at or near 1609 cm^{-1} is down-

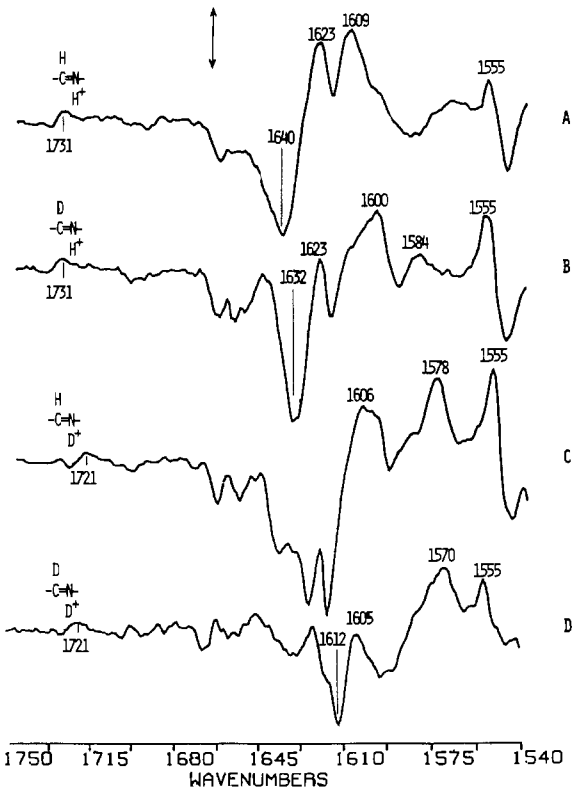

FIGURE 3: Comparison of 1540–1750-cm^{-1} region of FTIR difference spectra at 81 K for white membrane regenerated with *all-trans*-[10-^{13}C]retinal or *all-trans*-15-deuterio[10-^{13}C]retinal in the presence of either H_2O or D_2O. Conditions were similar to those described in Figure 1. The arrow corresponds to an absolute OD as follows: (A) 0.0018, (B) 0.0018, (C) 0.0019, and (D) 0.0011.

shifting due to Schiff base isotope substitutions. Furthermore, the relative order of the downshift parallels that found for the C=N stretch of bR570. We therefore assign at least a portion of the 1609-cm^{-1} band to the C=N stretch of K630.

We also note that a positive peak near 1731 cm^{-1} accompanied by a negative peak at higher frequency is evident in all samples exposed to H_2O shifting to 1719 cm^{-1} in D_2O. Such a frequency and shift upon D/H exchange is characteristic of COOH groups. We have previously found peaks in this region appearing in the bR570 → M412 transition (Rothschild et al., 1981) and the rhodopsin → meta II transition (Rothschild et al., 1983). In the present case the peaks are much smaller and may correspond to a slight shift in frequency of a COOH group due to an alteration in hydrogen bonding. A second possibility is the deprotonation of COOH and concomitant protonation of a COO^- to produce a COOH with a slightly different CO stretch frequency than the original COOH group. Additional peaks that may be assignable to protein groups appear between 1640 and 1700 cm^{-1}. In the normal PM, a positive peak at 1661 cm^{-1} (cf. Figure 1) is hidden by the negative peak at 1639 cm^{-1} which downshifts due to isotope substitutions relative to the unlabeled sample. It is less intense in samples in D_2O, raising the possibility it arises from the amide I mode of a peptide group(s).

Discussion

Isotopic substitutions at the Schiff base have been used as a means of assigning the C=N stretch vibration. In the case of bR570, the present work further confirms the assignment of the bR570 C=N stretch at 1639 cm^{-1}. In the case of K630, all isotope substitutions at the Schiff base examined indicate a downshift of a peak near 1609 cm^{-1}. For the ND substitutions (cf. Tables I and II) the magnitude of this shift is over

Table II: Isotopic Substitutions at the Schiff Base for Bacteriorhodopsin with *all-trans*-Retinal Containing a ^{13}C at the C(10) Position

	C=N stretch (cm^{-1})	
	BR570	K630
H –C=N– H$^+$	1640	1609
H –C=N– H$^+$	1632	1600 or 1584
H –C=N– D$^+$	~1625	1578
D –C=N– D$^+$	1612	1570

30 cm^{-1}. While this is larger than the downshift of the bR570 C=N stretch upon deuterium–hydrogen exchange, a deuterium-induced shift of over 40 cm^{-1} in the C=N stretching vibration (1628–1589 cm^{-1}) has also recently been observed in the room temperature O intermediate (Smith et al., 1983). Hence, the most plausible assignment of the 1609-cm^{-1} band is to the K630 C=N stretch of a protonated Schiff base.

