Low temperature FTIR study of the Schiff base reprotonation during the M to bR backphotoreaction

Asp 85 reprotonates two distinct types of Schiff base species at different temperatures

H. Takei,* Y. Gat,‡ M. Sheves,‡ and A. Lewis*§

*Department of Applied Physics, Cornell University, Ithaca, New York 14853 USA; ‡Department of Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel; and §Division of Applied Physics, The Hebrew University of Jerusalem, Jerusalem, Israel

ABSTRACT We have applied low temperature difference FTIR spectroscopy to investigate intermediates produced from the M intermediate upon blue light excitation (<480 nm). In agreement with an earlier report by Balashov and Litvin (1981), who studied these intermediates with low temperature visible absorption spectrophotometry, we have observed at least three stages in this backphotoreaction. The initial photoproduct is stable at 100 K, and two products of subsequent thermal reactions are observed upon raising the temperature to 130 and 160 K, respectively.

The alterations in the C=N stretching mode of the Schiff base have been identified by isotopically labeling the retinal chromophore, and changes in C=O stretching modes of amino acid residues with acidic side chains have been investigated. Analysis of the C=N stretching mode shows that the Schiff base remains unprotonated after the photochemical reaction at 100 K. Moreover, there are two types of Schiff bases, presumably associated with different bR species, that become thermally reprotonated at 130 and 160 K, respectively. Bands associated with the C=O stretching modes suggest that Asp 85 rather than Asp 96 reprotonates the Schiff base during the M to bR backphotoreaction. This conclusion is consistent with earlier observations that the polarity of electrical signals during this photochemical back reaction is reversed as compared to the thermal regeneration of bR from M.

INTRODUCTION

Bacteriorhodopsin, bR, is the only protein found in the purple membrane (pm) of a photosynthesizing bacterium called *Halobacterium halobium* (Oesterhelt and Stoeckenius, 1971). The bR molecules, which transverse the entire cross-section of pm, are vectorially oriented. The physiological role of bR is to pump protons across the membrane by the absorption of light thereby generating a proton concentration gradient across the pm. The light absorbing entity in bR is a complex of retinal covalently linked to an amino acid lysine in the single amino acid chain that is the bR protein. The covalent linkage of the retinal to the lysine is via a protonated Schiff base. During the light driven proton pumping process the retinal lysine complex in the initial pigment state undergoes a series of distinct absorption changes (see Fig. 1) that are classified in terms of a number of intermediates (Lozier et al., 1975). One of the intermediates that is associated with the proton pumping process is called M and this intermediate, which absorbs 158 nm to the blue of the 570 nm absorption of the initial pigment state, is thought to be a crucial intermediate in the proton pumping process since it is at this intermediate that the Schiff base loses its proton (Lewis et al., 1974). M, as with all the intermediates after the phototransformation of bR, can absorb light of the appropriate frequency and be returned to bR (Kalisky et al., 1977; Ohno et al., 1983). The reverse photoreaction of the M intermediate has attracted a great deal of attention and three distinct species have been identified using absorption spectroscopy (Balashov and Litvin, 1981). In this paper we focus on these distinct intermediates in the photoreaction of M to bR and we apply difference Fourier transform infrared (FTIR) spectroscopy in order to elucidate the protonation states of various chemical groups in the protein including the Schiff base in each of the intermediates in the photoreaction of M. The goal of this investigation is to understand this photoalteration in light of the considerable evidence that has been accumulated on these protonation changes in the forward photochemically driven steps that lead to bR mediated proton gradient generation.

The back photoreaction of M, which is stimulated by blue light within the M absorption, has led to a number of interesting observations in the past. The earliest observation that can be attributed to the M photoreaction was that of Oesterhelt and Hess (Oesterhelt and Hess, 1973). In this study, illumination of bleached bR was observed to accelerate the proton uptake. A spectrophotometric study at reduced temperatures where the thermal regeneration of bR from M was retarded showed that the decay was biphasic and both rate constants were accelerated upon blue illumination (Hess and Kuschmitz, 1977). Subsequently, a kinetic spectroscopic experiment revealed a blue shifted photoproduct of M which decayed into bR with a half-time of 200 ns (Kalisky et al., 1977).

Existence of the blue shifted photoproduct indicated that the photochemical and thermal regenerations of bR from M were distinct processes. The difference in these two pathways was also borne out by a number of studies on the effect of blue illumination on photoinduced electric signals in oriented bR samples. Due to the function of bR as a photoactivated proton pump, a layer of oriented bR molecules generates voltages under constant illumination. In terms of this photovoltage it was observed that in the presence of strong green illumination,

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simultaneous blue illumination leads to a reduction in the photovoltage (Karvaly and Dancshazy, 1977). In these early studies it was suggested that a blue absorbing species, in all probability M, functioned as either a proton sink or reverse proton pump under such illumination, thus inhibiting the photovoltaic response. Dancshazy et al. (1978) then performed a flash photolysis experiment whereby a green flash was followed by a blue (or green) flash with a time delay (Dancshazy et al., 1978). This study confirmed that the M intermediate was indeed responsible for the inhibitory effect caused by blue illumination by revealing that a time delay of 0.8 ms, corresponding to the maximum accumulation of M, resulted in optimal inhibitory effect on the observed electrical signal. Ormos et al. (1980) then investigated relative charge displacements induced by a green or blue flash. It was concluded that the charge displacement during the M to bR backphotoreaction was opposite in direction but equal in magnitude to that during the bR to M reaction. This made it clear that the M to bR backphotoreaction was distinct from the M-bR thermal regeneration which exhibited a positive electrical response.

In parallel with these studies low temperature visible absorption spectroscopy was applied to investigate intermediates in the M to bR backphotoreaction pathway. Litvin and Balashov (1977) were among the first to make detailed observation of intermediates found in the backphotoreaction. Studies by other workers using the same technique (Hurley et al., 1978; Kriebel et al., 1979) confirmed the existence of M photoproducts which were stable at low temperatures and the thermal regeneration of bR from these photoproducts at temperatures at which unbleached M is stable. The overall conclusion of these investigations were that blue illumination of the M intermediate at temperatures below 110 K produces a red shifted photoproduct with an absorption maximum peak ~420 nm. After a subsequent temperature increase to 130 K, a new thermal product was formed with an absorption maximum peak at ~565 nm and this species is believed to decay into another thermal product above 140 K with an absorption maximum peak ~585 nm.

It was assumed that the initial photoreaction below 110 K involves no reprotonation of the Schiff base because of the small shift in the absorption maximum as compared to M. Photoreisomerization was thought to be the action of light, due to the photoreversibility of this reaction (Hurley et al., 1978; Balashov and Litvin, 1981). Furthermore, it was suggested that the reprotonation of the Schiff base would occur only at temperatures above 110 K where a thermal product is observed with an absorption maximum ~565 nm.

To investigate the reprotonation process of the Schiff base during the backphotoreaction, we have employed vibrational spectroscopy that has proved to be powerful in elucidating the molecular structure of the bR molecule and its photointermediates. Resonance Raman spectroscopic techniques have yielded information on the protonation state of the Schiff base (Lewis et al., 1974) and isomerization of the retinal chromophore (Braiman and Mathies, 1980) among other things. FTIR techniques have not only provided information confirming results from resonance Raman spectroscopy but also revealed molecular structures of the protein moiety (Bagley et al., 1982; Rothschild et al., 1981; Siebert and Mantele, 1983). In this study we have applied the latter technique to investigate the Schiff base reprotonation in the course of the M to bR backphotoreaction as well as the identity of the groups that could be acting as donors for this reprotonation reaction.

