Goodridge for critical evaluation of the manuscript prior to submission.

Registry No. Monooxygenase, 9038-14-6; aniline hydroxylase, 9012-80-0; aniline, 62-53-3.

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Role of Water in Bacteriorhodopsin's Chromophore: Resonance Raman Study†

Peter Hildebrandt and Manfred Stockburger*

ABSTRACT: The influence of water on the structure and photochemical behavior of the retinylidene Schiff base (SB) chromophore of *bacteriorhodopsin* (BR) in the *purple membrane* (PM) of *Halobacterium halobium* was studied by means of resonance Raman (RR) spectroscopy. The width of the C=N stretching band in the RR spectra of the parent chromophore, BR_{570}, considerably narrowed when H_2O as a solvent was replaced by D_2O. This is interpreted in terms of resonance energy transfer from the C=N stretching vibration (1641 cm^{-1}) to the bending vibration (1635 cm^{-1}) of H_2O molecules that are located close to the SB group. When these molecules are removed by a rigorous dehydration (dh) procedure, RR spectroscopic evidence indicates that the covalently bound proton at the SB group of the chromophore is removed and the configuration of its retinal moiety is changed. On illumination this new species, BR_{530}(dh), runs through a photoinduced cyclic process. Two intermediates of this cycle could be characterized by their RR spectra. On this basis it was concluded that, in contrast to BR_{570}, the primary photoinduced step in the dehydrated PM is proton transfer from a neighboring base to the SB group of BR_{530}(dh). Our experiments suggest that in the natural chromophore, BR_{570}, an ion pair structure—the positive charge at the SB group and a negative counterion—is stabilized by surrounding water molecules. It seems that this structure is a prerequisite for BR's function as a light-induced proton pump initiated by photoisomerization of the chromophore.

Bacteriorhodopsin (BR),[1] the major component of the purple membrane (PM) of *Halobacterium halobium*, acts as a light-driven proton pump (Oesterhelt & Stoeckenius, 1973). The active transport of protons across the membrane establishes a proton gradient that is used by the cell to drive ATP synthesis (Racker & Stoeckenius, 1974). The absorption of BR in the visible is due to its chromophoric center which contains a retinal molecule bound to the protein via a Schiff's base (SB) linkage. On illumination BR runs through a cyclic process with various intermediates (Figure 1) and there is no doubt that proton pumping is controlled by this "photochemical cycle" [for a review, see Stoeckenius et al. (1979); Ottolenghi, 1980].

In the dark BR exists in the two equilibrated forms, BR_{570} and BR_{548}, with maximum absorption at 570 and 548 nm, respectively. Under light-adapted conditions BR_{570} predominates. It could be shown by extraction and reconstitution experiments that in BR_{570} the retinal moiety is in the all-trans configuration while in BR_{548} the 13-cis form is prevalent (Oesterhelt et al., 1973; Sperling et al., 1977; Pettei et al., 1977) (Figure 2). The big red shift of BR chromophores with respect to the absorption of related model compounds in solution is due to the strong interaction the protein exerts on the

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[1] Abbreviations: RR, resonance Raman; BR, bacteriorhodopsin; PM, purple membrane; FWHH, full width at half-height; (dh), dehydrated.

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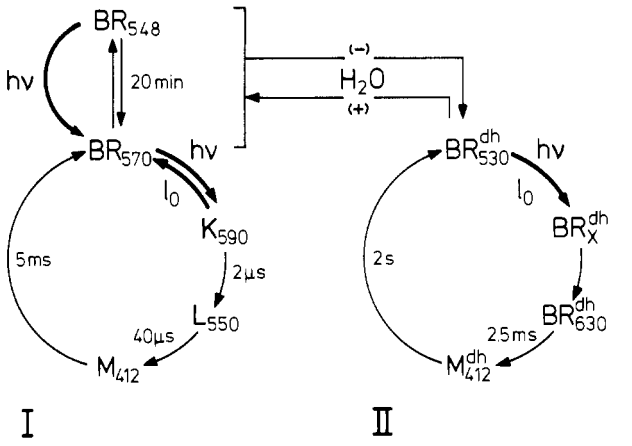

FIGURE 1: Photoinduced cyclic reactions of BR. (I) Hydrated PM: The different chromophoric states are assigned by capital letters in the conventional way and labeled by the wavelength of absorption maxima. The decay times are from literature data and refer to room temperature. Intermediates which occur during the back-reaction from M_{412} to BR_{570} were omitted. (II) Dehydrated PM: The parent BR_{530}(dh) and the two intermediates BR_{630}(dh) and M_{412}(dh) could be characterized in the present paper by their RR spectra. The decay times refer to room temperature.

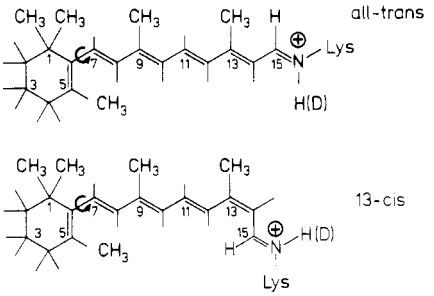

FIGURE 2: Retinylidene Schiff base chromophore of BR in the all-trans and 13-cis configuration. Retinal is bound to the ϵ-amino group of a lysine residue.

bound retinal chromophore (Kropf & Hubbard, 1958; Balogh-Nair et al., 1981).

It could be demonstrated by spectroscopic and kinetic measurements that water strongly influences the retinal–protein interaction (Korenstein & Hess, 1977a,b; Lazarev & Terpugov, 1980). Thus, when the content of water in the PM is decreased, the absorption of BR shifts to the blue and the decay of the M_{412} intermediate in the photochemical cycle is considerably slowed down (Korenstein & Hess, 1977a).

In the present paper the influence of water on the structure and function of the retinal chromophore is studied by resonance Raman spectroscopy which selectively probes the chromophoric site. Various BR chromophores could be characterized by their RR spectra, and valuable structural information was obtained from the vibrational bands of the spectra (Stockburger et al., 1979; Terner et al., 1979; Braiman & Mathies, 1980, 1982; Alshuth & Stockburger, 1981; Massig et al., 1982; Smith et al., 1983). Most of our measurements were performed with membrane films. From hydrated PM films we obtained RR spectra that were identical with the well-known spectra from aqueous diluted PM suspensions. The film technique allows one to regulate the water content and to obtain spectra of the hydrated and dehydrated membrane under identical excitation conditions. It is also an advantage of this technique that the strong bands of water do not perturb the spectra of PM films. Thus, for the first time the N–H stretching band of the protonated SB group of BR_{570} could be identified.

In the present work experimental evidence is provided that water molecules are located in close vicinity to the SB group of the chromophore. It seems that these molecules are essential for the function of BR's chromophore by stabilizing its configurational and conformational structure. After these molecules were removed by dehydration of the membrane, a new chromophore appeared, which in the fingerprint region of its RR spectrum closely resembles the 13-cis chromophore BR_{548}. The analysis of the characteristic modes of the SB group, however, indicates that this group is deprotonated in the dehydrated chromophore. It could be shown that this chromophore runs through a photochemical cycle whose intermediates are different from those of the proton pump controlling cycle of BR_{570} with the exception of the blue-absorbing intermediate M_{412} which in both cycles has a similar structure. In contrast to the hydrated membrane where the primary event is photoisomerization in case of the dehydrated membrane a photoinduced proton-transfer mechanism is suggested.

