The Photoreaction of Active-Site-Methylated Bacteriorhodopsin: An Investigation Using Static and Time-Resolved Infrared Difference Spectroscopy†

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ABSTRACT: The photoreaction of active-site-methylated, permethylated bacteriorhodopsin has been investigated by static and time-resolved UV–vis and infrared difference spectroscopy. Additional information on the isomeric composition of the initial state and of photoproducts was obtained by retinal extraction and subsequent HPLC analysis. The data show that the dark-adapted state contains only *all-trans*-retinal. Prolonged illumination produces a metastable state which contains essentially only 9-*cis*-retinal and which decays back to the dark-adapted initial state within 8 h. The time-resolved infrared difference spectra clearly demonstrate that laser flash excitation produces an intermediate that has all the characteristics of the L intermediate. It is demonstrated that the methyl group at the Schiff base nitrogen introduces a steric hindrance with the protein which inhibits a photoreaction at 80 K, but which allows the generation of an L-like intermediate at room temperature and 173 K.

The light-driven proton pump bacteriorhodopsin of the halophilic bacteria *Halobacterium halobium* is a retinal protein located in the plasma membrane. The chromophore, in its active conformation *all-trans*-retinal, is bound to the protein via a protonated Schiff base to lysine 216. The intermediates J, K, L, and M are well established for the photocycle (Stoeckenius & Bogomolni, 1982). Whereas in the former three species the Schiff base is still protonated, the M intermediate represents an unprotonated Schiff base (Aton et al., 1977; Marcus & Lewis, 1977). Most models for the proton-pumping mechanism assume that the deprotonation of the Schiff base and its reprotonation are directly linked to proton transport, i.e., the proton of the Schiff base is being pumped. However, so far there has been no direct proof for this hypothesis.

It has recently been shown that, if the deprotonation is blocked by methylation of the Schiff base, as in active-site-methylated BR (AMBR),[1] no proton pumping is observed, suggesting that the formation of M is essential for proton pumping (Longstaff & Rando, 1987). However, to strengthen this conclusion, it is important that the photoreaction is essentially unchanged up to the formation of M. In a recent publication, the photoreaction of AMBR was investigated in the visible and UV spectral ranges by millisecond time-resolved spectroscopy (Govindjee et al., 1988). The measurements indicated that, on the basis of the presence of a species with an absorption maximum around 550 nm, an L-like intermediate is formed from AMBR, which decays back to the initial state. However, the visible spectrum of an intermediate does not always allow an unequivocal structural assignment. Most importantly, from the data so far reported, it is not clear whether a trans/13-cis isomerization has taken place.

Vibrational spectra, such as resonance Raman and FTIR difference spectra, contain a great deal of structural information useful for understanding the molecular events that occur during proton pumping. The FTIR difference spectrum of the BR–L transition shows characteristic bands of the chromophore as well as of the protein, which can be used to identify an L-like intermediate (Engelhard et al., 1985; Gerwert & Siebert, 1986). The presence of strong hydrogen out-of-plane bending vibrations can be taken as an indication that at 80 K an intermediate similar to K can be formed (Rothschild & Marrero, 1982; Bagley et al., 1982; Rothschild et al., 1984). Therefore, we measured the infrared difference spectra for the photocycle intermediates of AMBR, using static and time-resolved techniques (Gerwert, 1988; Braiman et al., 1987; Siebert et al., 1980, 1981). In addition, as a control, static and time-resolved measurements have also been performed in the visible spectral range. Finally, additional information on retinal isomerization was obtained by extracting the chromophore and subsequent HPLC analysis.

The results show that an L-like intermediate can be formed at room temperature but that no photoreaction can be evoked at 80 K. In addition, data are presented which show that, because of the very long lifetime of this L intermediate, a secondary photoproduct is easily produced even at low light intensities and that this photoproduct contains a 9-cis chromophore. The time-resolved infrared data provide evidence for strong protein distortions already present earlier than 10 μs after the flash. These distortions decay within 5 ms and apparently do not influence the formation of the L-like intermediate. Finally, causes are discussed that explain the

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[1] Abbreviations: AMBR, active-site lysine monomethylated, non-active-site lysine permethylated bacteriorhodopsin; BR, bacteriorhodopsin; FTIR, Fourier transform infrared; HOOP, hydrogen out-of-plane vibrations; L, L550 intermediate of the photoreaction of BR; PMBR permethylated BR, all non-active-site lysines permethylated.

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unusually large red shift of AMBR as compared to active-site-methylated rhodopsin and methylated retinal Schiff base model compounds (Longstaff & Rando, 1985).

Materials and Methods

AMBR (active-site-methylated, permethylated BR) and permethylated BR (PMBR) were prepared as described previously (Longstaff & Rando, 1987). For the incorporation of [15-^{2}H]retinal, AMBR was bleached at 300 K with light from a slide projector with a 490-nm cutoff filter in the presence of 20 mM hydroxylamine hydrochloride, pH 7. Bleaching was complete after approximately 1 h. Bleached membranes were washed three times to remove excess hydroxylamine and resuspended in 10 mM phosphate buffer, pH 7. An ethanolic solution of [15-^{2}H]retinal was added to the suspension using a 3-fold excess of retinal. The yield of regeneration was rather poor, causing an absorption ratio A_{280}/A_{620} of at least 6. No increase in yield was obtained by a larger excess of retinal. Retinal oxime and excess retinal were removed by washing the membranes three times with a 2% solution of bovine serum albumin, fatty acid free (Sigma). [15-^{2}H]Retinal was prepared by the usual procedure of reduction of retinoic acid with $LiAl^2H_4$ and subsequent oxidation with MnO_2.

For evoking the photoreaction, AMBR was illuminated with light from a slide projector, using appropriate filters. In UV–vis and retinal extraction experiments, the intensity of the "red light" (640–800 nm) was 100 mW cm^{-2} and the intensity of the "green light" (500–550 nm) was 20 mW cm^{-2}. These approximate values were deduced from the photocurrent of a photodiode, by using the spectral sensitivity provided by the manufacturer. For the static infrared measurements, the intensities were lower. The illumination time was determined so that longer illumination did not cause further spectral changes.

The static FTIR difference spectra from hydrated film samples were obtained essentially as described previously (Engelhard et al., 1985; Gerwert & Siebert, 1986). The conditions of illumination are given in the text. Time-resolved FTIR difference spectra from hydrated films were obtained by the method described by Gerwert (1988) and Braiman et al. (1987). The time for collecting the 576 data points of one interferogram is 13.2 ms. The free spectral range was limited to 1950 cm^{-1} by a coated germanium long-pass filter. For excitation of the sample an excimer laser pumped-dye laser (rhodamine 6G, 590 nm, approximately 5 mJ) was used. Depending on the size of the spectral changes, the number of scans for the single-beam FTIR spectra varied between 256 and 2048. Spectral resolution was 2 cm^{-1} for the static and 4 cm^{-1} for the time-resolved FTIR difference spectra. Static spectra were measured either on a Bruker IFS 113v or on a home-built spectrophotometer equipped with an interferometer of the Bruker IFS 88 series. The time-resolved measurements were performed on the home-built apparatus. The spectrophotometers are equipped with MCT detectors. Higher time resolution was obtained with the flash photolysis apparatus with infrared monitoring beam (Siebert et al., 1980, 1981, 1982), using the same laser system for excitation. Here, usually 300 signals were averaged. Because of the long decay time (10 s), the repetition rate was 0.1 s^{-1}. In these experiments, depending on the infrared intensity, the spectral resolution varied between 6 and 8 cm^{-1}.