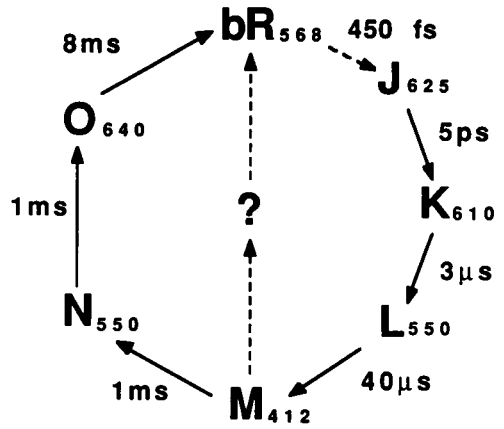

FIGURE 1 The photochemically generated cycle of intermediates involved in the pumping of a proton across the purple membrane. This investigation focuses on the back photoreaction from M to bR.

MATERIALS AND METHODS

Sample preparation

The S9 strain of *Halobacterium halobium* is cultivated under the standard conditions, and native pm is isolated by the standard procedure (Oesterhelt and Stoeckenius, 1974). Isotopically labeled retinal was synthesized using standard procedures and these samples were incorporated in the bR protein which was bleached to remove the native retinal chromophore. In one sample the hydrogen atom at C_{15} of the retinal chromophore is replaced by a deuterium; it will be referred to as 15-D. An additional sample was prepared by growing the bacterium on a defined medium containing lysine ^{15}N. In this sample the nitrogen atom of the Schiff base was replaced by ^{15}N.

For the difference FTIR measurements a PM solution is concentrated by blowing dry N_2 gas over the sample until the concentration is estimated to have an optical density of 4 for a 1 cm pathlength. A few drops of the concentrated pm solution are placed with a pipette on a 25 (diameter) × 4 mm (thickness) AgCl window (International Crystal Laboratories, Garfield, NJ), and the sample is gently dried with N_2 gas. Samples prepared in this manner exhibit amide I and II bands, ~1,660 and 1,550 cm^{-1}, respectively, with an intensity that can attenuate the IR signal by two-thirds and under these conditions good difference spectra can be obtained.

Before the sample is sealed with another AgCl window with a 2-mm thick Cu O ring in between, the liquid H_2O band in the 2,100–3,700-cm^{-1} region is monitored to insure an adequate level of humidity. If the integrated area in this region in the IR absorption corresponds to the integrated area of the amide I band at 1,660 cm^{-1}, a normal photocycle

is observed. In case a sample is to be studied in D_2O, the sample is first dried thoroughly in a dry N_2 atmosphere. While the sample is still under the dry N_2 atmosphere, a few drops of D_2O are placed on the sample, and the sample is allowed to dry again. This procedure is repeated a few times. During the last drying step, it is important to ensure that the sample retains an adequate humidity level rather than becoming excessively dehydrated. Absence of H_2O and proper deuteration are confirmed by taking an M/bR difference spectrum and confirming appropriate downshifts of aspartic acid bands in the 1,765–1,735-cm^{-1} region to the 1,755–1,725-cm^{-1} region. With a sample in D_2O, the liquid D_2O band in the 2,700–1,330 cm^{-1} is used to ensure an appropriate level of humidity.

The pair of AgCl windows are mounted on a cryostat (model 21 Cryodyne; Cryogenics), and the sample holder is suspended in an FTIR spectrometer (IBM IR 98) equipped with a liquid N_2 cooled HgCdTe detector.

FTIR spectra

The first step in recording the difference spectra is to accumulate the M intermediate at 100 K. This is accomplished by illuminating a light adapted sample with yellow light while the temperature is reduced from 275 to 100 K; as the temperature drops, M becomes the first intermediate to be thermally stabilized, and only insignificant amounts of other intermediates, K and L, become trapped in the process. After the M accumulation, the sample is exposed at 100 K to blue illumination. A photoproduct stable at 100 K is formed. Two subsequent thermal products are formed when the sample temperature is raised to 130 and 160 K, respectively. For difference FTIR spectra, it is important that a difference spectrum consists of two spectra obtained at the same temperature. Thus, for each of the thermally generated products, the sample temperature is returned to the initial value before the second spectrum is taken. In the appendix, detailed descriptions are given of a control experiment performed to confirm validity of the procedures. Below we give expanded procedures for obtaining difference FTIR spectra of the M to bR backphotoreaction. The light source used consists of a home made 100-W tungsten halogen lamp equipped with a pair of IR absorbing filters (Edmund Scientific) and an appropriate color filter.

I. M/M-1: The initial photoproduct at 100 K

(*a*) Light adaptation at 275 K with yellow illumination (GG495, Melles Griot) for 10 min. (*b*) Temperature lowered to 100 K under constant yellow illumination. (*c*) Illumination turned off after 100 K is reached. (*d*) 256 scans of M with 2 cm^{-1} resolution obtained (Spectrum M). (*e*) Blue illumination (BG12, Melles Griot) turned on for 1 min. (*f*) 256 scans of M-1 with 2-cm^{-1} resolution obtained (Spectrum M-1).

The M/M-1 difference spectrum is obtained by calculating the logarithmic difference of Spectra M and M-1. In such a difference spectrum, positive bands are characteristic of M and negative bands of M-1.

II. M-1/M-2: The thermal product formed at 130 K

This difference spectrum is obtained by continuing from I.*f* above. (*a*) Temperature raised to 130 K and maintained at this value for 10 min. (*b*) Temperature lowered to 100 K. (*c*) 256 scans of M-2 with 2 cm^{-1} resolution obtained (Spectrum M-2).

The M-1/M-2 difference spectrum is obtained by calculating the logarithmic difference of spectra M-1 and M-2. In such a difference spectrum, positive bands are characteristic of M-1 and negative bands of M-2.

III. M-2/M-3: The thermal product formed at 160 K

This difference spectrum could be obtained by continuing from above, but for ease of experiments, a slightly modified procedure is used. (*a*) Light adaptation at 275 K with yellow illumination for 10 min. (*b*) Temperature lowered to 130 K under constant yellow illumination. (*c*) Illumination turned off after 130 K is reached. (*d*) Blue illumination turned on for 1 min for the M to M-2 photoconversion. (*e*) 256 scans of M-2 with 2-cm^{-1} resolution are obtained (Spectrum M-2). (*f*) Temperature raised to 160 K and maintained at this value for 10 min. (*g*) Temperature lowered to 130 K. (*h*) 256 scans of M-3 with 2 cm^{-1} resolution are obtained (Spectrum M-3).

Note that M-1 is never trapped according to this procedure and the product formed in *d* is M-2. We have opted for the modified procedure because it is desirable to minimize the extent of the temperature change. Limiting the temperature change to 30 degrees rather than 60 allows us to obtain a good base line more readily.

The M-2/M-3 difference spectrum is obtained by calculating the logarithmic difference of Spectra M-2 and M-3. In such a difference spectrum, positive bands are characteristic of M-2 and negative bands of M-3.

