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# Removal of the 9-Methyl Group of Retinal Inhibits Signal Transduction in the Visual Process. A Fourier Transform Infrared and Biochemical Investigation†

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ABSTRACT: The photoreaction of opsin regenerated with 9-demethylretinal has been investigated by UV-vis spectroscopy, flash photolysis experiments, and Fourier transform infrared difference spectroscopy. In addition, the capability of the illuminated pigment to activate the retinal G-protein has been tested. The photoproduct, which can be stabilized at 77 K, resembles more the lumirhodopsin species, and only minor further changes occur upon warming the sample to 170 K (stabilizing lumirhodopsin). UV-vis spectroscopy reveals no further changes at 240 K (stabilizing metarhodopsin I), but infrared difference spectroscopy shows that the protein as well as the chromophore undergoes further molecular changes which are, however, different from those observed for unmodified metarhodopsin I. UV-vis spectroscopy, flash photolysis experiments, and infrared difference spectroscopy demonstrate that an intermediate different from metarhodopsin II is produced at room temperature, of which the Schiff base is still protonated. The illuminated pigment was able to activate G-protein, as assayed by monitoring the exchange of GDP for GTPγS in purified G-protein, only to a very limited extent (approximately 8% as compared to rhodopsin). The results are interpreted in terms of a specific steric interaction of the 9-methyl group of the retinal in rhodopsin with the protein, which is required to initiate the molecular changes necessary for G-protein activation. The residual activation suggests a conformer of the photolyzed pigment which mimics metarhodopsin II to a very limited extent.

Rhodopsin, the visual pigment of vertebrate rods, consists of the protein opsin and the chromophore 11-*cis*-retinal (Wald, 1968) which is bound to lysine-296 (Ovchinnikov, 1983) by a protonated Schiff base linkage (Oseroff & Callender, 1974). Upon absorption of light, the chromophore is converted to the all-trans isomer already in the primary photoproduct (Palings et al., 1987; Bagley et al., 1985; Ganter et al., 1988), and, through a series of intermediates, rhodopsin decays to *all-trans*-retinal and opsin. One of these intermediates, metarhodopsin II, possesses a distinct conformation which enables it to activate an enzyme cascade which finally results in the change of the electric potential of the cell (Stryer, 1986). Because of the multitude of proline residues in rhodopsin and in a number of structural-related hormone receptors, Brandl and Deber (1986) suggested that the change in the conformation of rhodopsin which activates the enzyme cascade is caused by a cis-trans isomerization of a proline residue. However, little is known of how the retinal isomerization is linked to the structural changes occurring in metarhodopsin II.

Resonance Raman and Fourier transform infrared (FTIR)[1] difference spectroscopy provides information on molecular events during the photoreaction [e.g., see Palings et al. (1987), Bagley et al. (1985), and Ganter et al. (1988)]. Because FTIR spectroscopy covers spectral changes of both the chromophore and the surrounding proteins, this method is well-suited for observing chromophore-protein interactions. Previously, we have reported spectra on the rhodopsin-isorhodopsin and rhodopsin-bathorhodopsin (Siebert et al., 1983) and on the rhodopsin-lumirhodopsin transitions (Ganter et al., 1988). During the rhodopsin-lumirhodopsin transition, three carbonyl groups are altered, two of which could be assigned to protonated carboxylic acids and the other to the amide I vibration of an amino acid bound to the amino terminus of a proline residue. De Grip et al. (1985) reported on changes occurring during the rhodopsin-metarhodopsin I and the rhodopsin-metarhodopsin II transitions in the region of the carbonyl stretching vibrations. Rothschild et al. (1987) studied the alterations in the protein during the decay of the metarhodopsin II species.

Detailed molecular information on the visual pigment was obtained by regenerating opsin with synthetic retinal analogues. In a recent review, Derguini and Nakanishi (1986) described

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[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; HOOP, hydrogen out of plane vibration; ROS, rod outer segment(s); 9-H, 9-demethyl; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

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most of the retinal analogues employed. 9-Demethylretinal (9-H-retinal) was prepared by Blatz et al. (1969), and the regeneration of rhodopsin with it yielded a pigment absorbing at 465 nm, 35 nm blue-shifted as compared to rhodopsin. Kropf (1976) and Eyring et al. (1980) reported on a red-shifted photoproduct obtained by illumination of 9-H-rhodopsin at 77 K. Resonance Raman studies (Eyring et al., 1980) showed that the unusual intense HOOP vibrations of bathorhodopsin are not present in the spectrum of this photoproduct of 9-H-rhodopsin. They therefore concluded that, in contrast to normal bathorhodopsin, the retinal is not twisted.