Time-resolved UV–vis difference spectra of suspensions of AMBR and PMBR were obtained with a conventional flash photolysis equipment with the same laser system for excitation. The optical density at 620 nm in a cuvette of 2-mm path length was adjusted to 0.5. Signals corresponding to the time-resolved transmission changes were stored in a modified Nicolet signal averager with a dual dwell time facility. Minimum dwell time was 10 μs. Usually 50 signals were averaged. Again, the repetition rate was 0.1 s^{-1}. The same kinetics were observed if, instead of the suspension, hydrated film samples were used. For measurements around 590 nm, the dye was exchanged for coumarin 153 (540 nm). The amplitudes of the signals obtained with 540-nm excitation were corrected by overlapping measurements above 620 nm. Static UV–vis spectra were taken with a Uvikon 810 (Kontron) or a Lambda 16 (Perkin-Elmer) spectrophotometer.

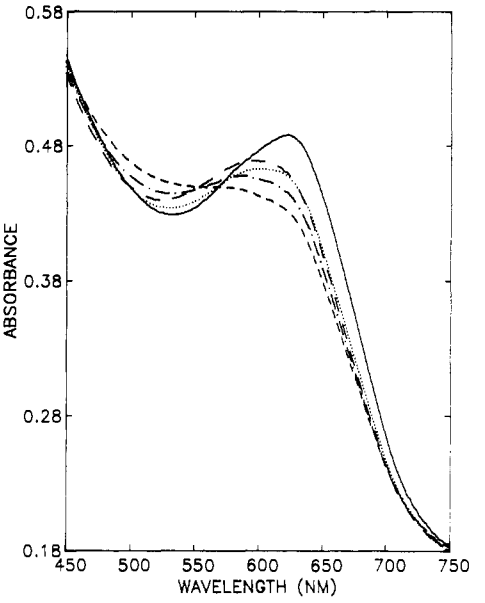

FIGURE 1: Spectra of AMBR obtained after various times of illumination with red light (>640 nm) at room temperature. Solid line, dark-adapted AMBR; dotted line, 5 s; long-dashed line, 20 s; dashed-dotted line, 1 min; short-dashed line, 5 min of illumination.

Extraction of retinal isomers from AMBR preparations was performed as follows: 2 nmol of AMBR (assuming an extinction coefficient of about 60 000 M^{-1} cm^{-1}) in 160 μL of an aqueous suspension were pipetted to 60 μL of 2-propanol and thoroughly mixed. Hexane (160 μL) was added to the mixture, which was again thoroughly mixed. The preparation was centrifuged in an Eppendorf centrifuge for 2 min at 12 000 rpm, and the organic layer was separated. The extraction procedure was repeated with a second portion of hexane. The combined organic layers were concentrated to a volume of about 15 μL with a gentle stream of nitrogen. This solution was injected into the HPLC system (Beckman 114M pump and Beckman 163 variable-wavelength detector adjusted to 360 nm, analytical column 25 × 0.4 cm with 3-μm Hypersil silica gel). A mixture of diethyl ether in hexane (8:92) was used as solvent. A flow of 1 mL/min was applied. Extractions were performed under dim red light or in entire darkness. All further manipulations were carried out under dim red light. Illumination conditions are given in the text.

Results

Figure 1 shows the effect of continuous illumination of AMBR with light of wavelengths longer than 640 nm. The strong absorbance increase in all the spectra below 480 nm is caused by light scattering of the rather turbid sample. Short-time illumination (up to 5 s) causes only an absorbance decrease together with a broadening of the absorption maximum toward shorter wavelengths, indicating that a blue-shifted photoproduct is formed (Figure 1, dotted trace). This

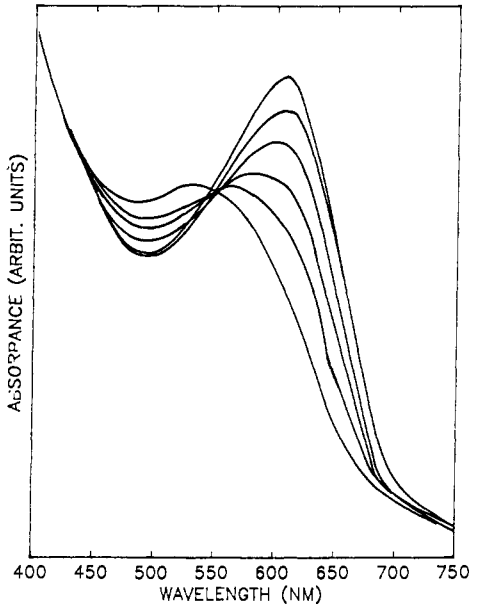

FIGURE 2: pH dependence of the spectrum of AMBR. From the top: pH 7.2, 8.9, 9.4, 9.8, 10.3, and 11.

becomes more clear upon prolonged illumination. However, together with the blue-shifted absorption maximum, an absorbance increase is observed around 530 nm. Consecutive traces obtained after prolonged illumination exhibit an approximate isosbestic point. However, the spectrum of the nonilluminated species and that obtained after 5 s of illumination do not share this isosbestic point. Thus, at least three species are involved in the total photoreaction. The first absorbance change goes back in the dark within approximately 25 s at room temperature. (It takes about 10 s to record the spectrum after illumination.) The product with an absorption maximum around 530 nm, however, is stable for at least 2 h and relaxes back to the initial state in the dark with a half-time of 8 h. Therefore, it will be called the metastable state. It can be driven back to the initial state absorbing at 620 nm by illumination with light around 530 nm, yielding a spectrum indistinguishable from the solid traces of Figure 1.

It is known that at alkaline pH the absorption maximum of BR is blue-shifted. A similar effect is observed for AMBR (Figure 2). Increase of the pH to 8.9 leads to a reduction of the absorbance. Further increase demonstrates the blue shift. An approximate isosbestic point is obtained for the spectra at pH 8.9 and higher, which is not shared by the spectrum obtained at pH 7.2. The absorption maximum of the alkaline species is around 520 nm.