In two previous examinations of the K630 C=N stretching vibration, both Bagley et al. (1982) and Siebert & Maentele (1983) argued that the small shift of the band at 1609–1605 cm^{-1} and the unchanged position for the different deuterium substitutions conflict with the assignment of the 1609-cm^{-1} band to the C=N vibration of K630. In addition, Siebert and Maentele questioned the conclusion that the K630 Schiff base is protonated. Both studies, however, did not examine in detail the 1570–1580-cm^{-1} region. As discussed in this paper, deuterium labeling of the Schiff base appears to cause the C=N vibration to shift into the 1570–1580 region, leaving a residual peak which is not due to the C=N vibration near 1605 cm^{-1}. Similar results are obtained for the substitution of ^{15}N for the Schiff base nitrogen (K. J. Rothschild, unpublished results) where a downshift to 1600 cm^{-1} is observed. Finally, the isotope-induced shifts for the different deuterium substitutions exhibit the relative frequencies expected on the basis of shifts observed for the bR570 C=N vibration.

The major question we now address is why the K630 C=N stretch frequency is so unusually low. In comparison, previously observed $\nu_{C=N}$ values in bacteriorhodopsin, in rhodopsin, and in retinylidene protonated Schiff base model compounds range between 1639 and 1660 cm^{-1}. Furthermore, deprotonation of the Schiff base such as in the case of M412 causes a downshift to only 1620 cm^{-1}.

One possible explanation of such a low-frequency C=N stretching mode is based on consideration of the effects of a counterion positioned near the proton of the Schiff base. Numerous theoretical analyses indicate that an increased separation of a negative charge from the Schiff base proton can result in a red shift of the visible absorption maximum (Hubbard & Kropf, 1958; Honig et al., 1976; Blatz et al., 1972; Suzuki et al., 1974). The mechanism of wavelength control in this case can be viewed qualitatively as due to variation in the extent of positive charge delocalization. When a counterion is close to the Schiff base proton, the positive charge is stabilized on the nitrogen. As it is moved away, resonances that involve positive charge further down the chain become more important. This delocalization has the effect of allowing the C=N to become more single bonded in character (i.e., lowers bond order) and thereby lowers the force

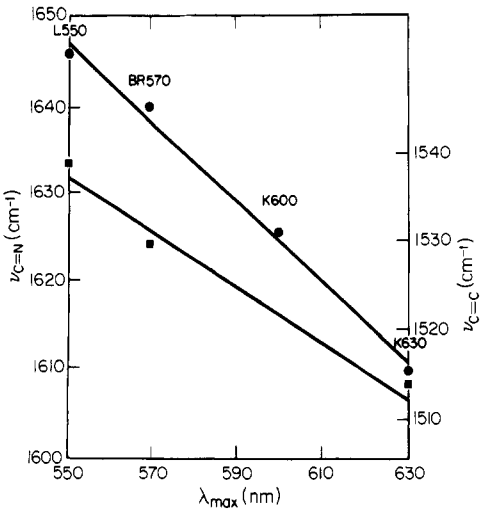

FIGURE 4: Plot of $\nu_{C=N}$ (dots) and $\nu_{C=C}$ (squares) vs. maximum absorption wavelength in visible for the early BR intermediates. The point for room temperature K $\nu_{C=C}$ is not shown due to the large uncertainty in its frequency (1517–1530 cm^{-1}) from RRS measurements.

constant of the bond. Honig et al. (1976) calculate that moving a negative charge from 2.8 to 3.4 Å away from the nitrogen results in a change in the bond order from 0.82 to 0.79. Using Gordy's rule to calculate the force constant, one predicts a shift of the C=N stretching frequency from 1641 to 1602 cm^{-1}.[4] Hence, a qualitative model of charge separation at the Schiff base leads to the prediction that $\nu_{C=N}$ will shift down by almost 40 cm^{-1} with only a small (0.6 Å) movement of the counterion.