For all of the spectra, the Fourier transforms are computed using Happ-Genzel apodization.

Low temperature visible absorption spectrophotometry

We have also performed low temperature visible absorption spectrophotometry on the native bR sample in order to confirm results obtained by earlier workers. The sample preparation procedure is basically identical to that for FTIR spectroscopy except that bR is suspended in a glycerol/water mixture (3:1 vol) and quartz plates are used as the substrate and cover window. The former measure is taken to reduce scattering of the measuring light by the sample. Nonetheless, we have obtained similar results with samples of bR that were dried according to the procedure described above for the FTIR sample. The only difference seen is an increase in the scattering background. Humidity of the sample is judged adequate when it is visibly moist; in contrast to FTIR spectroscopy where excessive IR absorption by liquid H_2O degrades the signal to noise ratio, no such problem is encountered with visible absorption spectroscopy, thus making this procedure adequate.

The pair of quartz substrate plate and window are mounted on the cryostat as with FTIR spectroscopy. Windows for the cryostat housing are quartz plates. The cryostat assembly is suspended in a spectrophotometer (model 17-D; Cary). Visible absorption spectra were obtained after the same sequence of illumination and temperature changes described earlier for the FTIR spectroscopy.

RESULTS AND DISCUSSION

Fig. 2 shows difference FTIR spectra showing three stages of the M to bR backphotoreaction, one photochemical and two thermal reactions. Note that M, M-1, M-2, and M-3 do not necessarily designate spectra from a single species. In fact, at room temperature it has been observed (Kalisky et al., 1977) that the decay of M after the absorption of blue light involves a biphasic decay and thus each transition may involve more than one species as will be discussed later.

A glance at the spectra in Fig. 2 reveals that M-1/M-2 and M-2/M-3 share a greater degree of overall similarity to each other than to M/M-1. The similarity is striking in the regions of 1,700–1,300 and 1,100–900 cm^{-1}. It may be suspected that the M-2/M-3 transition represents continuation of the M-1/M-2 transition; the thermal transition initiated at 130 K may not be complete until the temperature of 160 K is reached. There are, however, two aspects that preclude this possibility. First of all, the M-1/M-2 and M-2/M-3 spectra do indeed

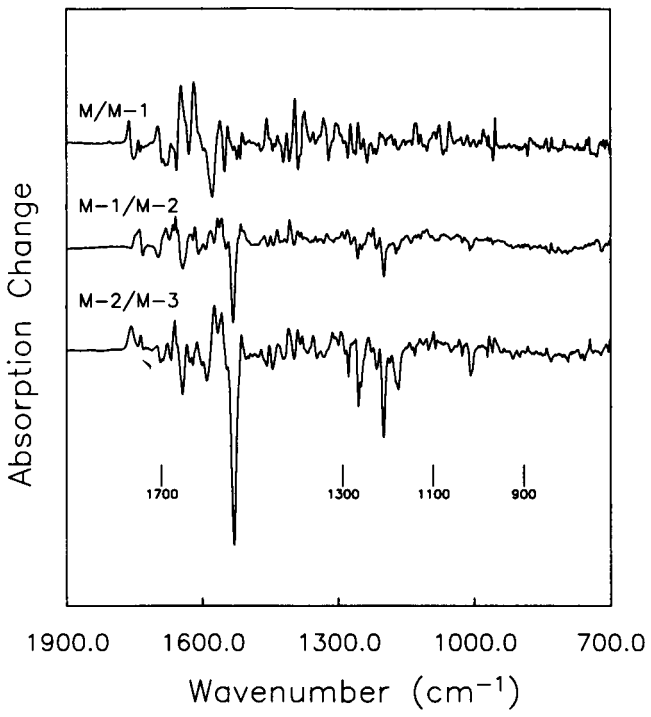

FIGURE 2 Difference FTIR spectra of the M/M-1, M-1/M-2, and M-2/M-3 transitions. The vertical scale, common to all three, is 0.06 OD from the top to bottom of the vertical scale.

exhibit clear differences in the regions of 1,800–1,700 and 1,300–1,100 cm^{-1}. Secondly, these transitions as studied with low temperature difference visible absorption spectroscopy are clearly different; while the maximum of the visible absorption increases to 546 nm as a result of the transition, that has been associated with M-1 to M-2, occurring between 130 and 160 K, the transition occurring at 160 K, that has been associated with M-2 to M-3, induces a further increase in the absorption to 563 nm and these alterations in the absorption can be seen in Fig. 3.

The similarity between the M-1/M-2 and M-2/M-3 difference spectra may indicate that there are difficulties with the simple sequential pathway that has been derived from the difference spectra of the absorption changes. These changes have been taken to indicate that each of the absorption transitions may be associated with disappearance of a single species and the simultaneous appearance of a new species. Such a simple sequential pathway should result in the FTIR difference spectra exhibiting matching negative/positive band pairs between one difference spectrum and the difference spectrum of the subsequent transition. For example, if a single species is thermally generated at 130 K and the same species in turn decays thermally into another species at 160 K, M-1/M-2 should exhibit a number of negative bands, corresponding to formation of this new species, while M-2/M-3 would show similar positive bands at the corresponding frequencies, indicating its disappearance. This is, however, not the case, and we will discuss the implications of this result below.

We are principally interested in bands that provide information on the proton transfer during the M to bR backphotoreaction. Such knowledge is expected to shed light on the Schiff base reprotonation process and the pathway followed by this process during the backphotoreaction. Identification of the Schiff base C=N stretching mode as well as aspartic acid C=O stretching modes is required if the goals of this investigation are to be achieved. In order to demonstrate that the spectra do indeed allow us to associate specific vibrational modes with these protonation changes the discussion below will be broken up into two sections. First, the spectra will be analyzed in order to assign the C=N stretching modes in the back photoreaction intermediates and then the assignments of the C=O stretching modes will be discussed.

I. C=N stretching mode of the Schiff base

The M intermediate is known to have an unprotonated Schiff base (Lewis et al., 1974), and previous difference FTIR spectroscopic studies at both low and room temperatures have assigned the positive band at 1,624 cm^{-1} in the M/bR spectrum to the C=N stretching mode of an unprotonated Schiff base (Bagley et al., 1982; Braiman et al., 1987). Thus, if the Schiff base undergoes

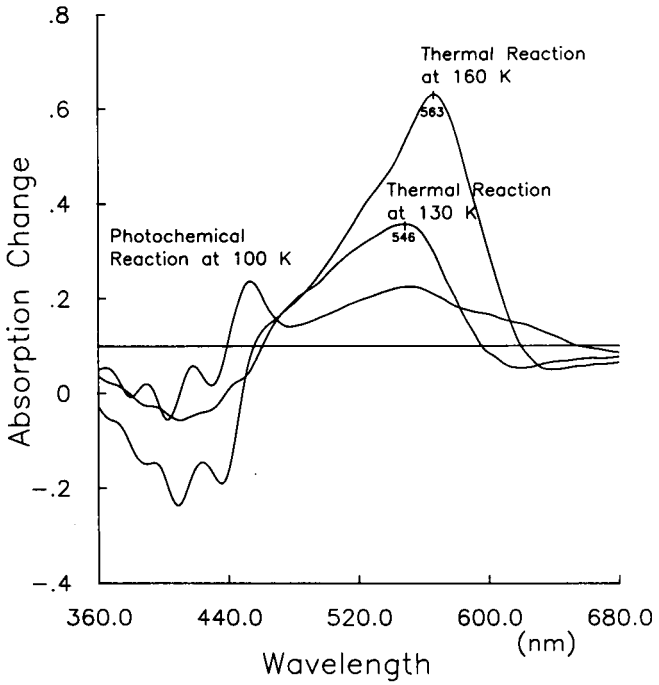

FIGURE 3 Difference visible absorption spectra associated with the M/M-1, M-1/M-2, and M-2/M-3 transitions. For each transition, the absorption spectrum obtained before the respective transition is subtracted from that afterward.