Figure 3, upper trace, shows the time-resolved transmission change at 625 nm of AMBR at 300 K. It is striking that the decay of the signal extends up to 10 s. Therefore, a low repetition rate of 0.1 s^{-1} had to be employed. Nevertheless, 80% of the signal amplitude recovered already after 0.5 s. The middle trace displays the corresponding signal at higher time resolution. Similar signals were obtained throughout the spectral range between 400 and 720 nm. All the signals can be fitted by four exponentials with rate constants of 100 ± 15 s^{-1}, 28 ± 3.5 s^{-1}, 5.3 ± 0.8 s^{-1}, and 0.25 ± 0.03 s^{-1}. At 625 nm, the relative amplitudes are 29%, 36%, 21%, and 14%, respectively. The difference between the signal and the fitted curve involving the first three exponentials is shown in the lower trace of Figure 3. Since no systematic deviations are observed, the fit is satisfactory.

The time-resolved difference spectra are depicted in Figure 4A for various times after the flash. Measuring conditions

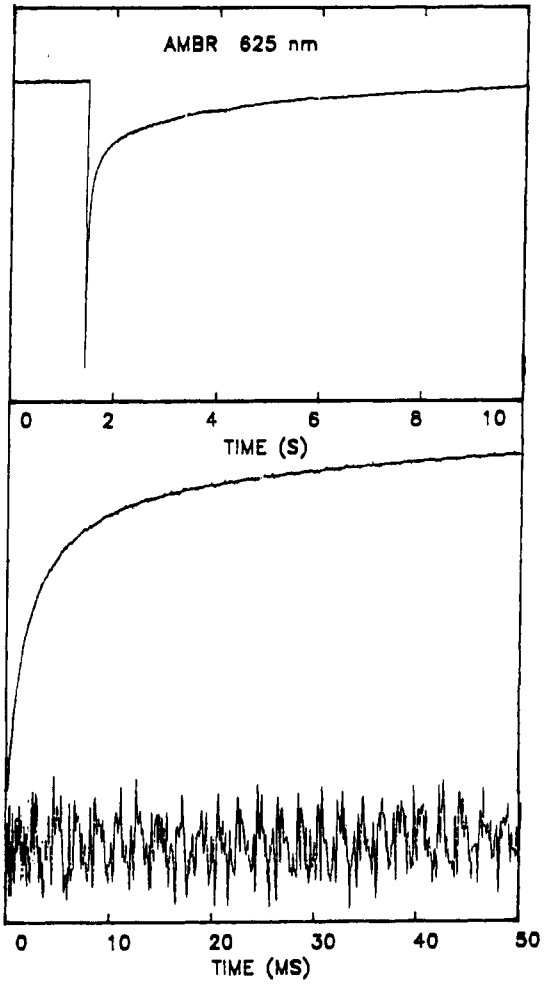

FIGURE 3: Time-resolved absorbance changes of AMBR at 625 nm. Upper trace: Dwell time 10 ms. Middle trace: The dual dwell time facility of the signal averager was employed. First 512 channels, 10 μs; last 512 channels, 100 μs. This trace contains also the fitted curve. Lower trace: Difference between signal and fitted curve in the time range of the middle trace. As compared to the middle trace, ordinate scale is expanded by a factor of 80.

are described under Materials and Methods. It should be noted that the evoked absorbance changes are very small as compared to those of BR: if the signals are normalized to equal absorbances at the excitation wavelength, the depletion signal is reduced by a factor of 10 and the absorbance rise at 410 nm by a factor of 20. In addition, it can be seen that the slower components dominate at lower wavelengths. The relative amplitudes (in order of increasing time constant) are as follows: at 695 nm, 35%, 39%, 18%, and 8%; at 575 nm, 15%, 28%, 29%, and 28%; at 545 nm, 0%, 10%, 55%, and 35%; and at 410 nm, 15%, 8%, 40%, and 37%.

Since by prolonged illumination with red light the metastable photoproduct is obtained with an absorption maximum around 530 nm, the question arises as to what degree the measuring beam in the flash photolysis experiments alters the composition of the sample. Therefore, the absorption spectra before and after the series of measurements above 600 nm were compared. No significant difference could be detected. Therefore, the amount of the metastable photoproduct is not significantly altered during the course of the measurement. In addition, the spectrum shows only a small deviation from the spectrum of completely dark-adapted AMBR. This indicates that less than 10% of the metastable state is present.

The time-resolved difference spectra exhibit a clear absorbance increase at 410 nm. To decide whether this increase might be due to the M intermediate formed from residual

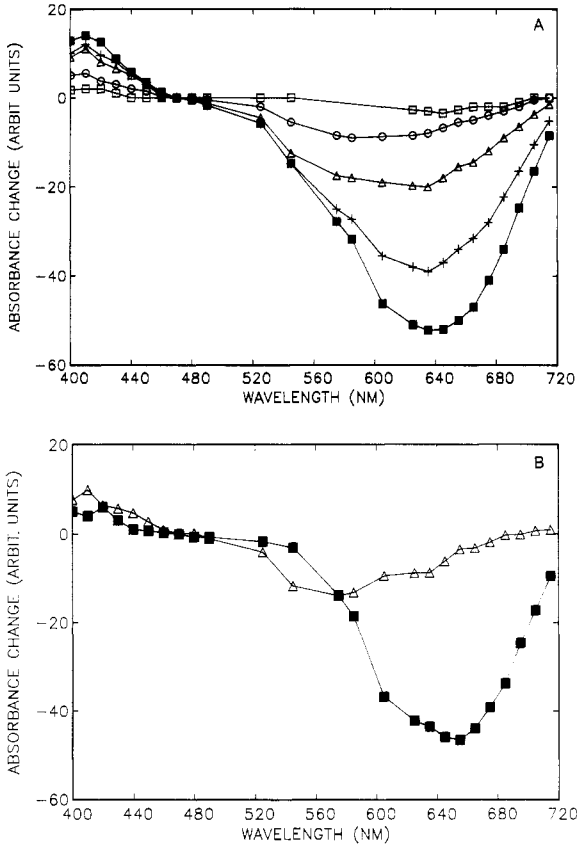

FIGURE 4: (A) Time-resolved UV–vis difference spectrum of AMBR for various times after the flash at room temperature: (■) 10 μs; (+) 10 ms; (△) 50 ms; (○) 500 ms; (□) 4 s. (B) Reconstructed difference spectra for the "pure" AMBR (■) and for the back-converted AMBR (△) species. Methods to obtain these spectra are given in the text.