All spectroscopic changes that were induced by dehydration were found to be completely reversible. However, when water was replaced by other polar molecules like HCl, NH_3, and CH_3OH, serious modifications of the retinal chromophore were induced.

Materials and Methods

Purple membrane (PM) which had been isolated from *Halobacterium halobium* R_1 cells by the convential procedure (Oesterhelt & Stoeckenius, 1974) was a gift from Dr. Oesterhelt's laboratory. Hydrated PM films were prepared from PM suspensions in H_2O and D_2O following the procedure of Korenstein & Hess (1977a). The optical density of the film was adjusted to ~1.2 at 570 nm. No buffer was added to the samples. Dehydration was achieved by exposing the film for several hours (usually 4 h) to a vacuum line at a pressure below 10^{-4} torr.

For time-resolved RR measurements the PM films were prepared in the circular nut (4 cm in diameter) of a metal disk which was fixed to a rotating axis. The circular frequency could be varied between 2 and 50 s^{-1}. The disk was placed into a home-built Plexiglas or stainless steel chamber at an angle of 45° to the incident laser beam, and the perpendicularly scattered light was focused to the entrance slit of the monochromator. When hydrated films were studied, a vessel filled with saturated KNO_3 solution (H_2O or D_2O) was put into the chamber to establish a high relative humidity (95%) of constant magnitude (Wexler & Hasegawa, 1954). For recording RR spectra of dehydrated films the stainless steel chamber was used, which was connected to the vacuum system.

In order to obtain RR spectra from PM films under photostationary conditions at low temperature, a quartz plate coated with a PM film was placed into a cryostat (Leybold, VSK 4-300). For dehydration this was connected to the vacuum system. The same scattering geometry was used as for the rotating disk. All measurements on dehydrated films in the cryostat were performed in a He atmosphere to avoid local heating in the laser focus. It was estimated that heat of conduction of the gas was sufficient to keep the local temperature of the illuminated spot below 110 K in all RR experiments which were performed at liquid nitrogen temperature.

Dehydrated PM films were exposed to vapors of dry HCl, DCl, CH_3OH, and NH_3 at partial pressures between 10 and 100 torr. RR spectra of such modified membranes were recorded at 300 K by using the rotating disk technique. For RR studies of PM in diluted aqueous suspensions the conventional rotating cell technique was used (Stockburger et al., 1979).

Raman spectra were recorded by conventional equipment consisting of an argon ion laser (Coherent CR 52), a Jar-

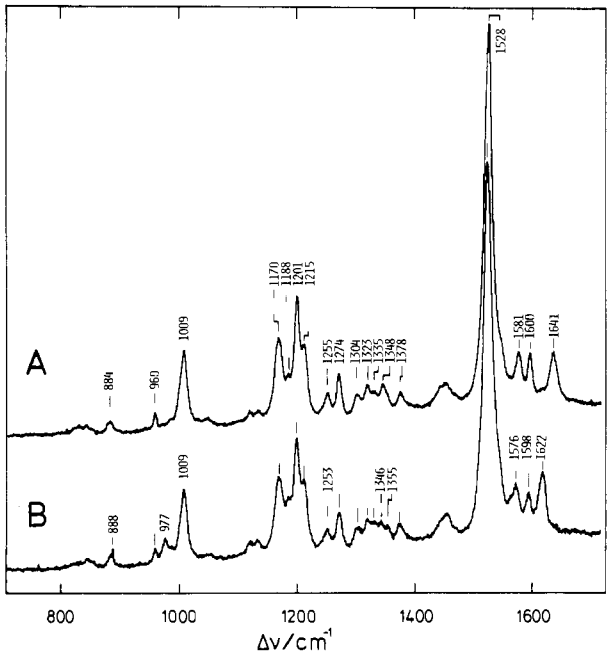

FIGURE 3: RR spectra of BR_{570} from a wet PM film at room temperature obtained with rotating disk technique. Photoconversion parameter [as defined by Stockburger et al. (1979)]: $I_0\Delta t \approx 0.1$, probe beam at 514 nm. Films were prepared from (A) H_2O and (B) D_2O suspensions.

rell-Ash 1-m double monochromator (25–100) provided with an RKB control system (125–610 polydrive) which allowed repetitive scanning, an ITT photomultiplier (FW 130), an Ortec detection system for photon counting, and a multichannel analyzer (Elscint, promeda). For excitation in the violet the 405-nm line of a Krypton laser (Coherent CR 2000 K) was used. In this case as well as for studies with aqueous PM suspensions the Raman equipment described by Massig et al. (1982) was applied. In all experiments the exciting laser beam was focused onto the sample (f = 150 nm). The applied laser power will be given in the figure captions. In studies with films the spectral bandwidth was 5 cm^{-1} while for the high-quality RR spectra from aqueous suspensions this was 3.7 cm^{-1}. Flash spectrophotometric measurements were performed with a computerized diodide array detection system (Uhl et al., 1984).

Results

Hydrated Membranes. It has been shown that the spectroscopic and photochemical behavior of the BR chromophore is the same in aqueous suspensions and hydrated films of PM (Korenstein & Hess, 1977a,b; Lazarev et al., 1980). One therefore would expect that also the RR spectra from the two types of samples are identical. Spectra of the unphotolyzed species BR_{570} from hydrated films (H_2O and D_2O) were recorded by using the rotating disk technique (Figure 3). As expected these are in all vibrational details identical with those obtained from aqueous suspensions (Alshuth & Stockburger, 1981; Massig et al., 1982).

The vibrational spectrum of BR_{570} has been comprehensively discussed (Stockburger et al., 1979; Massig et al., 1982; Braiman & Mathies, 1982). It is generally accepted that the retinal moiety in this chromophore is in the all-trans configuration. The pronounced isotopic shifts which occur on D_2O/H_2O exchange were ascribed to the fact that the SB nitrogen carries an exchangeable but covalently bound proton. Characteristic bands were assigned as C=N stretching and N–H in-plane (i.p.) hydrogen bending modes which move from 1641 and 1348 cm^{-1} to 1622 and 977 cm^{-1}, respectively, when

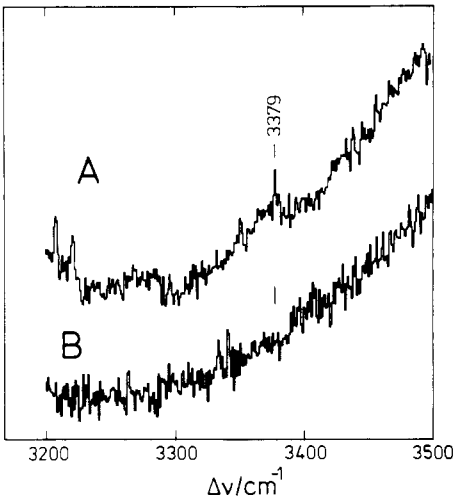

FIGURE 4: RR spectra of BR_{570} from a wet PM film in the N–H stretching region. Same conditions as in Figure 3. The film was prepared from (A) H_2O and (B) D_2O suspension.

the proton is replaced by a deuteron (Massig et al., 1982). However, up to now the N–H stretching vibration has not been observed, which leaves some uncertainty with respect to this interpretation. Instead, a hydrogen bond at the SB nitrogen was proposed (Leclercq et al., 1982). To clarify this point, we tried to find the N–H stretching vibration of the SB group.