change during the M/M-1 transition, an associated band is expected at ~1,624 cm^{-1}. Such a band should be made readily recognizable by isotopical labeling at the Schiff base nitrogen or deuterium substitution at C_{15} of the retinal chromophore. Resonance Raman spectroscopy studies have shown that the former labeling downshifts the C=N stretching frequency by a few wave numbers (from 1,619 to 1,614 cm^{-1} according to Lewis et al. (1978); from 1,622 to 1,618 cm^{-1} according to Argade et al. (1981)) while the latter substitution induces a downshift by an unspecified wavenumber (Ehrenberg et al., 1980; Stockburger et al., 1986).

In Fig. 4 the difference spectrum of the native sample shows such a positive band and this positive band at 1,619 cm^{-1} is downshifted to 1,616 and 1,615 cm^{-1} by ^{15}N and 15-D isotopic labeling. Identification of a corresponding negative band in the M/M-1 is difficult; there may be a negative band at 1,630 cm^{-1} which appears to be shifted to 1,624 and 1,625 cm^{-1} by ^{15}N and 15-D isotopic labeling, respectively. If indeed this band is the Schiff base frequency of M-1 it is unusual in its frequency both in terms of whether it corresponds to a protonated or unprotonated nitrogen. In spite of this uncertainty in the assignment of the Schiff base frequency of M-1, the lack of a significant change upon D_2O suspension in this region of the spectrum suggests strongly that the Schiff base remains unprotonated after the M/M-1 transition.

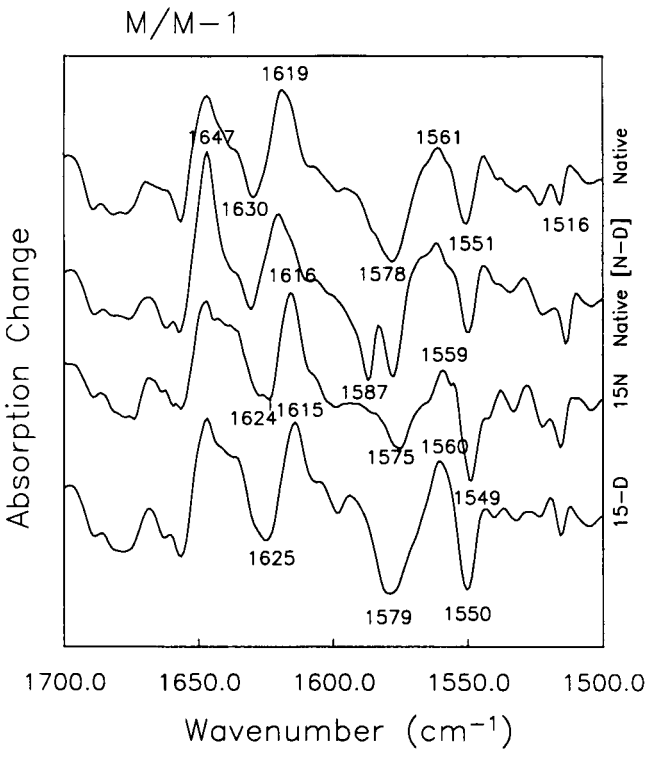

FIGURE 4 The 1,700–1,500-cm^{-1} region of the M/M-1 transition. Native [N-D] refers to the native sample in D_2O suspension while the remaining spectra are in H_2O.

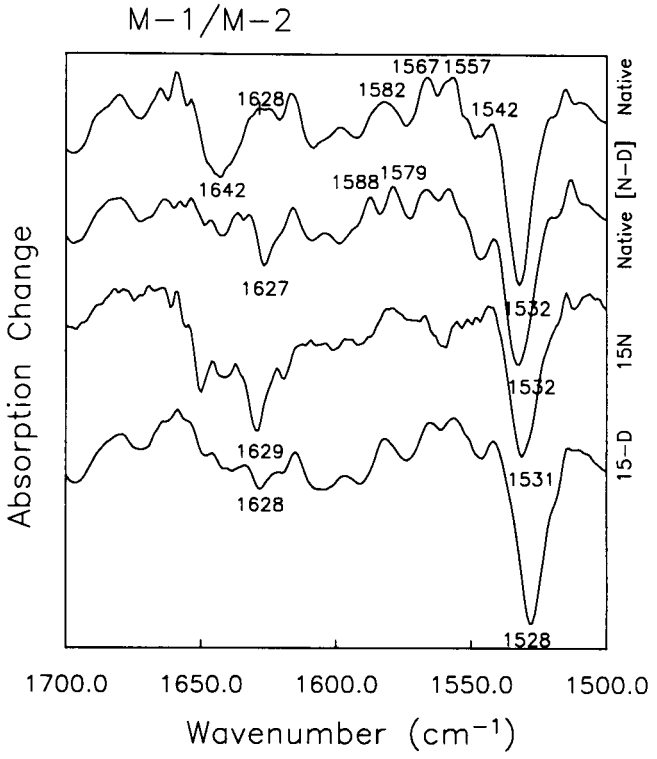

FIGURE 5 The 1,700–1,500-cm^{-1} region of the M-1/M-2 transition.

Fig. 5 shows the M-1/M-2 transition spectra. These spectra provide strong evidence that a protonated Schiff base is formed after the M-1/M-2 thermal transition. Note that the negative band at 1,642 cm^{-1} of the native sample is downshifted to 1,627 cm^{-1} in D_2O suspension. This is clear evidence for a protonated Schiff base. Moreover, the appearance of the same band at 1,629 and 1,628 cm^{-1} in ^{15}N and 15-D isotopic labeling supports formation of a protonated Schiff base (Lewis et al., 1978; Ehrenberg et al., 1980).

During the M-2/M-3 transition, whose spectra are shown in Fig. 6, the native sample again exhibits a negative band at 1,644 cm^{-1} which, in a manner quite similar to that during the M-1/M-2 transition, is downshifted to 1,627 cm^{-1} in D_2O suspension while ^{15}N and 15-D isotopic labeling result in the appearance of new negative bands at 1,629 and 1,630 cm^{-1}, respectively. Thus, it is clear, that this transition is also accompanied by formation of a protonated Schiff base. This suggests strongly that in the course of the backphotoreaction some Schiff base becomes reprotonated during the M-1/M-2 transition while another Schiff base does not become reprotonated until the M-2/M-3 transition.