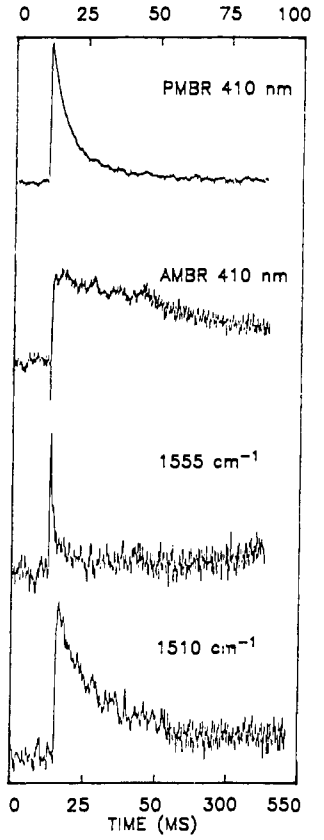

FIGURE 5: Signals corresponding to the time-resolved absorbance changes in the UV–vis and in the infrared spectral range. From the top: Signal at 410 nm of PMBR, dwell time 100 μs; signal at 410 nm of AMBR, dual dwell time facility, 100 μs first 512 channels, 1 ms last 512 channels; signal at 1555 cm^{-1}, same dwell time facility; signal at 1510 cm^{-1}, same dwell time facility.

Table I: Composition of the Retinal Isomers for Various States of AMBR Presented as Molar Percentage[a]

sample	13-cis	11-cis	9-cis	all-trans
dark adapted[b]	<6	<3	<3	>88
40-s illum[c]	14 ± 2	12 ± 2	9 ± 2	65 ± 2
15-min illum[d]	9 ± 2	9 ± 2	37 ± 2	45 ± 2
pH 10.3, dark[e]	30 ± 2		4 ± 1	66 ± 2
BR control, pH 10.5 dark[f]	54 ± 1			45 ± 1

[a] Light intensities are given under Materials and Methods. [b] Dark-adapted AMBR. [c] Illumination for 40 s with red light. [d] Illumination for 15 min with red light. [e] Dark-adapted AMBR at pH 10.3. [f] BR control, extraction at pH 10.5 in the dark.

permethylated BR, the decay of the corresponding signal was compared with that of PMBR. The result is shown in Figure 5, upper two traces. Such residual permethylated BR could arise by a slow thermal removal of the derivative of *o*-phthalaldehyde used to block the nonmethylated active-site lysine, and subsequent exchange of retinals. To discriminate this form from normal PMBR, it will be called back-converted AMBR. The time course of the signal decay at 410 nm of PMBR differs little from that of untreated BR (data not shown). The large difference in the decay rate constants between AMBR and PMBR (Figure 5) shows clearly that negligible amounts, if any, of PMBR contribute to the signal of AMBR. However, under Discussion, evidence will be presented that about 60% of the signal is caused by back-converted AMBR, which exhibits an M intermediate of which the decay was prolonged, probably by the *o*-phthalaldehyde/mercaptoethanol treatment.

The quantitative evaluation of the HPLC analysis of the retinal extraction is given in Table I. The unilluminated sample, kept for 24 h in the dark, contained essentially only *all-trans*-retinal (row 1 of Table I) when the retinal was extracted in the dark. Medium-time (40 s) illumination with light of wavelengths greater than 640 nm increases the amount of 13-*cis*-retinal and the 9-cis and 11-cis isomers (row 2 of Table I). Prolonged illumination (15 min) produces mostly the 9-cis isomer, and the amount of the 13-cis and 11-cis isomers is reduced (row 3 of Table I). Even at very low light intensities small, variable but measurable amounts of 9-cis and 11-cis isomers were produced. Due to this background, which is difficult to avoid, we were unable to detect the small changes of isomeric composition during the first 5 s of illumination, during which mainly a decrease of the absorbance is observed (Figure 1). The data show that the metastable photoproduct obtained after prolonged illumination contains essentially 9-*cis*-retinal. However, they do not allow conclusions on the isomerization during the primary photoreaction. The retinal extraction at pH 10.5, at which pH the absorption maximum is shifted to 520 nm (Figure 2), reveals a large increase of the 13-cis isomer (Table I, row 4). The all-trans/13-cis ratio approximates that of the thermal equilibrium of retinal.

Measurements in the visible spectral range showed that no photoreaction could be evoked by illuminating AMBR at 80 K with light of wavelengths above or below the absorption maximum. This was confirmed by FTIR difference spectroscopy. However, Figure 6A shows that, if first AMBR was illuminated at room temperature for 15 min with red light (>640 nm) to produce the metastable state and then rapidly cooled to 80 K, a photoreaction could be evoked by illumination with blue-green light (<540 nm). For comparison with the other difference spectra, the usual convention for the sign of the bands has been changed: bands of the initial state, i.e.,

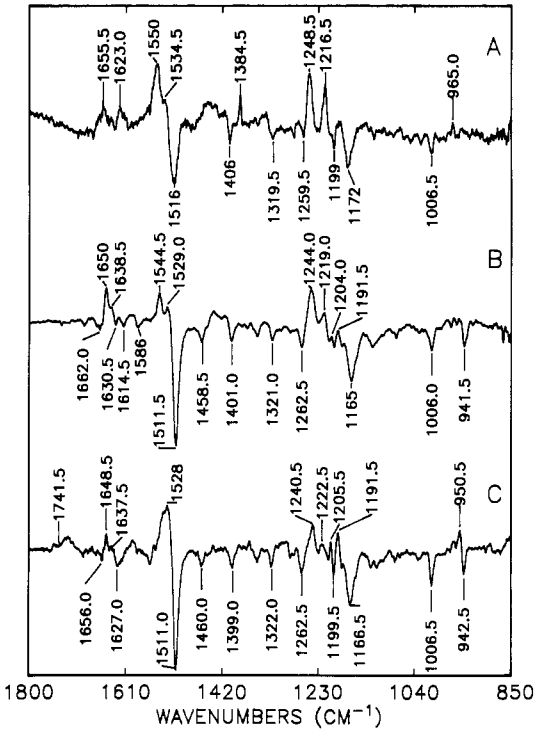

FIGURE 6: Static FTIR difference spectra of AMBR, spectral resolution 2 cm^{-1}. (A) Difference spectrum between the metastable photoproduct and its photoproduct obtained at 80 K. (B) Difference spectrum between dark-adapted AMBR and the metastable photoproduct at 300 K. (C) Difference spectrum between dark-adapted AMBR and its photoproducts at 173 K. Each single-beam spectrum was measured with 512 scans.

of the metastable species, point upward, whereas those of the photoproduct point downward. Control measurements in the visible showed that a species was obtained absorbing around 610 nm. This is confirmed by the ethylenic stretching frequency at 1516 cm^{-1}. Subsequent illumination with red light (>640 nm) does not evoke a photoreaction; i.e., the photoreaction is not photoreversible. Thus, one might argue that the original 620-nm species is produced. Figure 6B shows the difference spectrum between AMBR and the metastable photoproduct obtained at 300 K. Here, dark-adapted AMBR was illuminated for 10 min with red light (>640 nm). The comparison indicates that the overall appearance of the two difference spectra is very similar. Most of the bands have their counterparts. But there are also clear differences. The ethylenic mode of the red-absorbing species obtained at 80 K is at higher frequencies and causes a broader band, and there is an additional band of the metastable species at 1384.5 cm^{-1}. The band at 941.5 cm^{-1} of the 620-nm species at 270 K is not present in the 610-nm photoproduct produced at 80 K. Since the metastable state in both spectra is the same, the red-absorbing photoproduct obtained at 80 K must be somewhat different from dark-adapted AMBR. The absence of strong HOOP bands between 1000 and 850 cm^{-1} indicates that the chromophore in the three species is in a relaxed conformation. It should be mentioned that the photoreversibility of the metastable photoproduct shown by UV–vis spectroscopy (see above) could also be demonstrated by FTIR difference spectroscopy: the same spectrum as that of Figure 6B, but with opposite sign, was obtained, if the metastable state was illuminated for 5 min with blue-green light (<540 nm).