In RR spectra from aqueous PM suspensions the O–H stretching bands of water totally obscure the region between 3000 and 3500 cm^{-1} where one expects the N–H stretch. In hydrated films, however, the concentration of chromophoric sites is increased by a factor of 1000 while the concentration of water is decreased. The water bands therefore completely vanish in RR spectra of hydrated films. In the spectra of PM/H_2O films a very weak band superimposed on a huge background was detected at 3379 cm^{-1} and disappeared for PM/D_2O (Figure 4). —It should be noted that this effect was observed in three independent measurements and also with different samples.—The intensity of the weak feature at 3379 cm^{-1} is 1000 times smaller than that of the C=C stretch of BR_{570} at 1528 cm^{-1} (Figure 3). This would not be surprising for an N–H stretch since it is well-known that such vibrations have small intensity in resonance Raman transitions which can be explained by the small Franck–Condon factors involved (Massig et al., 1982). Since the amide-A N–H stretching vibrations of BR lie at 3315 cm^{-1} (Lazarev et al., 1980), it can be ruled out that the small peak at 3379 cm^{-1} originates from the protein skeleton. We therefore assign it to the N–H stretching vibration of the protonated SB group of the BR chromophore. The high frequency reflects a covalently bound proton and therefore excludes strong hydrogen bonding. This assignment is corroborated by the work of Lewis & Sandorfy (1982), who found that the N–H stretch in model compounds with a protonated SB group lies very close to 3379 cm^{-1}. It should be noted that overtones and combination bands of the retinal chromophore do not attain enough RR intensity to perturb the 3379-cm^{-1} region. This, however, is different for the N–D stretch which could not be identified since it is obscured by the strong combination bands around 2500 cm^{-1}.

Dehydrated Membrane. We found that dehydration of PM films leads to a blue shift of the absorption maximum from 570 and 530 nm at room temperature and to 520 nm at liquid nitrogen temperature. This is in agreement with reports of Korenstein & Hess (1977b) and Lazarev & Terpugov (1980).

In the following the unphotolyzed dehydrated chromophore is denoted by BR_{530}(dh). It was found that its RR spectrum

is completely different from that of BR_{570}. The degree of dehydration, therefore, could be checked via the RR spectrum. If no further spectral changes occurred, it was assumed that the dehydration process was complete. This final state was established when the sample stayed for several hours on a vacuum line (pressure $\leq 10^{-4}$ torr). The back-reaction to the hydrated form was achieved within less than 1 min in an atmosphere of relative humidity between 25 and 50%.

The transition to the dehydrated chromophore was found to occur from both the light-adapted and the dark-adapted membrane. Continuous illumination of the sample during dehydration by an unfocused laser beam at 514 nm with 100 mW of power had no effect on the degree of formation of the dehydrated chromophore. This only depended on the time the sample was exposed to the vacuum system.

The RR spectrum of the dehydrated membrane, recorded under stationary conditions at 110 K, is shown in Figure 5A. As Raman probe beam the 514-nm line was used. At the same time the sample was illuminated by a second beam at 647 nm. This had the purpose of converting the primary photoproduct which absorbs in the red—as will be shown later—back to the unphotolyzed state. Thus, one can be sure that the spectrum in Figure 5A reflects the dehydrated chromophore in its unphotolyzed form BR_{530}(dh).

The spectrum of BR_{530}(dh) shall now be compared with the spectra of the all-trans chromophore BR_{570} (Figure 3) and the 13-cis form BR_{548} (Figure 5D). These two forms can be easily distinguished by means of the intensity distribution among the three "diagnostic bands" at 1185, 1215, and 1255 cm^{-1}. It is immediately seen that in the fingerprint region from 1150 to 1300 cm^{-1} the spectra of BR_{530}(dh) and the 13-cis chromophore BR_{548} closely resemble each other, while the characteristic bands of the all-trans form, at 1215 and 1254 cm^{-1}, are of reduced intensity in BR_{530}(dh). We therefore conclude that in the dehydrated state, BR_{530}(dh), retinal mainly is in the 13-cis configuration, although a fraction of chromophores is still in the all-trans form.

The most surprising feature in the spectra of BR_{530}(dh), however, is the absence of isotopic shifts for samples that were prepared from H_2O and D_2O suspensions, respectively. This is demonstrated by a comparison of the spectra in parts A and B of Figure 5 which do not show any detectable change in the vibrational structure, while the related 13-cis chromophore BR_{548} exhibits significant changes in the spectra of Figure 5D,E which can all be ascribed to H/D exchange at the SB nitrogen. Thus, the C=N stretch shifts from 1635 to 1621 cm^{-1}, and the fairly strong band at 1344 cm^{-1}, which was assigned to a coupled in-plane (i.p.) bending mode of the two SB hydrogens, splits into a C_{15}-H i.p. mode at 1410 cm^{-1} and a N-D i.p. mode at a much lower frequency (Alshuth & Stockburger, 1981). Characteristic changes also occur in the out-of-plane bending region between 800 and 1000 cm^{-1}.

The lack of isotope effects in the two spectra of BR_{530}(dh) in Figure 5A,B might be explained in two different ways. (I) BR_{530}(dh) has a protonated SB group—an assumption that is favored by its absorption in the visible—but none of the characteristic SB vibrations attains enough RR intensity, or the deuterium shifts are too small to be detectable. (II) BR_{530}(dh) has a deprotonated SB group.

For resolving this problem we have also recorded the spectrum of a H_2O-dehydrated PM in which hydrogen at the C_{15} carbon atom of the SB group was replaced by a deuterium (Figure 5C). It can be seen that the vibrational features between 1620 and 1650 cm^{-1} in Figure 5A,B shift down by 10–20 cm^{-1} in the C_{15}-D spectrum. This region is represented