The above conclusion is consistent with results from analysis on the ethylenic mode, the C=C stretching mode of the retinal chromophore. Both M-1/M-2 and M-2/M-3 transitions are characterized by a prominent negative band ~1,530 cm^{-1} for the native sample in H_2O which is downshifted by a few wavenumbers by

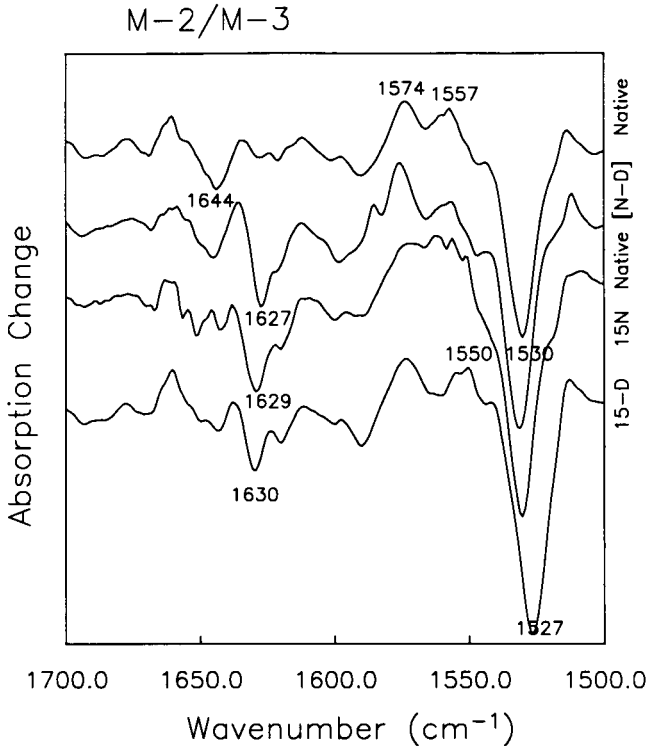

FIGURE 6 The 1,700–1,500-cm^{-1} region of the M-2/M-3 transition.

15-D substitution (see Figs. 5 and 6). It was observed by Ehrenberg et al. that the ethylenic band of bR, as observed by resonance Raman spectroscopy, was similarly affected by the 15-D substitution (Ehrenberg et al., 1980) so that it is clear that the negative bands at 1,532 and 1,530 cm^{-1} in the M-1/M-2 and M-2/M-3 transitions, respectively, are the ethylenic band. The difference of 2 cm^{-1} is significant in view of the fact that lower temperatures usually lead to lower frequencies in vibrational modes and this is contrary to the results obtained for the two transitions that are presently being discussed. With these two transitions, in spite of the fact that M-1/M-2 is measured at a lower temperature, the ethylenic stretch is in fact at a higher rather than at a lower temperature. Therefore, we feel confident in deducing that the ethylenic stretch observed in the difference spectra of these two transitions may in fact come from different species.

In support of this deduction is the fact that low temperature difference visible absorption spectroscopy also reveals formations of distinctly different species during the two thermal reactions and this is characterized by different maxima (see Fig. 3). Characterization of the M-1/M-2 thermal product by a higher IR wavenumber and its visible absorption maximum at a lower wavelength is consistent with the standard relationship between the ethylenic band frequency and the maximum visible absorption wavelength (Doukas et al., 1978).

Thus, we can summarize our deductions as follows: the Schiff base remains unprotonated after the M/M-1 transition, and two subsequent thermal reactions are accompanied by reprotonation of two distinct types of Schiff base species as well as formation of two similar yet clearly distinct ethylenic bands. This observation suggests that after the M/M-1 transition there are at least two species. One regains a state that has similarities to bR at 130 K while the other requires a higher temperature of at least 160 K to return to a protonated state.

II. Aspartic acid region

The 1,770–1,720-cm^{-1} region provides a wealth of information on the protonation state of carboxylic acid groups because the C=O stretching mode of protonated carboxylic acid shows a strong IR band in this region. In the case of bacteriorhdopsin, it is known that only aspartic acid residues undergo a change during the light adapted photocycle (Engelhardt et al., 1985; Eisenstein et al., 1987). In the discussion below of the changes we have observed on the M backphotoreaction in this region of the spectrum, we assume that this is also the case for these changes. This appears to be a good assumption in view of the fact that the M/M-3 spectrum (see Fig. 7 *b*) is very similar to what is observed for a M/bR spectrum in which M thermally decays to bR (Fig. 7 *a*). In addition, the mathematical sum (Fig. 7 *c*) of the three difference spectra for the three transitions associated with the backphotoreaction are also identical to what is normally observed for the M/bR difference spectrum.

Figs. 8 and 9 show the M/M-1, M-1/M-2, and M-2/M-3 difference spectra of the native sample in H_2O and

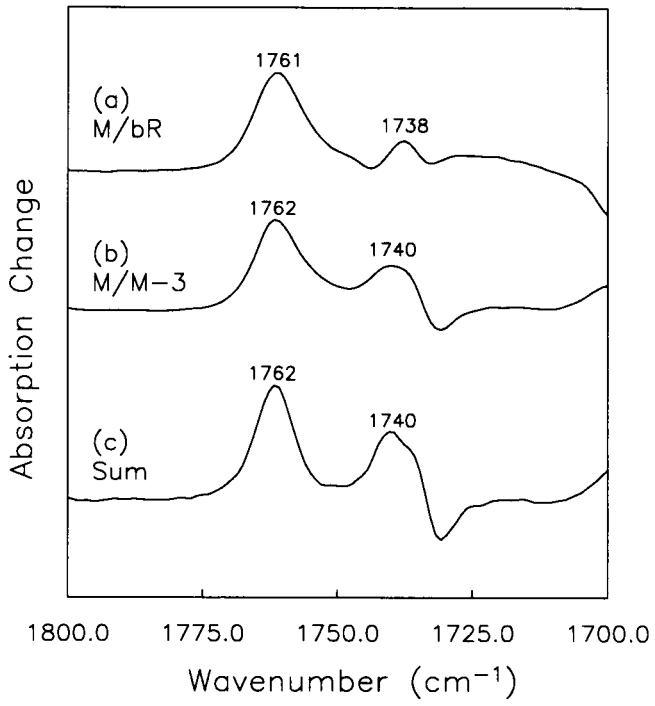

FIGURE 7 FTIR difference spectra of (*A*) M/bR, (*B*) M/M-3, and (*C*) the mathematical sum of M/M-1, M-1/M-2, and M-2/M-3.

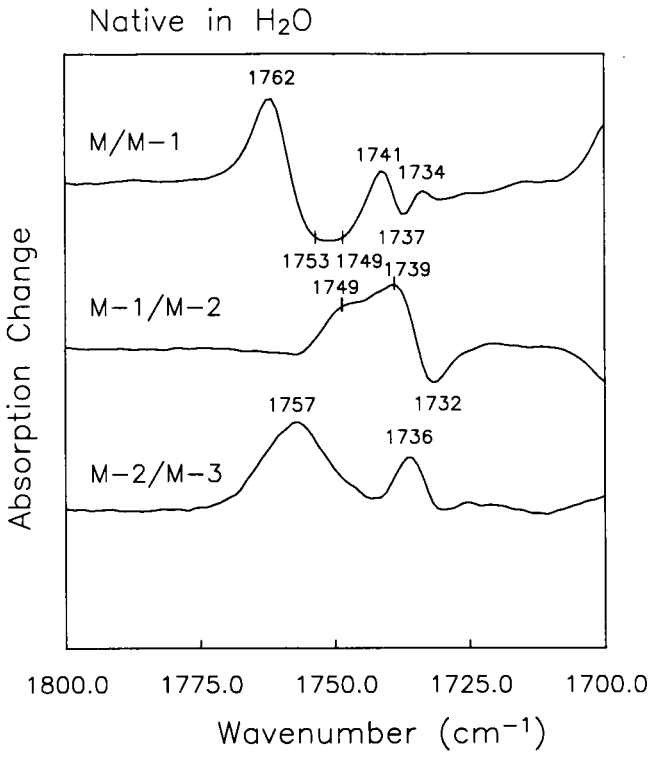

FIGURE 8 The aspartic acid region of the native sample in H_2O.