To determine whether an L-like intermediate can be produced at the temperature at which L can normally be stabilized (173 K), the corresponding difference spectrum was measured for AMBR (Figure 6C). At this temperature, a phototransition can now be evoked. Dark-adapted AMBR was illuminated for 5 min with red light (>640 nm). The spectrum shows great resemblance to that of trace B of Figure 6, suggesting that mainly the transition to the metastable species is observed. However, there are also clear deviations present. Positive bands can be seen between 1720 and 1755 cm^{-1}, the feature around 1650 cm^{-1} is shifted to lower frequencies, and the positive bands at 1191.5 and 950.5 cm^{-1} are increased. Thus, in addition to the metastable state, a different photoproduct must have been generated. The photoreaction, in contrast to the behavior at 80 K, is now completely photoreversible (data not shown). This means that both species produced at 173 K can be reconverted to the initial state absorbing at 620 nm.

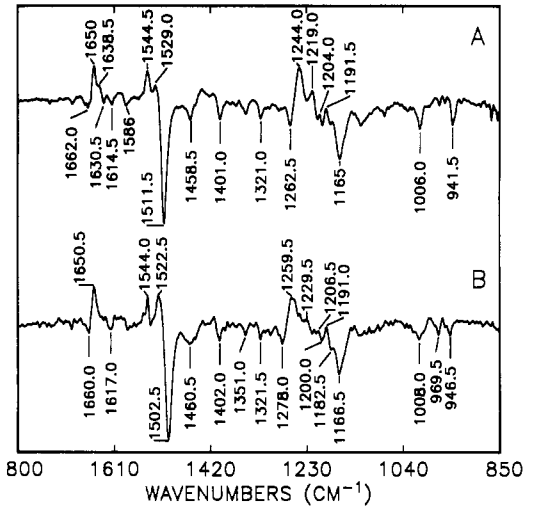

FIGURE 7: Static FTIR difference spectrum of AMBR and its metastable photoproduct at 300 K. (A) Native AMBR. (B) AMBR regenerated with [15-^{2}H]retinal.

To obtain information on the molecular changes occurring between AMBR and the metastable photoproduct, the difference spectrum of AMBR regenerated with [15-^{2}H]retinal was measured. As mentioned under Materials and Methods, the regeneration of bleached AMBR is rather poor, yielding an absorbance ratio A_{280}/A_{620} of only 6. Therefore, the noise in the difference spectra is larger. The two difference spectra are shown in traces A and B of Figure 7, for unmodified AMBR and [15-^{2}H]AMBR, respectively. As expected, deuteration at C_{15} causes a downshift of the ethylenic modes of AMBR (1502.5 vs 1511.5 cm^{-1}) and of the photoproduct (1522.5 vs 1529 cm^{-1}). In addition, the band structure between 1220 and 1270 cm^{-1} is shifted up, indicating that the corresponding modes are coupled to the 15-H in-plane bending vibration. A new negative band appears at 969.5 cm^{-1}, which corresponds probably to the 15-^{2}H rocking vibration of AMBR.

Due to the high probability of producing secondary photoproducts, it is difficult to obtain molecular information on the primary photoproduct by the static techniques employed. However, it is the single-photon photoproduct that should be compared with the L intermediate of normal BR. The time-resolved investigations in the UV–vis have shown that the photoproduct obtained within 10 μs has a long lifetime (Figure 3). Therefore, time-resolved fast-scan FTIR spectroscopy, which allows the acquisition of spectra within approximately 10 ms, can be applied for the investigation of this photoproduct (Gerwert, 1988; Braiman et al., 1987). Figure 8 shows the corresponding difference spectra for the first scan after the flash for normal AMBR (A) and [15-^{2}H]AMBR (C). Again, the noise is much larger in the spectrum of the

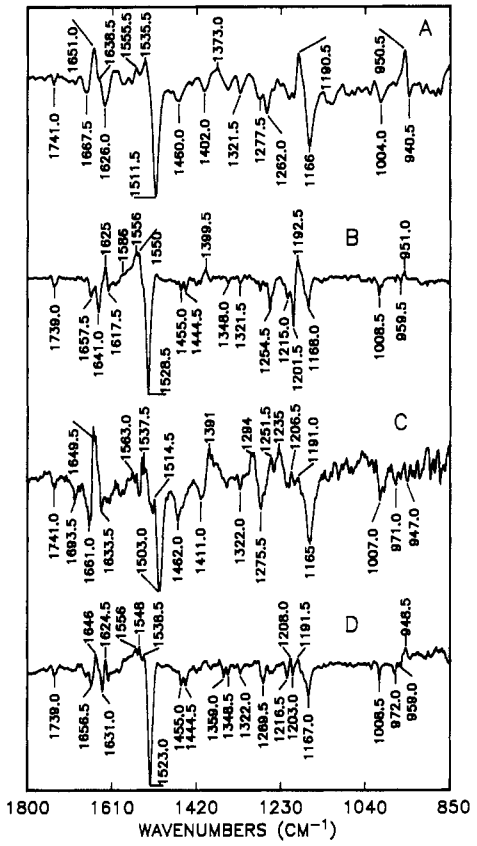

FIGURE 8: Time-resolved FTIR difference spectra of AMBR at 300 K and static BR – L difference spectra of normal BR at 173 K. (A) Time-resolved FTIR difference spectrum of native AMBR. (B) Static BR – L difference spectrum of native BR. (C) As in (A), but AMBR regenerated with [15-^{2}H]retinal was used. (D) As in (B), but BR regenerated with [15-^{2}H]retinal was used. Measuring conditions: (A) resolution 4 cm^{-1}; four difference spectra, of which the single-beam spectra were measured with 256 scans, were coadded; (B) resolution 2 cm^{-1}; the single-beam spectra were measured with 256 scans; (C) as in (A), but eight difference spectra were coadded; (D) as in (B).

latter compound. For comparison, the low-temperature static BR – L difference spectra of unmodified BR and [15-^{2}H]BR are depicted in traces B and D, respectively. The comparison with the difference spectra of Figures 6 and 7 clearly shows that the transient photoproduct is different from the metastable species. But great similarities exist between the time-resolved difference spectra and the BR – L difference spectra. The presence of strong positive bands at 1190.5 and 950.5 cm^{-1} in the spectrum of unmodified AMBR indicates that this intermediate is also present in the difference spectrum obtained at 173 K (Figure 6C), although at low yield. Thus, as in normal BR, an L-like intermediate can be produced and stabilized at 173 K which is photoreversible.