Protonated Schiff bases of 9-H-retinal do not exhibit unusual properties as compared to those of retinal. Therefore, the peculiar behaviour of 9-H-rhodopsin must be caused by the altered retinal–protein interaction. From the absence of large HOOP bands, it appears that especially the steric interaction is changed. One possibility of how the retinal isomerization could transmit the information to the protein is by means of steric interaction. Therefore, to attain information on the mechanism of rhodopsin activation, it is of interest to investigate the molecular changes occurring during the photoreaction of 9-H-rhodopsin. In this paper, the FTIR difference spectra for the different intermediates of the phototransition of 9-H-rhodopsin in $H_2O$ and $^2H_2O$ are presented. In addition, the capability of bleached 9-H-rhodopsin to activate the retinal G-protein is tested by measuring the binding of the nonhydrolyzable GTP analogue GTP$\gamma$S in exchange for the GDP to the active site of G-protein. The results are interpreted in terms of a close coupling of retinal isomerization and protein structural changes.

## Materials and Methods

*Spectroscopic Measurements.* All manipulations involving rhodopsin are performed in darkness or under dim red light. Rod outer segments were isolated as previously reported (Siebert et al., 1983). The 9-H-retinal was a generous gift from J. Lugtenburg. 15-Deuterio-9-H-retinal was prepared from an isomeric mixture of 9-H-retinal by 2-fold reduction with $NaB^2H_4$ to the retinol and subsequent reoxidation with $MnO_2$ to the retinal, yielding an isotopic enrichment of approximately 75% as tested by mass spectroscopy for normal retinal. The 11-cis isomer was separated by HPLC, using normal 11-*cis*-9-H-retinal as standard. Opsin was regenerated with 9-H-retinal as described (Ganter et al., 1988) with the modification that, because of the slow regeneration, a 10-fold molar excess of retinal was used.

*Formation of Photoproducts.* To obtain the bathorhodopsin, lumirhodopsin, metarhodopsin I, and metarhodopsin II photoproducts, the samples were illuminated with light of wavelengths between 435 and 470 nm. To obtain isorhodopsin from unmodified rhodopsin, the sample was illuminated for 20 min with light of wavelengths longer than 570 nm. For 9-H-rhodopsin, light with wavelengths longer than 515 nm was used. The difference spectra for the transitions to isorhodopsin and bathorhodopsin were measured at 80 K, to lumirhodopsin at 173 K, to metarhodopsin I at 243 K, and to metarhodopsin II at 270 K.

*FTIR and UV–Vis Measurements.* The method for the FTIR measurements has been described previously (Ganter et al., 1988): 0.1 mL of a suspension of ROS was dried onto a $CaF_2$ window and rehydrated with $H_2O$. The suspension contained 1 mg/mL rhodopsin, and, to increase the yield of metarhodopsin II, 5 mM phosphate buffer, pH 5.5. For UV–vis measurements of bathorhodopsin, isorhodopsin, lumirhodopsin, metarhodopsin I, and metarhodopsin II, the same samples were used.

*Flash Photolysis Experiments.* To monitor the generation of metarhodopsin II, time-resolved measurements were performed: the sample was excited with a 420-nm flash from a dye laser pumped by an excimer laser. The energy of the flash was approximately 1 mJ. The monitoring beam, tuned to 500 nm, was detected by means of a silicone photodiode. The light-induced signal from the photodiode was collected by a storage oscilloscope with dwell times of 50 $\mu$s and 2 ms. To block the actinic flash, a GG455 filter from Schott was used. To avoid excessive bleaching of the sample by the monitoring beam, a shutter opened within 0.5 s before the flash. For these measurements, ROS suspended in 100 mmol of phosphate buffer, pH 5.5, were used. Single-flash signals are shown.

*Determination of G-Protein Activation.* Guanosine 5′-[$\gamma$-$^{32}$P]triphosphate ([$\gamma$-$^{32}$P]GTP) was obtained from Amersham Inc. $^{35}$S-Labeled guanosine 5′-*O*-(3-thiotriphosphate) ([$^{35}$S]GTP$\gamma$S) was obtained from New England Nuclear. Bovine retinas were obtained from the W. E. Lawson Co. PIPES was obtained from Aldrich Chemical Co. Dodecyl maltoside was obtained from Calbiochem. Concanavalin A–Sepharose 4B and Sephadex G-25 were obtained from Sigma Chemical Co. Hydroxylapatite was obtained from Bio-Rad.

Rod outer segments were isolated according to the method of Papermaster and Dryer (1974), and rhodopsin was purified on a concanavalin A–Sepharose 4B column as described in Calhoon and Rando (1985). Retinal G-protein was purified by the method of Kühn (1980).