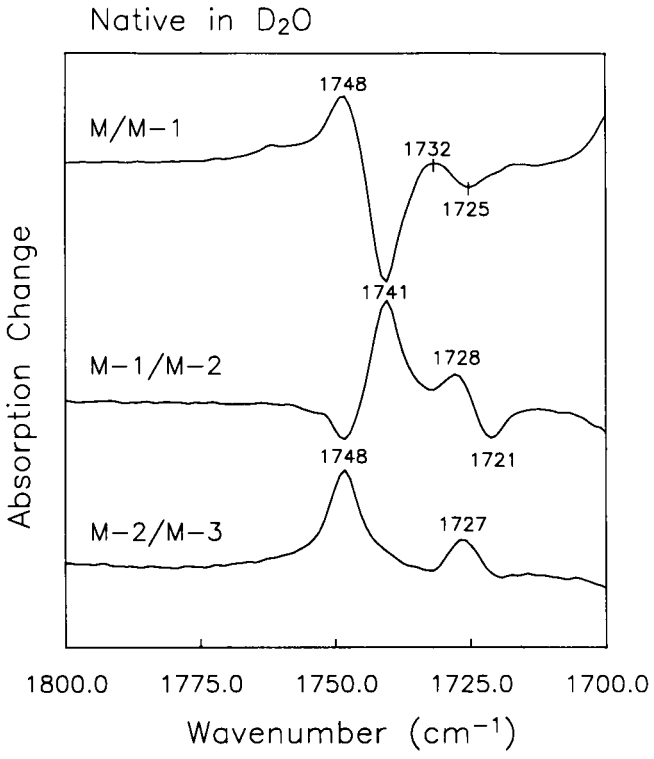

FIGURE 9 The aspartic acid region of the native sample in D_2O.

D_2O, respectively. Such a resuspension causes the protons connected with the carboxylic acid group of the aspartic acid residues to be readily replaced by a deuterium, downshifting the associated C=O stretching mode. Note that the D_2O effect does not simply result in a uniform overall shift of all participating bands, particularly for M/M-1 and M-1/M-2. The nonuniform shift must be caused by some bands in this region downshifting more (or less) than other bands. In the following discussion emphasis will be placed on trying to elucidate these shifts in relation to the protonation state of the Schiff base.

When the spectra in Figs. 8 and 9 are compared most of the bands are downshifted by $\sim$10 cm^{-1} in D_2O. The M-2/M-3 spectrum provides a good example. The alterations seen in this transition seem to indicate that the D_2O effect can be explained as a 9 cm^{-1} downshift of the positive bands at 1,757 and 1,736 cm^{-1}. In the M-1/M-2 transition, the positive/negative pair at 1,739 and 1,732 cm^{-1} seems to be downshifted by 11 cm^{-1}, while in the M/M-1 transition a pair at 1,741 and 1,737 cm^{-1} are apparently downshifted to 1,732 and 1,725 cm^{-1}, respectively. In contrast to these bands, the prominent positive 1,762 cm^{-1} band in M/M-1 is unique in being apparently downshifted by as much as 14 cm^{-1} to 1,748 cm^{-1}.

To explain this observation and the associated result in Fig. 8 which shows an unusually broad negative peak stretching from 1,749 to 1,753 cm^{-1}, we hypothesize that in this region in the M/M-1 spectrum there are in fact two bands that are undergoing a simultaneous change in environment. To support this hypothesis we have prepared a diagramatic representation in Fig. 10 that shows how this explanation could account for the spectra seen in Figs. 8 and 9. To rationalize the sharp negative band for M/M-1 at 1,741 cm^{-1} in D_2O two bands are considered in Fig. 10.1. One of these bands has a positive peak at 1,762 cm^{-1} and a negative peak at

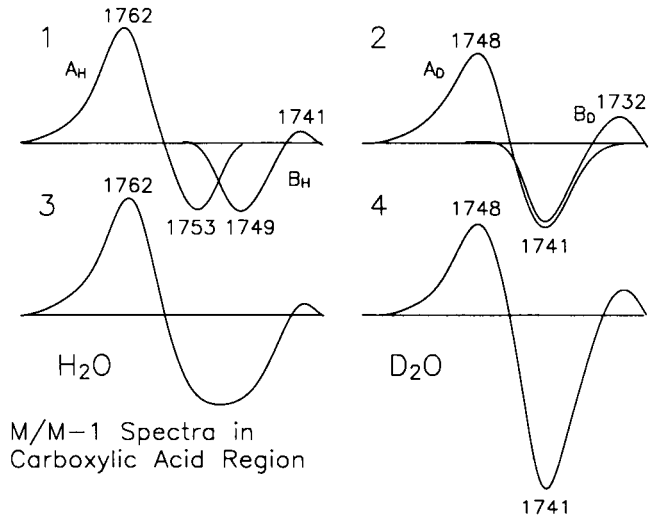

FIGURE 10 Nonuniform downshifting of the aspartic acid C=O stretching bands in H_2O and D_2O during the M/M-1 transition.

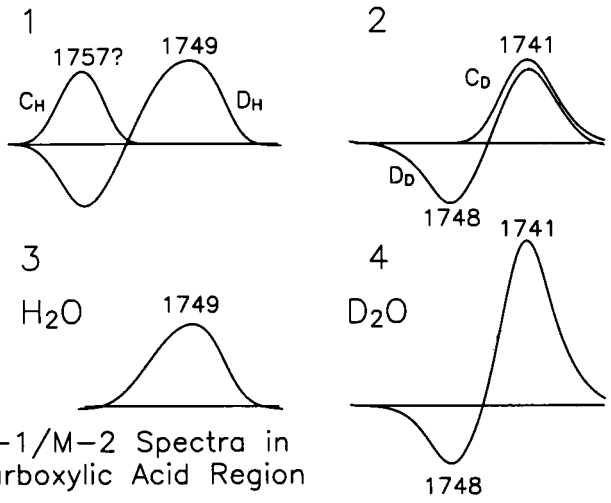

FIGURE 11 Nonuniform downshifting of the aspartic acid C=O stretching bands in H_2O and D_2O during the M-1/M-2 transition.