The difference spectrum obtained for the second scan after the flash (ca. 100-ms delay) shows the same spectral features, only reduced by a factor of approximately 5 (data not shown). Thus, the species that is still present after the fast decay of the signal (Figure 3) is essentially the same as that present during the first 10 ms after the flash. Earlier intermediates cannot be detected by the fast-scan FTIR technique. Therefore, we employed our infrared flash photolysis setup to AMBR (Siebert et al., 1980, 1981). The technique is essentially the same as a normal flash photolysis apparatus but employs an infrared monitoring beam from an infrared spectrophotometer, and the difference spectrum is sampled point by point by measuring every five wavenumbers. Here, the time resolution is only limited by the detector response and by the electronics. For these investigations the time resolution

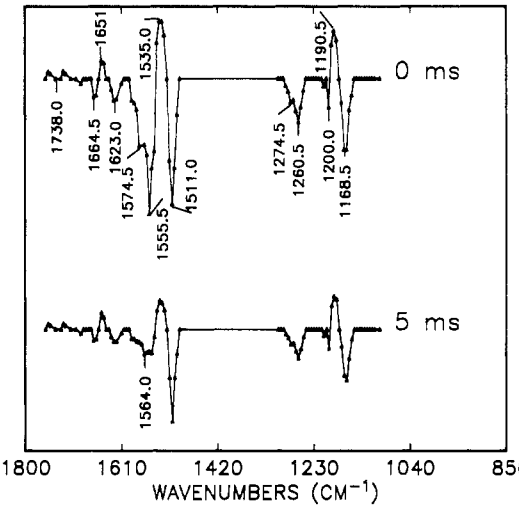

FIGURE 9: Time-resolved IR difference spectra of AMBR obtained with the infrared flash photolysis apparatus. Upper trace: 100 μs after the flash. Lower trace: 5 ms after the flash. Spectral resolution varies between 5 and 8 cm^{-1}. For each point in the spectra 300 signals were averaged, and the obtained traces were further averaged manually.

was 100 μs, but within the limits imposed by the noise, no additional features appeared for measurements with a dwell time of 10 μs. The corresponding difference spectrum is shown in Figure 9 for 100 μs (denoted as 0 ms) and 5 ms after the flash. Since the measurement is very time-consuming, the range between 1500 and 1300 cm^{-1} was omitted. Within noise limits, the spectrum obtained 5 ms after the flash and the time-resolved FTIR spectrum (Figure 8) are indistinguishable. However, a clear difference is observed for the spectrum obtained immediately after the flash. Here a strong negative band is present at 1555 cm^{-1}. The decay of this band is very fast, as shown in Figure 5, lower two traces. Here, the signals corresponding to the absorbance changes at 1510 and 1555 cm^{-1} are compared. The rate constant is approximately 1000 s^{-1}, faster than any of the rate constants observed in the UV–vis.

Discussion

The minimum in the time-resolved UV–vis difference spectrum (Figure 4A, first spectrum after the flash) is clearly red-shifted (635 nm) as compared to the absorption maximum of AMBR (620 nm). This indicates that the spectra of the initial state and of the photoproduct overlap. This is further supported by the small size of the signals. The 10-fold reduction of the depletion signal of AMBR as compared to BR or PMBR cannot be explained by a reduced quantum efficiency, since the flash-evoked infrared difference spectra (Figure 8) exhibit comparable absorbance changes if normalized to equal absorbances at the wavelength of the exciting laser (590 nm). It is striking that the minimum of the depletion band becomes very broad for longer times, extending from 625 to 575 nm in the spectrum taken 500 ms after the flash. This suggests that an additional, blue-shifted component undergoes a photoreaction with a slow decay time constant. The two fast components are almost absent at 525 and 545 nm, indicating that these two fast components are not involved in the photoreaction of this additional species. However, the two components comprise 74% of the signal at 695 nm, 65% of the signal at 625 nm, and 23% of the signal at 410 nm. Assuming that the signal observed at 695 nm is only due to the photoreaction of the 620-nm species, one can calculate the total (fast and slow) difference spectrum for the photoproduct from the 620-nm species alone [difference between spectrum

1 (10 μs) and spectrum 3 (50 ms) divided by 0.74]. The result is shown in Figure 4B (solid squares). The remaining difference spectrum (difference between spectrum 1 of Figure 4A and the difference spectrum of the 620-nm species) reflects then the photoreaction of the additional species (Figure 4B, open triangles). It resembles very much a normal BR − M difference spectrum. Due to the large noise present in the signal at 410 nm (Figure 5), which would be larger by a factor of 3 if it would be measured with the maximum time resolution (dwell time 10 μs), we were unable to resolve a component characteristic for the rise of an M intermediate. But the similarity of the difference spectrum, especially the ratio of the maximum to the minimum, indicates that it reflects the photoreaction of back-converted AMBR. Since the decay time constants are very long, one has to assume that the additional manipulation of permethylated bacterioopsin with *o*-phthalaldehyde/mercaptoethanol influences the protein to some extent, in addition to the modification of the active-site lysine. From the small size of the depletion signal mentioned above and from the two spectra in Figure 4B one can estimate the amount of back-converted AMBR to be less than 4% and, thus, negligible for the further investigations. The difference spectrum of the 620-nm species (Figure 4B) shows even more clearly the overlap between the initial state and the photoproduct. The minimum is now further red-shifted to 650 nm. Between 460 and 525 nm the absorbance changes are very small. The residual absorbance increase around 420 nm can be explained by a somewhat larger absorbance of the blue-shifted photoproduct. It is not clear why the slow kinetics of the photoreaction of back-converted AMBR are also observed in the photoreaction of the 620-nm species. It could be that it is also influenced by the *o*-phthalaldehyde/mercaptoethanol treatment.

Table I demonstrates that completely dark-adapted AMBR contains essentially *all-trans*-retinal only. This is in contrast to normal dark-adapted BR, for which a 13-cis:all-trans ratio of 2:1 prevails (Scherrer et al., 1989). The preference of the all-trans isomer is probably due to the steric interaction of the methyl group at the Schiff base in AMBR with the protein. In line with this hypothesis, this interaction inhibited the formation of a photoproduct at 80 K. The protein is too rigid to allow for a trans/cis isomerization. On the other hand, at alkaline pH the isomeric composition approaches that of the thermal equilibrium between *all-trans*- and 13-*cis*-retinal in solution. Thus, alkaline pH opens the conformation of the protein, thereby releasing the proposed steric interaction. A blue-shifted alkaline species of BR has also been investigated by Druckmann et al. (1982), and the spectral shift was explained by the deprotonation of the Schiff base. However, in the case of AMBR, no deprotonation can occur and the reversible blue shift must be caused by an altered chromophore–protein interaction. The lack of an isosbestic point (Figure 2) suggests two molecular causes for the spectral changes. The first step of alkalinization, resulting mainly in a reduction of the absorbance, opens the protein conformation and allows for the new isomeric equilibrium. Further alkalinization probably changes the interaction of the positively charged Schiff base with its environment and, thus, causes the blue shift.