It is important to note that the above calculation ignores the coupled vibrations of other group modes such as the NH in-plane bending mode and is thus likely to be quantitatively unreliable. Qualitatively, however, we still would expect an increased charge separation at the Schiff base to cause a lowering of the C=N stretch frequency and an associated red shift of the λ_{max}. In fact, such a correlation between ν and λ is implied for the first three states in the bR photocycle as shown in Figure 4. An accurate $\nu_{C=N}$ for L550 was recently obtained from resonance Raman data (Argade & Rothschild, 1983) by using a spinning cell and the method of Voigtian profile analysis which eliminated the need for arbitrary subtraction factors used in previous analyses. Two other resonance Raman studies of L550 (Narva et al., 1981; Terner et al., 1979) also indicate a $\nu_{C=N}$ which is 4–6 cm^{-1} above the frequency in bR570. The point for bR570 is well established by both resonance Raman and FTIR-difference spectroscopy. Interestingly, a line drawn through these points also predicts correctly a fourth experimental point, since at room temperature K absorbs between 590 and 610 nm (Nagle et al., 1982), relative to 630 nm at 77 K. From the correlation plot this leads to a $\nu_{C=N}$ = 1625 cm^{-1}, in good agreement with the resonance Raman room temperature data of Terner et al. (1979).[5] The O intermediate also falls near the line with $\nu_{C=N}$ = 1626 cm^{-1} (Smith et al., 1983a) and λ_{max} 590–600 (Nagle et al., 1982).

Figure 4 also shows the well-known linear correlation between λ_{max} and the eythlenic (C=C stretching) frequency in the retinyl model compounds and in retinal-based pigments such as bacteriorhodopsin and rhodopsin (Doukas et al., 1978) which has a slope that is 70% of the C=N correlation. This difference in slope suggests that the change in the bond delocalization at the Schiff base is greater than at the C=C bond(s) during the early part of the phoyocycle. In contrast, a secondary interaction occurring nearer to the β-ionone ring would be expected to affect the ethylenic modes to a greater extent relative to the C=N. In support of this idea, recent calculations by Kakitani et al. (H. Kakitani, T. Kakitani, H. Rodman, B. Honig, and R. Callender, unpublished results) show that an external point charge introduced over the ionone ring (Nakanishi et al., 1980) produced a smaller change in the C=N bond order relative to the C_{11}=C_{12} bond. Hence, we conclude our data are consistent with wavelength regulation in the initial steps of the bR photocycle through a mechanism which directly perturbs the Schiff base.

It should be noted that while the C=N correlation applies to all of the early protonated states in the bR photocycle, it is not expected to be as general as the C=C correlation plot which also includes points from rhodopsin, its bleaching intermediates, and model compounds such a retinal SB (both protonated and deprotonated) (Aton et al., 1980). This fact stems from consideration of electron delocalization in the polyene chain which directly influences both λ_{max} and the C=C stretching frequency (Heyde et al., 1971). A general correlation is therefore expected for all systems *independent of the actual source of delocalization.* In the case of the $\nu_{C=N}$, no such general correlation is expected with λ_{max} since all factors that alter $\nu_{C=N}$ will not necessarily change λ_{max} in the same way. For example π-electron delocalization due to a proton/counterion interaction will cause a drop in $\nu_{C=N}$ and red shift in λ_{max}. On the other hand, deprotonation of the Schiff base removes the NH bending mode, causing a drop in the $\nu_{C=N}$ but a blue shift of λ_{max} (Aton et al., 1980). This explains why the M intermediate ($\nu_{C=N}$ = 1620 and λ_{max} 412 nm), which contains a deprotonated Schiff base, deviates from the $\nu_{C=N}$ vs. λ_{max} plot in Figure 4. The predicted presence of an external point charge interaction near the carbon 12 of retinal (Honig et al., 1979a,b) in rhodopsin which becomes altered during an 11-cis to the all-trans isomerization would also account for apparent lack of correlation between the λ_{max} of rhodopsin, bathorhodopsin, and isorhodopsin and $\nu_{C=N}$ which is approximately constant (Eyring & Mathies, 1979; Aton et al., 1980).