1,753 cm^{-1} corresponding to the hypothetical environmental change while the other band has negative and positive peaks at 1,749 and 1,741 cm^{-1}, respectively. If the former pair of positive and negative peaks are downshifted by 12 cm^{-1} as a result of resuspension in D_2O, while in the latter pair of peaks the D_2O effect is only 8 cm^{-1}, then it is a natural consequence that the sample in D_2O exhibits a sharper and larger negative band at 1,741 cm^{-1} (see Fig. 10.2). Moreover, this suggests that the negative 1,753-cm^{-1} band forms a pair with the positive 1,762-cm^{-1} band shown as A_H because both downshift in D_2O significantly more than other bands. If this explanation is indeed correct the M/M-1 transition does not involve any observed change in the protonation state of the aspartic acid residues. Instead this explanation would suggest that in the photochemical alteration associated with the M to M-1 transition two aspartic acids are undergoing a change in their environment.

The M-1/M-2 spectra can be analyzed in a similar vein. For this analysis the reader is referred to Fig. 11. For convenience in our discussion the positive band at 1,749 cm^{-1} in H_2O which is the only band above 1,739 cm^{-1} is reproduced in Fig. 11.3, whereas in D_2O the region above 1,728 cm^{-1} is reproduced in Fig. 11.4. The discrepancy in the number of bands can be explained as follows. In D_2O there are actually two overlapping positive bands at 1,741 cm^{-1} (see Fig. 11.2) whereas in H_2O, in which there is a single positive band, the spectrum detected can be formed (see Fig. 11.1) by a single peak arising from one aspartic acid residue at 1,757 cm^{-1} and a negative/positive pair of peaks at 1,757 and 1,749 cm^{-1} arising from a second aspartic acid residue. In view of our earlier observation that most aspartic acid bands are shifted by either $\sim$10 cm^{-1} or by as much as 14 cm^{-1}, it is reasonable to suppose that the observed bands at 1,748 and 1,741-cm^{-1} bands in D_2O arise from the 1,757-cm^{-1} positive component in Fig. 11.4 shifting to 1,741 cm^{-1} and the negative/positive pair at 1,757 and 1,749 cm^{-1} moving in unison to 1,748 and 1,741 cm^{-1}.

Using the above as a basis for discussion the reader is referred once again to Fig. 10.1 and 10.2, and Fig. 11.1 and 11.2. The transition from M to M-1 leaves us with two bands in H_2O in the M-1 intermediate. These bands occur at $\sim$1,753 which is at 1,762 cm^{-1} in M (a positive peak in Fig. 10.1) and at 1,749 cm^{-1} which is the positive peak at 1,741 cm^{-1} in M. The 1,753-cm^{-1} band is thought to be identical with the positive band at 1,757 cm^{-1} that is deduced to be present in M-1 and this band, which is derived from the 1,762-cm^{-1} band in M, disappears in the M-1/M-2 transition. The positive 1,762-cm^{-1} band in M/bR has been assigned to Asp 85 (Braiman et al., 1988; Gerwert et al., 1989). Thus, it is our suggestion that Asp 85 undergoes an environmental change in the M to M-1 photoreaction and then is deprotonated in the M-1 to M-2 reaction in which the Schiff base gets reprotonated.

The M-2/M-3 spectrum is characterized by two prominent positive bands, indicating that two aspartic acid residues become deprotonated (see Figs. 8 and 9). The 1,757-cm^{-1} band is probably identical to a positive shoulder observed at the same frequency in the standard M/bR spectrum. This shoulder was eliminated by the replacement of Asp 85 by glutamic acid (Braiman et al., 1988), and it has been tentatively assigned to Asp 85 (Braiman et al., 1988; Gerwert et al., 1989). Thus, it appears that Asp 85 is involved in this transition. Earlier we concluded that the M-1/M-2 transition is accompanied by deprotonation of Asp 85. Thus, as with the Schiff base, it appears that there are also two types of Asp 85. One type undergoes an environmental change during the M/M-1 transition and subsequently becomes deprotonated during the M-1/M-2 transition. The other type becomes deprotonated only during the M-2/M-3 transition.

The 1,736-cm^{-1} band in the M-2/M-3 spectrum probably corresponds to a small positive band observed at $\sim$1,738 cm^{-1} in the M/bR spectrum. This band has not been positively identified, but it has been suggested to be due to either Asp 212 or 115 (Braiman et al., 1988; Gerwert et al., 1989). On the other hand, Braiman et al. showed quite clearly that Asp 115 has a 1,740-cm^{-1} bR band in the standard K/bR spectrum. When we consider that only the M/M-1 spectrum exhibits a similar (in terms of shape and intensity) negative band at 1,737 cm^{-1}, we may conclude that the positive/negative band pair at 1,734 and 1,737 cm^{-1} in the M/M-1 represents the sole environmental change that Asp 115 undergoes during the backphotoreaction. This could suggest that the positive band at 1,736 cm^{-1} in the M-2/M-3 spectrum is due to Asp 212. Thus, based on this suggestion, the M-2/M-3 transition involves Asp 85 and 212 though it is not clear whether Asp 212 becomes deprotonated.

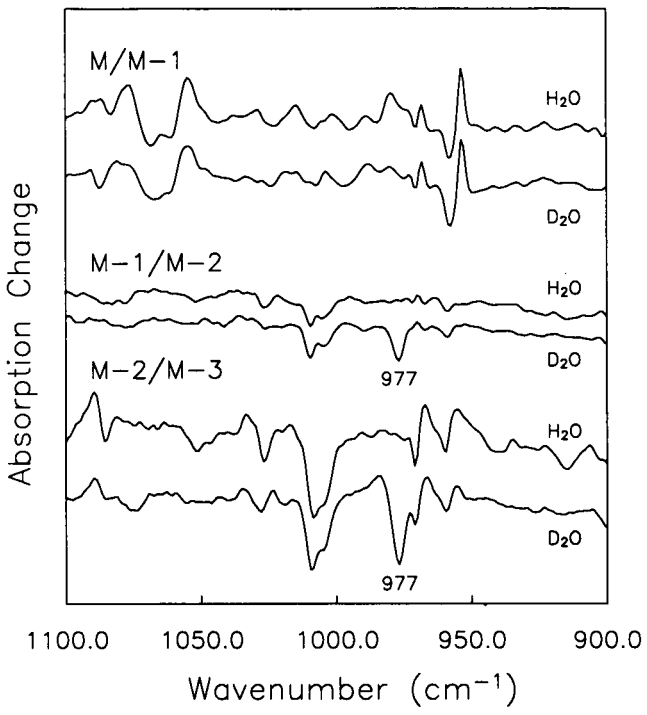

FIGURE 12 The 1,100–900-cm^{-1} region of M/M-1, M-1/M-2, and M-2/M-3 in H_2O and D_2O.

Mutants of bR will have to be investigated in order to clarify these deductions.

Asp 96, that is known to affect the Schiff base reprotonation during the thermal decay of M (Otto et al., 1989; Butt et al., 1989), does not have a clear role during the M to bR backphotoreaction because its IR band has not been assigned unambiguously. It has been observed, however, that the substitution of Asp 96 by asparagine results in an overall increase in the amplitude in the 1,755–1,740-cm^{-1} region in the standard M/bR spectrum (Braiman et al., 1988; Gerwert et al., 1989). This can be explained by the elimination of a negative Asp 96 band from this region. During the M to bR backphotoreaction, the M/M-1 transition is the only transition exhibiting negative bands in the corresponding region. The 1,753-cm^{-1} negative band that is correlated with the appearance of M-1 (see Fig. 10.1) has been assigned to Asp 85 so that the negative 1,749-cm^{-1} band associated with M-1 may be due to Asp 96. This would suggest that Asp 96 does not become deprotonated during this transition.