*Preparation of 9-H-Rhodopsin.* To obtain 9-H-rhodopsin, native rhodopsin in 10 mM PIPES, pH 6.5, 100 mM NaCl, and 6 mM dodecyl maltoside was bleached in the presence of 10 mM $NH_2OH$ for 15 min with intense light at 4 °C. Excess $NH_2OH$ was removed by gel filtration on G-25 Sephadex. Opsin was regenerated by incubation with a 6–8 molar excess of 11-*cis*-9-H-retinal for 60 min at 20 °C. 9-H-Rhodopsin was purified by hydroxylapatite chromatography: 9-H-rhodopsin in 20 mM $KH_2PO_4$ at pH 6.5 was loaded onto a hydroxylapatite column and eluted with 370 mM $KH_2PO_4$ at pH 6.5. Reconstituted rhodopsin was prepared as 9-H-rhodopsin but with normal 11-*cis*-retinal.

*GTP$\gamma$S Exchange Assays.* The GTP$\gamma$S binding assay was modified from a procedure described by Wessling-Resnick and Johnson (1987). In our conditions, the assay was performed with solubilized rhodopsin instead of with membranes containing rhodopsin prepared from rod outer segments. The assay mixture contained 25 nM rhodopsin, 450 nM G-protein, 176 nM [$^{35}$S]GTP$\gamma$S (0.125 $\mu$Ci/pmol), 110 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.6 mM dodecyl maltoside, 0.1 mM EDTA, 4% glycerol, 1 mM PIPES, and 10 mM Tris-HCl in a final volume of 250 $\mu$L at pH 7.4.

Rhodopsin in 10 mM PIPES, pH 6.5, 100 mM NaCl, and 6 mM dodecyl maltoside was irradiated for 10 s or 1 min at 4 °C with intense light filtered through water and a yellow filter (Corning 3-68, cutoff of 540 nm), or with white light, and then added to the assay mixture. Values for unirradiated rhodopsin were obtained by performing the assay under dim red light.

Assay mixtures without G-protein were incubated for 1 min at 20 °C. Reactions were started by addition of G-protein, and 50-$\mu$L aliquots were taken at timed intervals, filtered through nitrocellulose filters, and immediately washed with two 4-mL aliquots of ice-cold buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM EDTA). Filters were dissolved in scintillation fluid, and the radioactivity was counted in a Beckman LS 330. Assay were done in

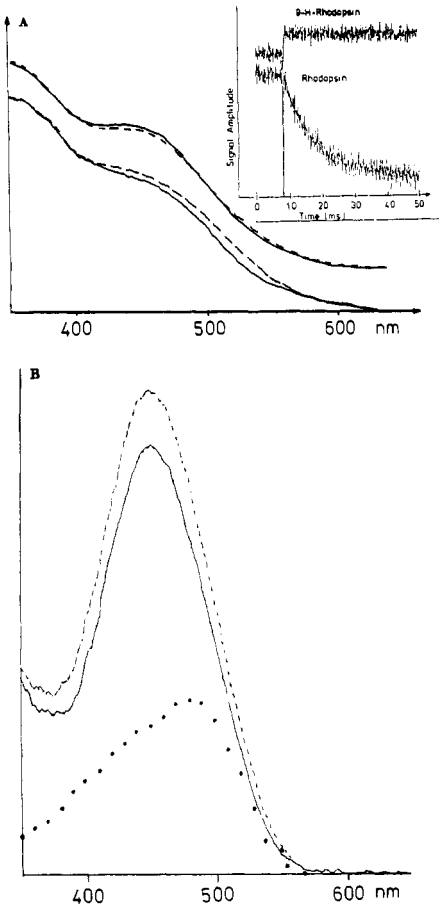


FIGURE 1: UV–vis spectra 9-H-rhodopsin (solid lines) and its photoproducts (dashed lines). (A) Spectra of hydrated films: lower two traces, spectra at 80 K; upper two traces, spectra at 270 K. Insert shows time-resolved measurements at 300 K of a suspension of 9-H-rhodopsin (ROS) (upper trace) and unmodified rhodopsin (ROS) (lower trace). Wavelength of monitoring beam 500 nm. (B) Spectra of solubilized 9-H-rhodopsin in the reaction mixture of the G-protein activation assay (solid line) and of its room temperature photoproduct (dashed line); dotted line, difference spectrum formed manually, absorbance scale expanded 3 times.

triplicate. The rate of exchange of GDP bound to the retinal–G-protein for GTP was calculated from the slopes of the plots of the GTPγS binding versus time in their linear portion.