Implications for Proton Transport and Energy Transduction. Several models of proton translocation and energy transduction in bacteriorhodopsin have been proposed which involve as a key feature the movement of the Schiff base relative to one or more negative counterions in the binding pocket. While the molecular groups involved in this interaction

[4] Following the treatment of Blatz & Mohler (1975), we have used Gordy's rule (Gordy, 1946) to calculate the force constant K:

$$K = ap(X_a X_b/d^2)^{3/4} + b \quad (1)$$

where a = 1.67, b = 0.3, p is the bond order, d is the bond length, and X_a and X_b are the Pauling electronegativity of the two atoms. p and d were from Honig et al. (1976). The frequency, $\nu_{C=N}$, was calculated from

$$\nu_{C=N} = (1/2\pi)\sqrt{K(M_C + M_N)/(M_C M_N)} \quad (2)$$

where M_C and M_N are the mass of carbon and nitrogen, respectively. It should be noted that this calculation neglects all other parts of the retinal such as the NH group which may have an appreciable effect on $\nu_{C=N}$. For example, it has been demonstrated that the NH bending mode of the Schiff base can mix strongly with the C=N stretch, thereby affecting its frequency (Aton et al., 1980). Hence, changes in NH bending frequency due to perturbation of an external charge would also be expected to strongly influence the C=N stretch frequency. It is not clear to what extent inclusion of these factors would alter the prediction based on the simple calculation mentioned above.

[5] At room temperature, the first measured intermediate is "J" which decays within 6 ps to K610 (Applebury et al., 1978). Thus, the first intermediate step due to motion of the SB proton may not be K but J.

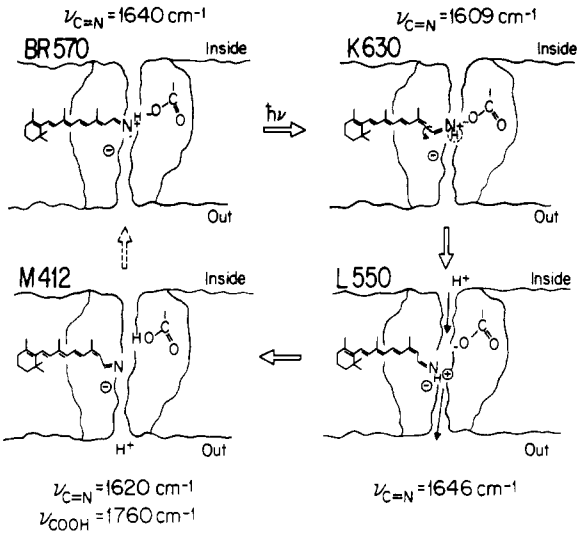

FIGURE 5: Schematic diagram depicting possible changes in the Schiff base during the first three steps of the photocycle which are consistent with the data presented in this paper. Retinal isomerization around the 13–14 double bond during the primary step causes the initial charge separation which is reduced in the second step by either a protein conformational change or further rotation of the chromophore about single bonds. In the third step the proton moves to an acceptor group, and the original COO^- becomes protonated. These events may actually involve several additional steps that are not shown here.

have not yet been defined, our data are consistent with a generalized switching model involving charge separation as shown in Figure 5 [see Nagle & Mille (1981) for a general discussion of switching models].

In the first step, the isomerization of the retinal chromophore around the 13–14 double bond causes a movement of the SB away from a counterion such as an aspartate or glutamate. A recent resonance Raman study (Smith et al., 1984) indicates that the C=N bond remains in the trans configuration during the bR570 to K step. Hence, the isomerization about the 13–14 double bond could easily cause a translation of the nitrogen's position and result in a movement of the SB proton away from a counterion facing the cytoplasmic surface and toward a second counterion facing the external surface. The K630 state would then correspond to an intermediate state in this movement of the SB proton.[5] As pointed out by several groups (Honig et al., 1979a,b; Warshel & Barboy, 1982), such a charge separation would lead to a significant increase in the stored energy in the K state which has recently been measured at 77 K by laser calorimetry (Birge & Cooper, 1983) to be close to 15 kcal/mol (one-third of the total photon energy absorbed).