III. Schiff base N-D hydrogen out-of-plane vibration

The changes in the Schiff base protonation state that have been deduced above can also be confirmed by looking for the appearance of the N—D out-of-plane mode. Resonace Raman spectroscopic studies have detected such a mode at 977 cm^{-1} for bR in D_2O and a similar band is observed in M/bR FTIR difference spectra. Fig. 12 shows the FTIR difference spectrum associated with the M/M-1 transition in D_2O. In this transition there is no band observed at 977 cm^{-1}, suggesting the absence of such a band in the M/M-1 transition. It is clear on the other hand that during both M-1/M-2 and M-2/M-3, a new promient negative band appears at 977 cm^{-1} in D_2O suspension. Thus, we can conclude, as from the analysis of the C=N stretching mode, that there are two different groups of bR molecules and the Schiff bases of these molecules become reprotonated during the M-1/M-2 and M-2/M-3 transitions, respectively.

CONCLUSIONS

We have studied intermediates formed during the M to bR backphotoreaction with low temperature difference FTIR spectroscopy. The C=N stretching mode of the Schiff base has been identified by isotopically labeling the Schiff base nitrogen and the hydrogen at C_{15} of the retinal chromophore. Analysis of the above C=N stretching mode and the N—D in-plane bending mode shows that the Schiff base remains unprotonated immediately after the initial photochemical reaction of M at 100 K. This photochemical reaction is accompanied by formation of two distinct species. Of the two, one species has its Schiff base reprotonated when the temperature is raised to 130 K, while the Schiff base of the other species becomes reprotonated only at 160 K.

Analysis of aspartic acid bands shows that Asp 85 is the likely proton donor during the reprotonation of the first species at 130 K; Asp 85 in one set of bR molecules first undergoes an environment change during the photochemical reaction at 100 K and then becomes deprotonated at 130 K. In the second set of bR species, the Asp 85 has not undergone any change until 160 K, when Asp 85 in this set of bR apparently reprotonates the Schiff base in this group of bR.

Fig. 13 summarizes the M to bR backphotoreaction pathway. After the photochemical reaction at 100 K

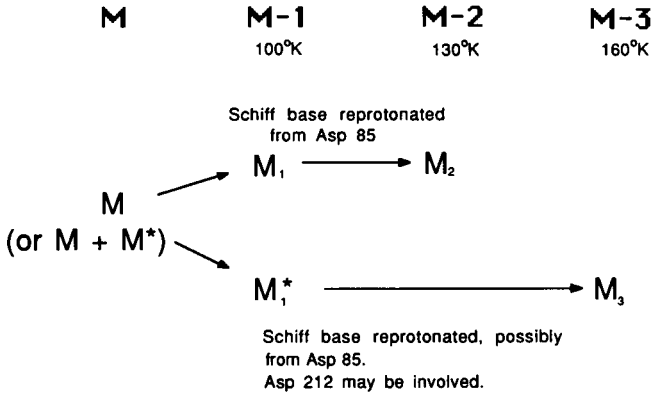

FIGURE 13 Proposed M to bR backphotoreaction pathway. Note that the convention used in this paper is to designate even those species in the backphotoreaction with a protonated Schiff base with the letter M. Nonetheless, alternate conventions exist in which these protonated species are called bR with appropriate superscripts.

there are two types of species present and these are labeled as M_1 and M_1^*. It is not clear in the current investigation whether these form from one or two forms of M, but our own earlier study on multiple M forms at room temperature (Takei and Lewis, 1993), suggesting two distinct M forms could indicate that M_1 and M_1^* arise from two different M species. M_1 and M_1^* decay thermally into bR-like species, M_2 and M_3, at 130 and 160 K, respectively. Presence of two bR-like species suggests that the light adapted bR state could consist of two species; such a possibility has previously been suggested by other workers (Dancshazy, et al., 1988).

APPENDIX

A control experiment was performed in which an FTIR spectrum was accumulated at 130 K, a second spectrum was recorded after the sample was raised in temperature to 160 K and returned to 130 K and then a difference of these two spectra were recorded. This experiment was completed to check for the absence of two potential problems: first, that the temporary temperature change does not induce artifacts in the difference spectrum. Within the temperature range where a sample does not undergo any thermal decay, a spectrum representing the difference induced by a transitory change in the temperature should exhibit a flat base line if the initial and final temperatures are the same; second, that the M accumulation procedure does not result in formation of any other species that either undergoes a photochemical reaction upon blue light illumination or a thermal decay when the temperature is raised to 160 K. Specifically, presence of K and L would be problematic even though K is not expected to form under the M accumulation procedure used in this study.

Both potential problems can be checked if the M accumulation procedure used in this study is subjected to a 130 → 160 K temperature change. The range is limited to 30 degrees because all difference spectra reported in this paper were subjected to at most a 30 degree temperature change. In addition, this temperature range was chosen since any residual K should be observed to decay to L at 160 K. The bottom spectrum in Fig. 14 is such a difference spectrum; an M enriched sample is subjected to 130–160 K temperature change, and the experiment is designed to reveal any change induced in this process. The lack of any features proves that no artifacts are formed. Moreover, no other species are observed to decay thermally. For reference, the top spectrum in Fig. 14 is an unaveraged difference spectrum between two consecutive spectra from a sample maintained at a constant temperature.

It should be mentioned that the FTIR spectrometer needs to be carefully stabilized to successfully acquire a difference spectrum such as the one at the bottom of Fig. 14; it is not unusual for the spectrometer to require more than half a day for complete thermal stabilization.

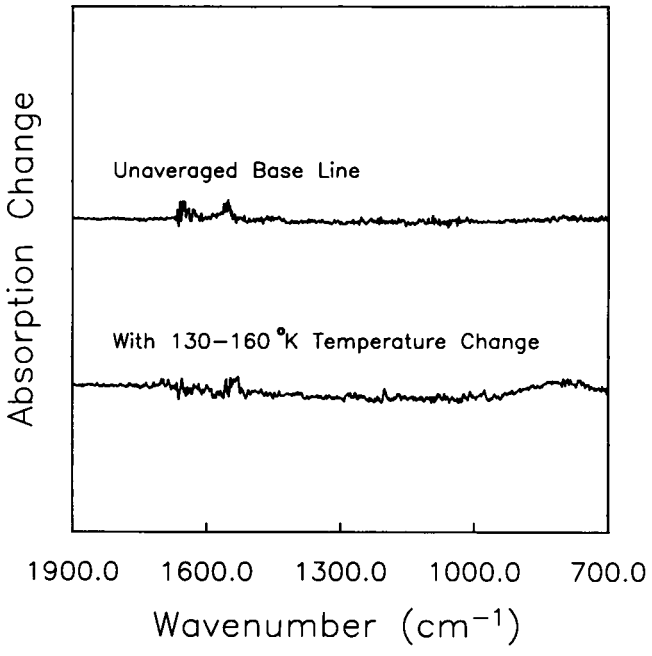

FIGURE 14 Difference FTIR spectra of a control experiment. Neither K thermal decay nor the formation of other intermediates or artifacts due to the temporary temperature change are observed.

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