At 173 K, however, the protein is flexible enough to allow for a photoreaction (Figure 6C). The state produced under these conditions is essentially the metastable state containing predominantly 9-*cis*-retinal, which is produced at room temperature upon prolonged illumination with red light (Figures 6B and 7). But the small additional bands indicate that at 173 K another state can be stabilized (see below). The photoreaction is completely photoreversible at this temperature. If the metastable state is cooled to 80 K, a photoproduct can now be produced by illumination with blue-green light (Figure 6A), which has some similarities to dark-adapted AMBR, containing essentially *all-trans*-retinal only. Therefore, this photoproduct also contains predominantly *all-trans*-retinal. But the deviations in the difference spectrum from that of Figure 6B show that the two states involved are different from those of Figure 6B. Apparently, some band positions in the fingerprint region of the metastable state exhibit a small temperature dependence (1248.5 vs 1244 cm^{-1} and 1216.5 vs 1219 cm^{-1}). Such an effect is not observed in normal BR. The higher frequency of the ethylenic mode (1516 cm^{-1}) indicates that the absorption maximum of this photoproduct is around 610 nm, i.e., less red-shifted than that of AMBR (620 nm). In view of the discussion presented above, the more open protein conformation of the metastable state does allow for a photoreaction even at 80 K, leading to the isomerization of the chromophore from 9-cis to all-trans. But since the protein is rigid, the photoproduct cannot relax to dark-adapted AMBR, although it resembles it to a certain degree. The lack of strong HOOP modes shows that, nevertheless, the chromophore adopts a rather planar geometry in both states. Therefore, the difference between the 610-nm photoproduct obtained from the metastable state at low temperature and dark-adapted AMBR must be due to the different protein structures.

The UV–vis data together with the retinal extraction experiments suggest that the metastable state, containing essentially 9-*cis*-retinal, is produced by a secondary photoprocess. The state produced by short-time illumination (Figure 1) relaxes back in 25 s, i.e., more slowly than the signal produced by a flash (Figures 3 and 5). Therefore, this state also may already contain a secondary photoproduct, which, however, is different from the metastable one. The retinal extraction data obtained for low light intensities indicate that this state contains 9-cis and 11-cis isomers. It is well-known that BR can, under special conditions, also accommodate 9-*cis*-retinal (Chang et al., 1987). But the presence of 11-*cis*-retinal upon medium-time illumination (Table I) appears to be unique. It is conceivable that the methyl group at the Schiff base nitrogen provides more space for the chromophore by steric interaction with the protein during the photoreaction at room temperature. In view of the complex isomeric compositions evident from Table I, the apparent isosbestic point in Figure 1 must be regarded as fortuitous.

The red shift of the absorption maximum of AMBR (50 nm) is much larger as compared to that of active-site-methylated rhodopsin or methylated retinal Schiff bases (Longstaff & Rando, 1985). This might be due either to electronic effects such as reduced interaction of the positively charged Schiff base with its counterion or to geometric effects. The low frequency of the ethylenic mode is in accord with the red-shifted absorption maximum, solely indicating greater charge delocalization, which may be due to either causes. The C=N stretching vibration, however, could perhaps discriminate between the two effects. The electronic effects should reduce the C=N frequency, whereas the geometric effects should not alter it appreciably. To identify the C=N mode, the difference spectrum of the metastable state was measured with AMBR regenerated with [15-^{2}H]retinal. The comparison of the two difference spectra in Figure 7 indicates some changes between 1620 and 1630 cm^{-1}, but a clear band cannot be identified. This is probably due to the fact that the C=N

frequencies of the initial state and of the photoproduct overlap. The frequency range, however, would be similar to the position of the C=N stretch of the deuterated Schiff base of BR. A more detailed discussion of this result will be postponed until the description of the time-resolved difference spectra, where the C=N stretch can more accurately be identified.

Besides causing the downshift of the ethylenic mode from 1511.5 to 1502.5 cm^{-1}, somewhat larger than in normal BR, deuteration at C-15 has pronounced effects on the fingerprint modes in Figure 7, of which most are upshifted. This indicates a coupling of these modes with the 15-H rocking mode. Only the 1165-cm^{-1} band of dark-adapted AMBR is only little affected. In comparison to normal BR, this indicates that this band can be assigned to the C_{10}–C_{11} stretching vibration. Since the metastable state still contains a mixture of retinal isomers (Table I), the photoproduct fingerprint bands cannot be assigned. The ethylenic mode of this state exhibits nevertheless a single band, which is shifted from 1529 to 1522.5 cm^{-1} upon 15-^{2}H labeling. This indicates an absorption maximum around 560 nm. As in normal BR, the 15-^{2}H rocking vibration of AMBR can be assigned to the band at 969.5 cm^{-1} (Figure 7b).

The comparison of the time-resolved FTIR difference spectra of normal and labeled AMBR with the corresponding BR – L difference spectra reveals many similarities (Figure 8). A feature around 1740 cm^{-1}, which was attributed to protonated aspartic acids (Engelhard et al., 1985; Eisenstein et al., 1987) and later to the specific residues Asp96 and Asp115 (Braiman et al., 1988; Gerwert et al., 1989), can be seen in all the spectra. The fingerprint region reveals again the band at 1166 cm^{-1} of AMBR (Figure 8A), which was already assigned to the C_{10}–C_{11} stretching mode due to its lack of sensitivity to 15-^{2}H labeling (Figure 8C). It corresponds to the bands at 1168 and 1167 cm^{-1} in traces B and D of Figure 8. The positive band at 1190.5 cm^{-1} corresponds to the band at 1192.5 cm^{-1}, which represents the C_{10}–C_{11} stretching mode of the L intermediate. As in the BR – L difference spectra, it loses intensity upon 15-^{2}H labeling. The negative band at 1262 cm^{-1} with a shoulder at 1277 cm^{-1} is similar to a band structure of BR with deuterated Schiff base (Siebert & Mäntele, 1983). The corresponding band of the protonated Schiff base is at 1254.5 cm^{-1} (Figure 8D). The further upshift upon deuteration at C-15 (Figure 8C) is again also observed in normal BR (Siebert & Mäntele, 1983). The upshift produced upon deuteration of the Schiff base is explained by the release of the coupling of this mode with the N–H bending vibration. Therefore, it is not surprising that in AMBR this mode is also upshifted as compared to normal BR. The main difference in the fingerprint region is the reduced intensity of the band around 1200 cm^{-1}, which, in normal BR (1201.5 cm^{-1}, Figure 8B), was assigned to the C_{14}–C_{15} stretching mode (Smith et al., 1987; Gerwert & Siebert, 1986). But it is still clearly visible, as is the mode at 1215 cm^{-1}, which was assigned to the C_8–C_9 stretching vibration (Smith et al., 1987). The fingerprint region clearly shows that the flash-induced photoreaction of AMBR involves an all-trans/13-cis isomerization and that a state very similar to normal L is generated. The low frequency of the ethylenic mode (1535 cm^{-1}, Figure 8A), which shifts down upon 15-^{2}H labeling, indicates that this L-like intermediate is comparably red-shifted with respect to L as AMBR with respect to BR.