The next step in the photocycle (K to L) produces an apparent upshift in the $\nu_{C=N}$ from 1609 to 1646 cm^{-1} (Argade & Rothschild, 1983) which is accompanied by a blue shift of the λ_{max} from 630 to 550 nm. This change is consistent with an additional movement of the Schiff base (Honig, 1978; Stoeckenius, 1978) so that the proton is now brought closer to a second negative group. Finally, the L to M step of the photocycle involves a deprotonation of the Schiff base. Both the FTIR difference spectrum of bR570 to M412 (Rothschild et al., 1981; Bagley et al., 1982) and kinetic IR spectroscopy (Siebert et al., 1982) reveal the protonation of one or more COO^- groups at this stage. If a proton moves from the Schiff base to an acceptor group such as aspartate, this would result in the net production of one COOH group. Alternatively, the original counterion might consist of a COO^- which would be expected to reprotonate at some step prior to completion of the photocycle and thus be responsible for the observed peak.

It is important to recognize that our finding of a K C=N stretch frequency at 1609 cm^{-1} does not conclusively confirm the model of charge separation as has been proposed on theoretical grounds by a number of groups. Such a confirmation will most likely only be possible through three-dimensional high-resolution models of bR and its intermediates. Our results are, however, consistent with charge separation models and in general with those models that hypothesize a change in the electrostatic interaction of specific protein groups near the Schiff base during bR570 → K transition. Further FTIR studies and particularly those involving isotope labeling of specific protein groups are likely to provide additional information concerning the nature of these SB interactions and the overall mechanism of proton pumping in bacteriorhodopsin.

Acknowledgments

We thank G. Rosa for preparation of the regenerated PM films, S. Smith for providing assistance in isolation of white membrane, H. Jurgen Weber for supplying the retinal-deficient strain of *H. halobium*, and C. Winkel for assistance in organic synthesis of the labeled retinals. We also thank P. Ahl, M. Braiman, B. Honig, and R. Mathies for useful discussions about the manuscript.

Registry No. Deuterium, 7782-39-0; carbon-13, 14762-74-4.

References

Alshuth, Th., & Stockburger, M. (1981) *Ber. Bunsenges. Phys. Chem. 85*, 484–489.

Applebury, M. L., Peters, K. S., & Rentzepis, P. M. (1978) *Biophys. J. 23*, 375–382.

Argade, P., & Rothschild, K. J. (1983) *Biochemistry 22*, 3460–3466.

Argade, P. V., Rothschild, K. J., Kawamoto, A. H., Herzfeld, J., & Herlihy, W. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1643–1646.

Aton, B., Doukas, A. G., Narva, D., Callender, R. H., Dinur, U., & Honig, B. (1980) *Biophys. J. 29*, 79–94.

Bagley, K., Dollinger, G., Eisenstein, L., Singh, A. K., & Zimanyi, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4972.

Becher, B. M., & Cassim, J. Y. (1975) *Prep. Biochem. 5*, 161–178.

Birge, R. R. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 315–354.

Birge, R. R., & Cooper, T. M. (1983) *Biophys. J. 42*, 61–69.

Blatz, P., & Mohler, J. (1975) *Biochemistry 14*, 2304–2309.

Blatz, P. E., Mohler, J. H., & Navangul, H. V. (1972) *Biochemistry 11*, 848.

Braiman, M., & Mathies, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 403–407.

Braiman, M., & Mathies, R. (1983) *Biophys. J 41*, 14a.

Clark, N. A., Rothschild, K. J., Luippold, D., & Simons, B. (1980) *Biophys. J. 31*, 65–96.

Curry, B., Broek, A., Lugtenburg, J., & Mathies, R. (1982) *J. Am. Chem. Soc. 104*, 5274–5286.

Dinur, U., Honig, B., & Ottolenghi, M. (1981) *Photochem. Photobiol. 33*, 523–527.

Doukas, A. G., Aton, B., Callender, R. H., & Ebrey, T. G. (1978) *Biochemistry 17*, 2430–2435.

Eyring, G., & Mathies, R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 33–37.

Gordy, W. J. (1946) *J. Chem. Phys. 14*, 305.

Heyde, M. E., Gill, D., Kilponen, G., & Rimai, L. (1971) *J. Am. Chem. Soc. 93*, 6776.

Honig, B. (1978) in *Energetics and Structure of Halophilic Microorganisms* (Caplan, S. R., & Ginzburg, M., Eds.) pp 109–121, Elsevier/North-Holland Biomedical Press, New York.

Honig, B., Greenberg, A. D., Dinur, U., & Ebrey, T. G. (1976) *Biochemistry 15*, 4593–4599.