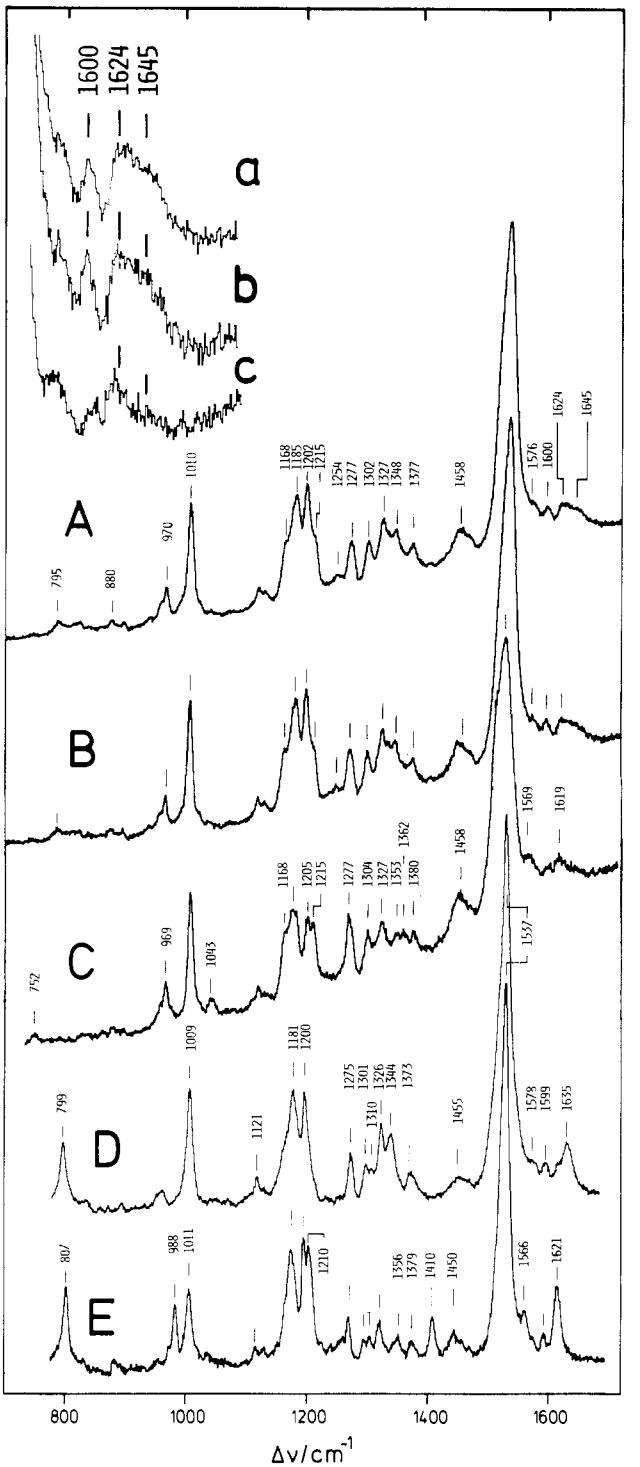

FIGURE 5: RR spectra: (A) BR_{530}(dh) from dehydrated PM film; stationary sample at 110 K, probe beam at 514 nm, additional pump beam at 647 nm. Sample prepared from H_2O suspension. (B) The same as in (A), but prepared from D_2O suspension. (C) The same as in (A), but with the retinal chromophore deuterated at the C_{15} position. (D) BR_{548} from H_2O PM suspension as described by Alshuth & Stockburger (1981). (E) The same as in (D), but from D_2O suspension. (a), (b), and (c) are sections from the spectra in (A), (B), and (C) on an enlarged scale.

by an extended scale as an insert in Figure 5. We therefore assign these features to C=N stretching vibrations of BR_{530}(dh). Indeed, shifts of similar magnitude for the C=N stretch were already found for various C_{15}-D-modified BR chromophores (Braiman & Mathies, 1980; Massig et al., 1982).

The heterogeneous structure in the C=N stretching region—at least two different peaks at 1624 and 1645 cm^{-1}

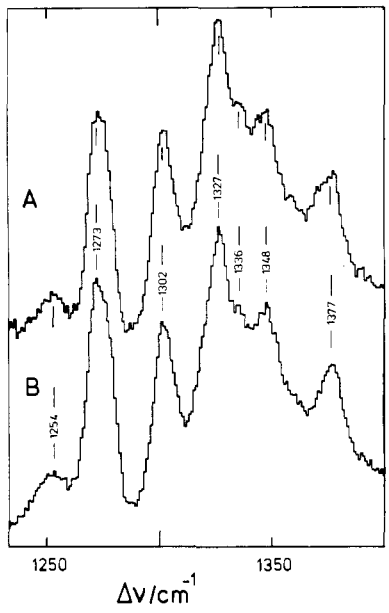

FIGURE 6: (A) and (B) refer to sections of the spectra in Figure 5A,B on an enlarged scale and under improved signal-to-noise ratio.

can be discerned—is due to contributions from various C=N structures which may be induced by environmental effects at the binding site and/or by the 13-cis/all-trans mixture in the configuration of the retinal chain of BR_{530}(dh) which we have found.

Once the C=N stretch of BR_{530}(dh) is identified, one has to ask for the isotopic shifts that one would expect for deuterium exchange at the nitrogen. For the two hydrated chromophores BR_{570} and BR_{548} shifts of 19 and 14 cm^{-1}, respectively, can be deduced from the spectra in Figures 3 and 5. As was pointed out by Aton et al. (1980), part of this shift is due to reduced coupling between the C=N stretch and the N-H i.p. bending motion which follows on H/D exchange. When C=N/N-H coupling does not exist, the residual shift is solely due to an increase of the reduced mass. Indeed, it was found by Argade et al. (1980) that the C=N stretch of BR_{570} is only shifted down by 13 cm^{-1} when ^{14}N is replaced by ^{15}N. The residual mass effect as a consequence of H/D exchange should be of similar magnitude. The inspection of the C=N region in Figure A,B, however, reveals that within the accuracy of our measurements no change of frequency or intensity distribution occurs between the two spectra. This behavior strongly suggests that in BR_{530}(dh) the nitrogen of the SB group is not protonated.

Additional support for this conclusion is obtained from the analysis of hydrogen bending modes of the SB group. Deuteration at the C_{15} carbon induces considerable changes between 1348 and 1370 cm^{-1} in the spectra of BR_{530}(dh) while a new band arises at 1043 cm^{-1} (Figure 5A,C). This is in close analogy to the spectra of BR_{548} where a new band appears at 1047 cm^{-1} in the C_{15}-D-modified chromophore (Th. Alshuth, private communication). On this basis we assign the bands at 1348 and 1043 cm^{-1} in Figure 5A,C to C_{15}-H/C_{15}-D i.p. bending modes, respectively. On the other hand, it is well established that the corresponding N-H mode of BR chromophores is also close to 1348 cm^{-1} (Massig et al., 1982). If the nitrogen were protonated but the N-H bend would not attain sufficient RR intensity, one would at least expect that the two SB hydrogen modes are coupled to some extent. In BR_{548} this coupling is rather strong. However, even when the coupling is weak, one would expect some influence on the C_{15}-H i.p. bend at 1348 cm^{-1}. We therefore have recorded

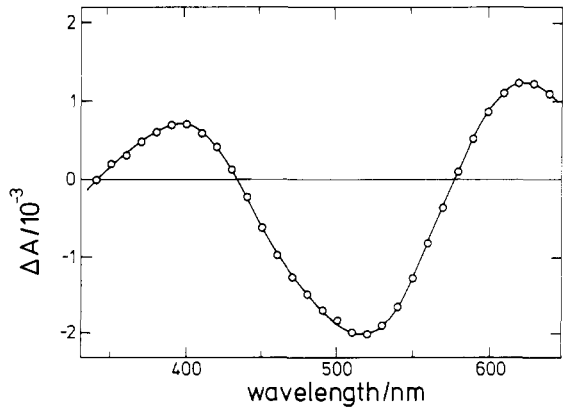

FIGURE 7: Absorbance changes, ΔA, of a dehydrated PM film, whose absorbance was 0.3 at 530 nm, measured 1 ms after illumination by a photographic flash of 100-μs duration.

spectra in this region at an extended scale (Figure 6). However, no relevant frequency or intensity variations are seen in the spectra when going from H_2O- to D_2O-dehydrated samples. This result again is in favor of an unprotonated nitrogen in the SB group of BR_{530}(dh).