The comparison of the Schiff base region of spectra A and C of Figure 8 shows that 15-^{2}H labeling of the retinal removes a negative band around 1626 cm^{-1}, suggesting that this band can be identified with the C=N stretch of AMBR. It is not completely clear where the band is shifted to. Since the left-hand part of the broad positive band at 1600 cm^{-1} of Figure 8A is missing in Figure 8C, the band may be shifted to approximately 1608 cm^{-1}. But the noise present in this figure makes the assignment of the shifted band not completely satisfactory. But there is a general tendency that the intensity of low-frequency C=N stretches is very weak [e.g., the modes of the deuterated Schiff bases of the K intermediate (Gerwert & Siebert, 1986) and of the deionized blue membrane (Fahmy & Siebert, 1989)]. The disappearance of the negative band around 1626 cm^{-1} is, however, clear. It is interesting to compare the position of the C=N stretch of AMBR with those of other methylated Schiff bases. In Figure 10, the infrared spectrum for regions of the C=N and C=C stretches of methylated retinylidene butylamine Schiff base is shown. (The compound was prepared by condensing retinal with the BF_4^- salt of methylbutylamine, with a small excess of the free amine, in CH_2Cl_2.) Since no carbonyl band around 1660 cm^{-1} can be seen, the Schiff base was formed quantitatively. The spectrum shows that the C=N stretch is located at 1630 cm^{-1}, in agreement with resonance Raman data (Marcus et al., 1979). Our investigations on active-site-methylated rhodopsin allow the assignment of the C=N stretch to a band at 1624 cm^{-1} (U. M. Ganter, C. Longstaff, R. R. Rando, and F. Siebert, unpublished results). Thus, for all three compounds the C=N stretches of the methylated Schiff bases are located at positions of the corresponding deuterated Schiff bases. The model compound and active-site-methylated rhodopsin show only slightly red-shifted absorption maxima [5 and 25 nm, respectively (Longstaff & Rando, 1987)] as compared to the corresponding protonated Schiff bases. Therefore, it can be expected that the C=N force constant is not appreciably altered. Thus, the observed downshift of the C=N band of the methylated Schiff bases must be caused by altered couplings of the C=N mode with other vibrations. To investigate which mode couples most strongly, we performed simplified normal-mode calculations of a model compound truncated at C_{12}, using the force field developed for protonated retinal Schiff bases (Smith et al., 1985). The N–C_{methyl} force constants were adopted from the N–C_{amine} force constants, whereas the C_{methyl}–H force constants were taken from the methyl group at C_{13}. The calculations show that the N–C_{methyl} stretching mode couples most strongly. Depending on the value of the C=N/N—C coupling, the C=N mode can be shifted easily between 1660 and 1620 cm^{-1}. Thus, without additional vibrational investigations using isotopically labeled retinals and methyl groups, an explanation for the coincidence of the positions of methylated and deuterated Schiff bases cannot

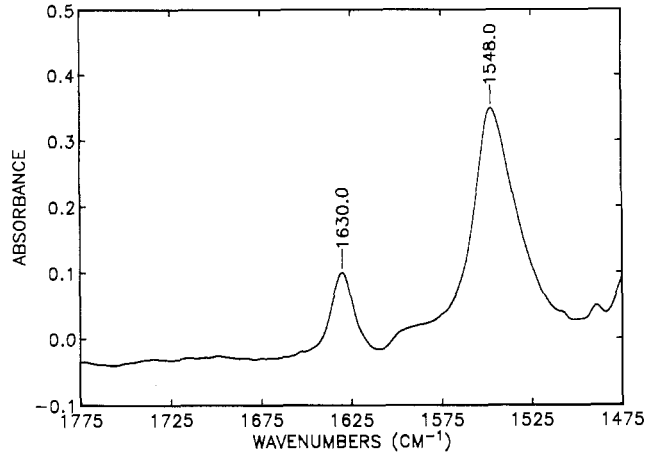

FIGURE 10: Infrared spectrum of methylated retinylidene butylamine Schiff base in CH_2Cl_2.

be given. However, from the results obtained for active-site-methylated rhodopsin and the methylated Schiff base model compound and from the equal positions of the C=N stretches of AMBR and deuterated BR it can be deduced that the large red shift observed for AMBR does not greatly alter the C=N force constant. For the deionized blue membrane, which has an absorption maximum at 605 nm, the C=N frequency was determined to 1627 cm^{-1} (Fahmy & Siebert, 1989). This suggests that the C=N force constant of the deionized membrane is probably smaller than the force constant of AMBR. Therefore, the mechanisms of the red shifts in those two systems are probably different. Altered steric interactions could contribute to the red shift. Indeed, it was recently shown that the chromophore in BR is twisted around the 14–15 and 10–11 single bonds (Fahmy et al., 1989). Interestingly, no further red shift could be observed upon deionization of AMBR. The low-frequency modes of AMBR (940.5 cm^{-1}) and its L-like photoproduct (950.5 cm^{-1}) may be due to either the methyl group (e.g., rocking vibration or $N-CH_3$ stretching vibration) or the 14-H or 15-H HOOP modes. Without further labeling a decision between those alternatives cannot be made.

The origin of the strong negative band at 1555 cm^{-1} in Figure 9 (0 ms) is not completely clear. Its position suggests that it is caused by an amide II mode of the protein backbone. But there is no corresponding large amide I mode visible. Nevertheless, in addition to the position, the fast decay time constant, which is observed neither in the UV–vis nor for the signal of the ethylenic mode at 1510 cm^{-1}, indicates that this band is actually caused by the protein. Again, this protein distortion may be caused by the methyl group of the Schiff base nitrogen.

Taken together, the data show that methylation of the Schiff base in BR introduces an additional steric interaction with the protein which inhibits the photoreaction at low temperature. The distortion caused by the introduction of this methyl group, however, is weak enough to allow the formation of an L-like intermediate at room temperature and even, to some extent, at 173 K. Therefore, the conclusions drawn by Longstaff and Rando (1987) that deprotonation of the Schiff base is a prerequisite for proton pumping are correct.

Registry No. Retinal, 116-31-4.

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