Honig, B., Dinur, V., Nakanishi, K., Balogh-Nair, V., Gawinowicz, M. A., Arnaboldi, M., & Motto, M. G. (1979a) *J. Am. Chem. Soc. 101*, 7084–7086.

Honig, B., Ebrey, T., Callender, R. H., Dinur, U., & Ottolenghi, M. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2503–2507.

Hubbard, R., & Kropf, A. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 130–139.

Hurley, J. B., & Ebrey, T. G. (1978) *Biophys. J. 22*, 49–66.

Lewis, A. (1982) *Methods Enzymol. 88*, 561–617.

Lewis, A., Spoonnhower, J., Bogomolni, R. A., Lozier, R. H., & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4462–4466.

Lozier, R. H., Bogomolni, R. A., & Stoeckenius, W. (1975) *Biophy. J. 15*, 955–962.

Nagle, J. F., & Mille, M. (1981) *J. Chem. Phys. 74*, 1367–1372.

Nagle, J. F., Parodi, L. A., & Lozier, R. H. (1982) *Biophys. J. 38*, 161–174.

Nakanishi, K., Balogh-Nair, V., Arnaboldi, M., Tsujimoto, K., & Honig, B. (1980) *J. Am. Chem. Soc. 102*, 7945–7947.

Narva, D. L., Callender, R. H., & Ebrey, T. G. (1981) *Photochem. Photobiol. 33*, 567–571.

Oseroff, A. R., & Callender, R. D. (1974) *Biochemistry 13*, 4243–4248.

Pande, J., Callender, R. H., & Ebrey, T. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7379–7382.

Pardoen, J. A., Neijenesch, H. N., Mulder, P. P. J., & Lugtenburg, J. (1983) *Recl. Trav. Chim. Pays-Bas 102*, 341–436.

Pardoen, J. A., Winkel, C., Mulder, P. P. J., & Lugtenburg, J. (1984) *Recl. Trav. Chim. Pays-Bas 103*, 135–141.

Rothschild, K. J., & Marerro, H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4045.

Rothschild, K. J., Zagaeski, M., & Cantore, B. (1981) *Biochem. Biophys. Res. Commun. 103*, 483–489.

Rothschild, K. J., Sanches, R., & Clark, N. A. (1982) *Methods Enzymol. 84*, 696–714.

Rothschild, K. J., Cantore, W., & Marrero, H. (1983) *Science (Washington, D.C.) 219*, 1333–1335.

Rothschild, K. J., Marrero, H., Braiman, M., & Mathies, R. (1984) *Photochem. Photobiol.* (in press).

Schulten, K. (1978) in *Energetics and Structure of Halophilic Microorganisms* (Caplan, S. R., & Ginzburg, M., Eds.) pp 331–340, Elsevier/North-Holland Biomedical Press, New York.

Schulten, K., & Tavan, P. (1978) *Nature (London) 272*, 85.

Siebert, F., & Maentele, W. (1983) *Eur. J Biochem. 130*, 565.

Siebert, F., Maentele, W., & Kreutz, W. (1982) *FEBS Lett.*

Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. (1983a) *Biochemistry 22*, 6141–6148.

Smith, S., Braiman, M., & Mathies, R. (1983b) *Proc. First Int. Conf. Time-Resolved Vibrational Spectrosc.*, 219–230.

Smith, S. O., Myers, A. B., Pardoen, J. A., Winkel, C., Mulder, P., Lugtenburg, J., & Mathies, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2055–2059.

Stockburger, M., Klusmann, W., Gattermann, H., Massig, G., & Peters, R. (1979) *Biochemistry 18*, 4896–4899.

Stoeckenius, W. (1978) in *Energetics and Structure of Halophilic Microorganisms* (Caplan, S. R., & Ginzburg, M., Eds.), pp 185–198, Elsevier/North-Holland Biomedical Press, New York.

Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 52*, 587–616.

Stoeckenius, W., Lozier, R. H., & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta 505*, 215–278.

Suzuki, H., Komatsu, T., & Kitajima, H. (1974) *J. Phys. Soc. Jpn. 37*, 751–758.

Terner, J., Hsieh, C. L., Burns, A. R., & El-Sayed, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3046–3050.

Warshel, A., & Barboy, N. (1982) *J. Am. Chem. Soc. 104*, 1469–1476.