One might object that the lack of isotopic shifts is due to the fact that in our samples H/D exchange at the SB nitrogen has not taken place. This would imply that resuspension of PM in D_2O, a procedure we have used, did not accomplish such an exchange. This, however, can be rejected for various reasons. Thus, a complete H/D exchange by just the same procedure was established in hydrated membranes as can be seen in the spectra of Figure 3. In the case of dehydrated membranes no spectral changes could be observed between samples prepared from PM suspended in D_2O for a few days or for several months. Finally, we point out the consecutive sections where spectroscopic evidence for H/D exchange in a dehydrated membrane will be presented.

Photochemical Cycle of BR_{530}(dh). We found that the dehydrated chromophore like the hydrated one is photolabile and runs through a reversible cyclic process. This process was investigated by time-resolved RR experiments as well as flash photolysis in a time domain between 100 μs and 1 s (Figure 1).

The existence of a primary photoproduct was inferred from photostationary low-temperature RR experiments (110 K). Following Oseroff & Callender (1974) single (514 nm) and double (514 and 647 nm) beam experiments were carried out to obtain the spectra of the photostationary mixture and of the unphotolyzed species. Broadening of the C=C stretching band and its shift to lower frequency in the spectrum of the mixture (not shown) indicate the existence of a red-shifted photoproduct "BR_x(dh)".

In Figure 7 the optical difference spectrum is shown at a delay time of 1 ms after the photoflash. Obviously the primary photoproduct relaxes to an intermediate which absorbs at 630 nm. The time of formation of this product was below the 100-μs time resolution of the flash photolysis device. BR_{630}(dh) then relaxes to the blue-absorbing intermediate M_{412}(dh) (rate constant 4×10^2 s^{-1}) which finally very slowly (0.5 s^{-1}) converts to the parent species.

Intermediate BR_{630}(dh). This intermediate could be well characterized by time-resolved RR experiments at room temperature. A mixture of the parent species and the two intermediates BR_{630}(dh) and M_{412}(dh) was probed. The main spectral features of BR_{630}(dh) were obtained by subtracting the contribution of the parent which amounted to about 60% (Figure 8).

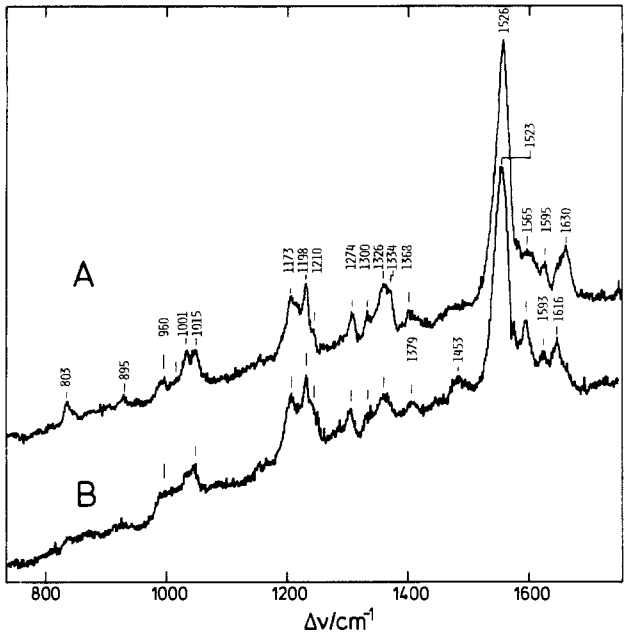

FIGURE 8: RR spectra of the intermediate BR_{630}(dh) in the photochemical cycle of dehydrated PM. Probe beam at 514 nm, rotating disk at 25 s^{-1}, photoconversion parameter $l_0\Delta t = 5$, $\Delta t = 19$ μs. The contribution of the parent BR_{530}(dh) was subtracted on the basis of the C=C stretching bands which are 15 cm^{-1} apart. The contribution of M_{412}(dh) with its strongest band at 1565 cm^{-1} was not subtracted. The dehydrated film was prepared from (A) H_2O and (B) D_2O suspension.

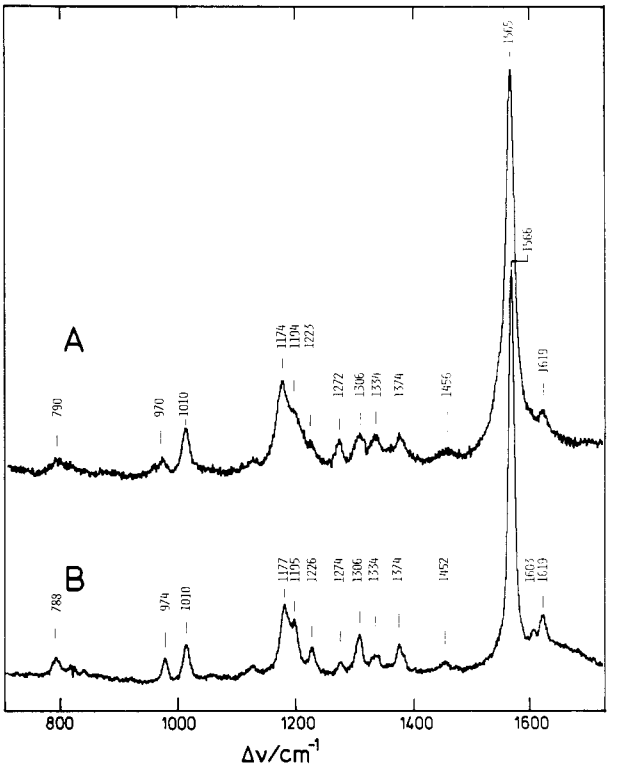

FIGURE 9: RR spectra: (A) M_{412}(dh) from a dehydrated PM film. (B) M_{412} from aqueous suspension. A 514/405-nm pump/probe configuration was used, combined with the rotating sample technique. The photoconversion parameters were analogous to those reported by Stockburger et al. (1979).

The spectra in the fingerprint region do not allow an unequivocal conclusion concerning all-trans or 13-cis configuration. They rather suggest that a mixture of both is prevalent or that the retinal chain is twisted around the C_{13}–C_{14} double bond. A distorted structure of the retinal chain is suggested by the fairly strong bands in the hydrogen out-of-plane bending region between 800 and 1000 cm^{-1} (Eyring et al., 1982; Massig et al., 1982).

Significant isotopic effects could be observed in the spectra of H_2O- and D_2O-dehydrated samples (Figure 8A,B). thus, a distinctive band in the C=N stretching region shifts from 1630 to 1616 cm^{-1}. Another band at 1334 cm^{-1} in the N-H i.p. bending region disappears while the intensity in the N-D i.p. region between 970 and 980 cm^{-1} increases. In the out-of-plane region a fairly distinct band at 803 cm^{-1} vanishes. These are clear pieces of evidence for a protonated Schiff base in BR_{630}(dh). It should be noted that the significant red shift with respect to the parent BR_{530}(dh) is another strong piece of evidence for the protonation of the SB group in this intermediate (Honig et al., 1976).

Intermediate M_{412}(dh). The spectrum of this intermediate was recorded in a pump/probe experiment using the two laser lines at 514 and 405 nm, respectively. Since the probe line at 405 nm coincides with the maximum of absorption of M_{412}(dh) but on the other hand is far away from the absorption maxima of all other chromophores, a spectrum of the pure M intermediate could be obtained. In Figure 9 this is compared with the spectrum of hydrated M_{412} which was obtained from aqueous PM suspension under identical pump/probe conditions. The two spectra are fairly similar, which leads us to the conclusion that the M_{412} chromophores have a similar structure in the hydrated and dehydrated membrane. A more refined inspection reveals a few differences such as small intensity changes and broadening effects. This is nicely seen for the strongest band at 1565 cm^{-1} which is probably due to a delocalized C=C stretching mode in which the C atoms of various double bonds move in phase. For the hydrated chromophore this band was recorded at higher spectral resolution. We found that it can be well fitted by a Lorentzian line shape with a FWHH of 10 cm^{-1}. In the case of M_{412}(dh) the width increases to 19 cm^{-1}, and the band is no longer Lorentzian but shows strong wings on both sides, which give the band an asymmetric shape. This behavior points to inhomogeneous broadening. This could be effected by the superposition of various conformational states of the retinal moiety contributing to the band shape. The broadening would be due to the fact that π-electron delocalization and thus the C=C frequency vary as a function of conformational distortion. Such effects are also indicated by the broadening of bands in the fingerprint region which involve C–C stretching coordinates coupled with hydrogen bending motion, whose frequencies would also vary with conformational distortion. Broadening in the out-of-plane bending region (750–1000 cm^{-1}) as well as intensity changes of some bands might also be explained by conformational variation.

The comparison of the two spectra of Figure 9 then leads to the following characterization of M_{412}(dh). Like the related M_{412} of the hydrated membrane, it has a deprotonated Schiff base, and its double-bond configuration is identical with that of M_{412}. On the other hand, a variety of conformational substates of the retinal moiety seem to be accessible in the dehydrated membrane while in the presence of water a well-defined conformation predominates. This implies that water molecules contribute to keep the retinal chromophore in a fairly fixed position.

It is of great interest to compare the spectra of the two dehydrated forms BR_{530}(dh) and M_{412}(dh) which according to the present analysis and previous work on M_{412} (Pettei et al., 1977; Stockburger et al., 1979; Braiman & Mathies, 1980) are both deprotonated and have the retinal moiety in the 13-cis

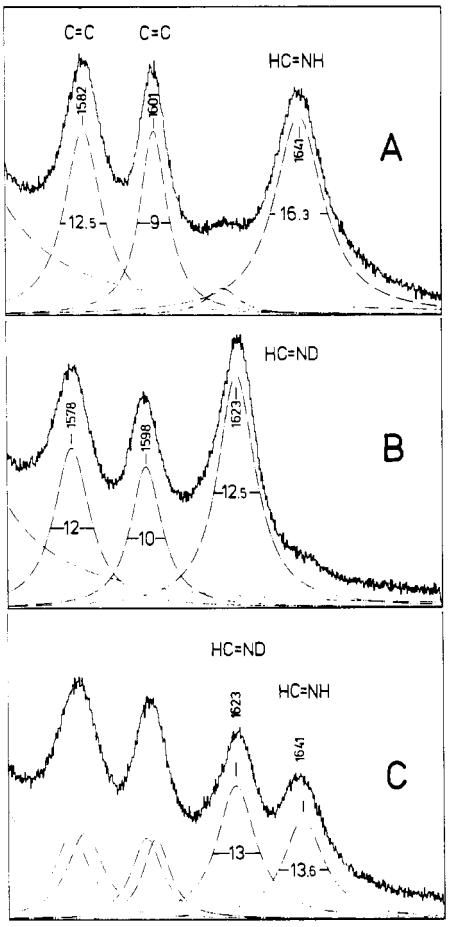

FIGURE 10: RR spectra of BR_{570} from aqueous PM suspension. Rotating cell, probe beam at 514 nm, photoconversion parameter $I_0\Delta t$ = 0.1. (A) H_2O; (B) D_2O; (C) H_2O/D_2O = 1:1.

configuration. The big differences in the fingerprint region between the two spectra (Figures 5 and 9) suggest that the two chromophores are in considerably different conformational states. Also the big red shift of BR_{530}(dh) would require a strong interaction with charged groups, which in the case of M_{412}(dh) is much less important. Consequently π electrons are more delocalized in BR_{530}(dh) than in M_{412}(dh). The concomitant changes in coupling of C=C and C–C stretching motion could also be a reason for the spectral differences in the fingerprint region.

Water Molecules Near the SB Group. We have demonstrated that water controls the structure and function of BR chromophores. Here we present RR spectroscopic evidence that in a hydrated membrane water molecules are placed in close vicinity to the SB group. In the spectra of BR_{570} from aqueous suspensions the C=N stretching band not only shifts up from 1623 to 1641 cm^{-1} but also is significantly broadened when D_2O is replaced by H_2O. This latter effect could not be observed for any other band. Spectra of high quality were recorded in the C=N stretching region from suspensions of H_2O, D_2O, and a 1:1 mixture of both. They are depicted in Figure 10. It could be shown that the strong C=C band of BR_{570} at 1529 cm^{-1} whose wing extends into the spectral region of Figure 10 can be ideally fitted by a Lorentzian line shape. This implies that the line width is homogeneous and determined by dephasing processes of the final vibrational level (Laubereau & Kaiser, 1978). Lorentzians therefore were used to fit the weak vibrational bands.

The parameters of the band-fitting procedure with Lorentzian line shapes are also given in Figure 10. The line width of the C=N stretch is broadened from 12.5 to 16.3 cm^{-1} on

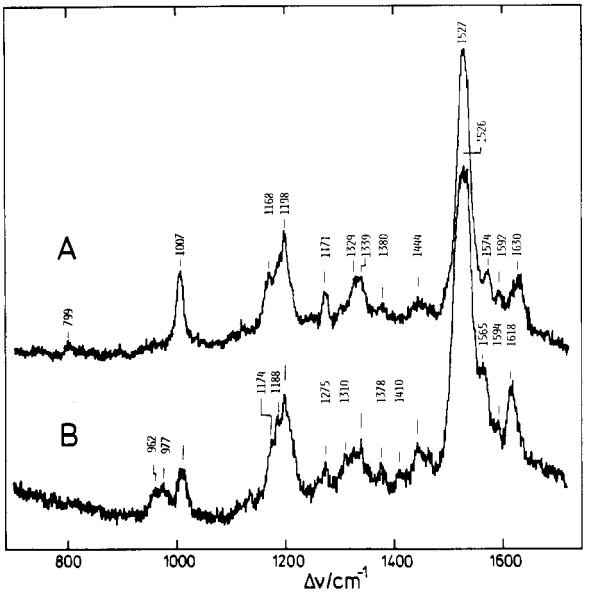

FIGURE 11: RR spectra: (A) BR_{535}(HCl); (B) BR_{535}(DCl); at room temperature, probe beam at 514 nm, rotating sample. Other conditions are given in the text.

D_2O/H_2O exchange while the width of the two neighboring C=C bands remains unchanged. The question is if this effect is due either to the D/H exchange at the SB nitrogen or to the exchange of the solvent. An answer can be obtained from the analysis of the spectrum of a 1:1 mixture of H_2O/D_2O (Figure 10). It was found that the line widths of the C=N bands of the mixture are 13 and 13.6 cm^{-1} for the deuterated and protonated nitrogen, respectively, and thus are nearly equal. This means that the broadening cannot be ascribed to the D/H exchange at the nitrogen. On the other hand, the widths of the two C=N stretching bands of the mixture are only slightly above the value of 12.5 cm^{-1} in D_2O. This suggests that it is only the H_2O isomer which has a statistical weight of 25% in the mixture which is responsible for broadening but not D_2O or HOD.

In this respect it is of importance that the bending vibration of H_2O at 1635 cm^{-1} is nearly in resonance with the C=N stretch at 1641 cm^{-1}, while the bending frequencies of HOD and D_2O lie at 1440 and 1205 cm^{-1}, respectively. From the frequency match in H_2O suspension it is suggested that the broadening is due to vibrational energy transfer from the SB group to a water molecule which would shorten the lifetime of the C=N stretching vibration. This would require that at least one water molecule must be closely attached to the SB group since the vibrational energy exchange mechanism is a short-range effect (Legay, 1977). Broadening of the C=N band in H_2O was also observed for other BR chromophores, e.g., for BR_{548} (Figure 5D,E).

Replacement of Water. The next step in our experiments was to replace water by other polar molecules. This was achieved by exposing dehydrated PM films to molecular vapors. At a partial pressure of 50 torr of dry HCl the dehydrated chromophore BR_{530}(dh) was converted to a new complex with a slightly shifted absorption maximum at 535 nm. The RR spectrum of this new form, BR_{535}(HCl), was recorded at room temperature and with the rotating sample technique in order to avoid photochemical decomposition. Under analogous conditions a spectrum of the complex BR_{535}(DCl) was obtained. A comparison of the spectra of the two forms in Figure 11 reveals clear isotopic effects which can be assigned to the exchange of the SB proton. Thus, the C=N stretch at 1630 cm^{-1} shifts to 1618 cm^{-1} in the presence of DCl, and

also the big changes between 900 and 1000 cm^{-1} can be ascribed to the isotopic substitution at the SB nitrogen. In contrast to the dehydrated chromophore these findings clearly point to a protonated SB group in the presence of HCl vapor.

We now compare the spectra of BR_{535}(HCl, DCl) with those of the hydrated BR_{570} chromophores in Figure 3. From the intensity distribution of the three diagnostic bands at 1255, 1215, and 1185 cm^{-1}, it is concluded that in BR_{535}(HCl) a 13-cis configuration similar to that of the dark-adapted form BR_{548} is dominating. The spectral data are interpreted as follows. HCl molecules that are adsorbed by the membrane from the vapor phase are able to accept the role of the water molecules in the vicinity of the SB group stabilizing predominantly the protonated 13-cis configuration.

The situation is different when the dehydrated PM film is exposed to dry NH_3. Under the influence of the exciting laser radiation (514 nm) a yellow brownish complex is formed, which indicates that the retinal–protein linkage is disrupted. We suppose that the first step in this reaction is the photochemical addition of NH_3 to the C=N double bond. If, however, NH_3 is added to hydrated PM films or to PM suspensions, no significant changes of the chromophoric structure are induced. The application of CH_3OH vapor on the other hand results in a disruption of the SB linkage for both hydrated and dehydrated films.

Discussion

Water at the Chromophoric Site. It was found in the present work that water molecules exert a strong influence on the structure and function of BR chromophores. One might argue that the observed phenomena are due to changes in the secondary and tertiary structure of the protein, which frequently occur when water is removed (Levin, 1974). At this point it is important to realize that irreversible denaturation of the protein can be ruled out, since all dehydration effects we have observed were completely reversible.

Neutron diffraction experiments of 7-Å resolution did not show any structural changes of the protein on dehydration of PM (Rogan & Zaccai, 1981). Lazarev & Terpugov (1980, 1981) have found that the infrared amide bands, which are known to depend sensitively on conformational changes, also are not influenced by dehydration. This implies that the removal of water can only induce minor structural changes in the protein backbone. From this behavior it is suggested that the important structural and functional modifications at the chromophoric site—trans–13-cis isomerization of the retinal chain, deprotonation of the SB group, and different photochemical behavior—are due to the removal of those water molecules that are localized in the immediate vicinity of the chromophore.

In this context we mention the work of Terpugov et al. (1982), who also have investigated PM films by RR spectroscopy. On the basis of isotope shifts in the spectra of H_2O/D_2O dehydrated samples, they concluded that the SB group remains protonated after the removal of water. In our experiments we have also found residual isotope effects under conditions where water was not completely removed. Obviously the water molecules at the chromophoric site are tightly bound, and it requires, as stated under Materials and Methods, a prolonged exposure to a high vacuum line to remove them completely from their binding site.

At this point it is also important to cite the RR spectroscopic studies of Doukas et al. (1981) on H/D exchange at the SB nitrogen in BR. They found that in aqueous PM suspension this mechanism takes place in times shorter than 3 ms. Since base-catalyzed dissociation followed by reprotonation cannot account for such a short reaction time, they proposed that a water molecule directly interacts with the SB group which would enable a concerted H/D exchange reaction. In the model they propose a water molecule is directly attached to the SB group.

There is convincing experimental evidence that in the intact BR chromophore a negatively charged counterion is located near the positively charged SB group (Fischer & Oesterhelt, 1979, 1980). Obviously the structure and function of the BR chromophore is largely determined by this ionic interaction. From the present paper it then is suggested that the ion pair structure is stabilized by water molecules.

We support this point of view by further evidence. There are good reasons to assume that the negative counterion is a carboxylate side group of a glutaric or aspartic amino acid residue (Fischer & Oesterhelt, 1979; Herz et al., 1983). It was found that by addition of hydrochloride or hydrofluoride to an aqueous PM suspension the carboxylate ion can be protonated ($pK_a \approx 2.9$) (Mowery et al., 1979; Fischer & Oesterhelt, 1979). At a pH value of 2 one obtains a new complex, BR_{605}(acid), in which the internal counterion is completely neutralized. When the ionic strength is further increased, new complexes, $BR_{563}(Cl^-)$ or $BR_{545}(F^-)$, are formed in which the internal counterion seems to be replaced by Cl^- or F^- anions. The RR spectra of these chromophores were studied in our laboratory by Massig (1982). It turned out that $BR_{563}(Cl^-)$ or $BR_{545}(F^-)$ consists of a mixture of two species whose structure is closely related to that of BR_{570}(all-trans) and BR_{548}(13-cis) in the natural membrane.

From these studies it is suggested that, under conditions where the internal counterion is neutralized and externally introduced anions (Cl^-, F^-) are available in excess, a chromophoric structure is built up, which is very similar to that in the natural membrane. This is the case although the charge density of the carboxylate anion is quite different from that of chloride or fluoride anions. It is thus suggested that a shell of water molecules is located between the positive SB group and the counterion which is leveling the charge distribution of the individual counterions.

Finally, it is interesting to note that for the visual pigment rhodopsin Rafferty & Shichi (1981) have postulated that water is directly involved in the interaction of the retinylidene group and opsin. Their conclusions were based on dramatic absorption changes that follow the dehydration of the membrane.

There is thus convincing evidence that water molecules are located in the vicinity of the retinal chromophore. But evidently the chromophore cannot be immersed completely in a pool of water since otherwise the SB bond would not be stable. The fact that the unprotonated intermediates, M_{412} and M_{412}(dh), in the hydrated and dehydrated membrane have a similar conformational structure implies that in the absence of ionic interactions with the SB group water molecules do not determine the chromophore's structure but rather the nonpolar interactions with the protein environment (Warshel & Barboy, 1982) are largely responsible. This is why we believe that water molecules are confined to the vicinity of the terminal SB group, where they stabilize ionic structures and also contribute to keep the chromophore in a fixed position within the protein pocket.

Chromophoric Structure and Photochemical Reactivity. The different structure in the hydrated and dehydrated membrane also gives rise to a different photochemical behavior. It was one of the most striking conclusions from the present RR studies that the parent chromophore in the dehydrated membrane, BR_{530}(dh), does not carry a covalently bound

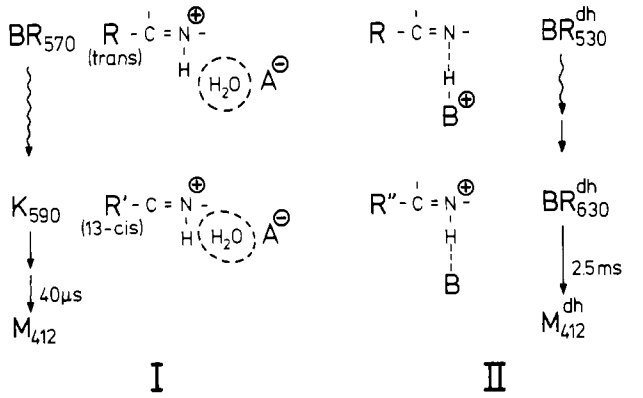

FIGURE 12: Primary photochemical events in (I) the hydrated and (II) the dehydrated PM. Assignments of the various chromophoric states are the same as in Figure 1. R refers to the retinal moiety, and R′ and R″ are conformationally distorted states with respect to R. A^- is a counterion, probably a carboxylate anion, and B is a basic side group of the protein. Water molecules are assumed to be located within the encircled areas.

exchangeable proton at its SB group. One might argue that in the absence of water the ion pair structure which was suggested for BR_{570} (Figure 12) is unstable, and instead a neutral configuration is formed where the SB proton has migrated to the negative counterion. However, the fact that dehydration only slightly changes the color of the chromophore—the absorption maximum is shifted from 570 to 530 nm—is not in favor of such an assignment. It is well-known from model calculations that the SB proton exerts a strong influence on the red shift of the retinylidene chromophore (Honig et al., 1976; Favrot et al., 1979; Balogh-Nair et al., 1981). It is thus suggested that in BR_{530}(dh) a proton is in the neighborhood of the SB group. Indeed, it could be shown by model calculations that a proton which is bound to a basic group "B", thus leaving a positive charge at this group, is able to induce a red shift of the chromophore if this proton is attached to the unprotonated Schiff base via a hydrogen bond (Favrot et al., 1979). It is therefore appealing to assign BR_{530}(dh) to such a configuration (Figure 12).

We found that important structural components as well as the photochemical behavior of BR_{530}(dh) can be understood on the basis of ideas which had been put forward by Sandofy & his co-workers when they discussed the primary event in visual pigments (Favrot et al., 1979; Dupuis et al., 1981). They argued that in a hydrophobic environment the ion pair structure formed by the positively charged SB group and a negative counterion cannot be stable but instead a "proton bridge" between the SB nitrogen and a neighboring proton donor or acceptor was suggested. Applied to BR_{530}(dh) this would imply that the proton is attached to the base B and hydrogen bonded to the SB nitrogen (Figure 12). This bond, however, cannot be a strong one since we could not observe any isotope shifts in the RR spectra of BR_{530}(dh) upon H/D exchange in the membrane. The structure we propose for the parent BR_{530}(dh) thus requires that B is a stronger base than the Schiff base. If one accepts that the proton moves in a double well potential between the bridge heads, the well closer to B would be the deeper one.

Candidates for B are the nitrogen bases of the protein environment. The most favored one would be the ϵ-amino group of lysine with its high basicity (pK_a = 10.5). However, since we do not know the strength of the Schiff base in BR_{530}(dh), other nitrogen bases like the guanidino group of arginine or even the indole ring of trypthophan had to be taken into account. [For the amino acid sequence we refer to the work of Ovchinnikow et al. (1979) and Khorana et al. (1979).]

The first intermediate in the photochemical cycle of the dehydrated membrane which could be well characterized by its RR spectra [BR_{630}(dh) in Figure 8] has a protonated Schiff base. This means that proton transfer to the SB group is triggered by photoexcitation. It is very likely that this process occurs during the primary photochemical step from BR_{530}(dh) to BR_x(dh). The photoinduced proton transfer is just the essential point in Sandorfy's model. Thus, it is well-known that the basicity of the SB group is much higher in the excited than in the ground state. This has been shown explicitly for model compounds by Schaffer et al. (1974) on the basis of Förster's (1950) and Weller's (1957) original work. In the excited state, therefore, the potential well which is closer to the SB nitrogen is the one, and the proton can migrate to the nitrogen (Figure 12). This configuration must be stabilized by concomitant changes in other coordinates, probably conformational distortion of the retinal moiety. The proton transfer to the SB group is also reflected by the big red shift from 530 to 630 nm.

When the proton is detached from the SB group during the photochemical cycles, the two intermediates M_{412} and M_{412}(dh) are formed, which, as was argued above, have a similar conformational structure. This implies that in this intermediate state water is no longer the structure-determining factor, but nonpolar interactions seem to be the dominating ones.

The main structural and kinetic features of the two photochemical cycles (Figures 1 and 12) can be summarized as follows. The structure of the parent chromophore BR_{570} in the hydrated membrane is largely determined by an ion pair configuration which is stabilized by an internal hydration shell, and there is general agreement that all-trans → 13-cis photoisomerization is the primary event. In the dehydrated membrane, however, a different charge distribution is stabilized, which gives rise to the parent chromophore BR_{530}(dh) with an unprotonated SB group. In this case the primary step is photoinduced proton transfer.

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Registry No. HCl, 7647-01-0; DCl, 7698-05-7; NH_3, 7664-41-7; CH_3OH, 67-56-1; H_2O, 7732-18-5; 13-*cis*-retinal, 472-86-6; *all-trans*-retinal, 116-31-4.

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