All UV–vis spectra related to the measurement of G-protein activation were taken with a Perkin-Elmer 552A UV/VIS spectrophotometer between 650 and 250 nm in quartz semimicrocuvettes. Spectra of 9-H-rhodopsin and its room temperature photoproduct in the reaction mixture are shown.

## Results

*UV–Vis Spectra.* The UV–vis spectra of the infrared samples (hydrated films) of 9-H-rhodopsin are shown in Figure 1A. Because of the large excess of retinal used for regeneration, the 9-H-rhodopsin appears as a shoulder at the absorption band of free retinal. The absorption maximum of 9-H-rhodopsin is at 465 nm (Blatz et al., 1969). Illumination at 77 K causes a small red-shift (Figure 1A, lower two traces). Because of strong light scattering, which varies with temperature, the exact absorption maxima of lumirhodopsin and metarhodopsin I, obtained by warming up the sample, cannot be ascertained. It appears that the red-shift observed in bathorhodopsin is partially reverted in luminrhodopsin but the absorption maximum is still red-shifted by approximately 5 nm as compared to the initial state, whereas that of metarhodopsin I is nearly unshifted (data not shown). By illumination with light of wavelengths longer than 515 nm, no

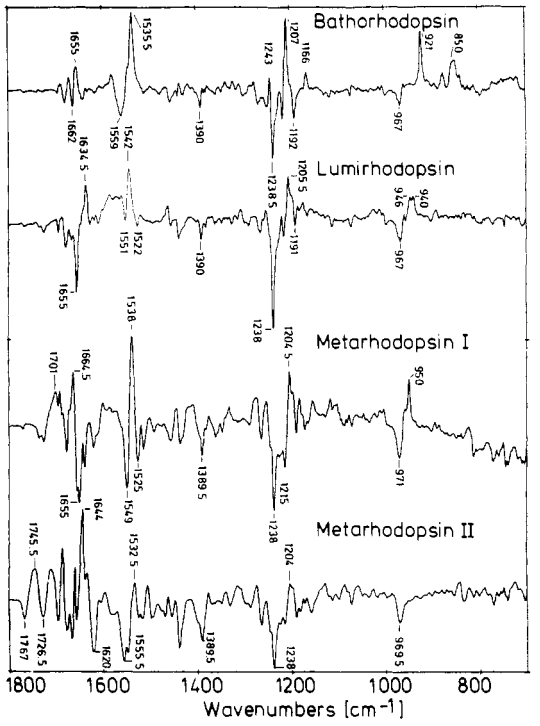


FIGURE 2: Infrared difference spectra of the phototransitions of unmodified rhodopsin. From the top: bathorhodopsin, lumirhodopsin, metarhodopsin I, and metarhodopsin II.

isorhodopsin could be produced (data not shown). At 270 K, the light scattering is reduced. After illumination, a small increase of the absorption is observed above 475 nm and a decrease around 465 nm (Figure 1, upper two traces), indicating again a red-shift of the absorption maximum. Further warming to 293 K does not change the spectrum significantly, and even after 14 h, no absorption increase around 360 nm could be detected. In Figure 1B, the spectra of 9-H-rhodopsin (dark and illuminated) from the assay for G-protein activation are presented. Since the protein is solubilized and the excess retinal removed, the effect of illumination can be ascertained more precisely. It is clear that also in dodecyl maltoside a red-shifted photoproduct of 9-H-rhodopsin is produced at room temperature. In addition, an overall increase in absorbance is observed. The small deviation as compared to the spectra of the hydrated films can probably be explained by light-scattering changes in the latter case. Forming manually the difference spectrum (Figure 1B, dotted trace) does not provide evidence for the presence of an additional blue-shifted photoproduct.

*Flash Photolysis Experiments.* The light-induced signal corresponding to the absorbance change at 500 nm of unmodified rhodopsin is shown in the insert of Figure 1A. The formation of metarhodopsin II, i.e., the deprotonation of the Schiff base, can be inferred from the large absorbance decrease. Since the absorbances of rhodopsin and metarhodopsin I are almost equal at 500 nm, no change caused by this transition can be seen. In the upper trace of the insert of Figure 1, the corresponding signal of 9-H-rhodopsin, also measured at 500 nm, is shown. Here, an increase in absorbance is observed, the kinetic of which is not resolved at this time resolution. Further measurements at 440, 455, and 465 nm did not reveal any decrease of the absorption. In a separate experiment, the signal was measured with a dwell time of 2 ms, increasing the time range to 2 s. No further reaction could be detected (data not shown).

*Infrared Spectra.* The difference spectra of the different phototransitions of unmodified rhodopsin are shown in Figure

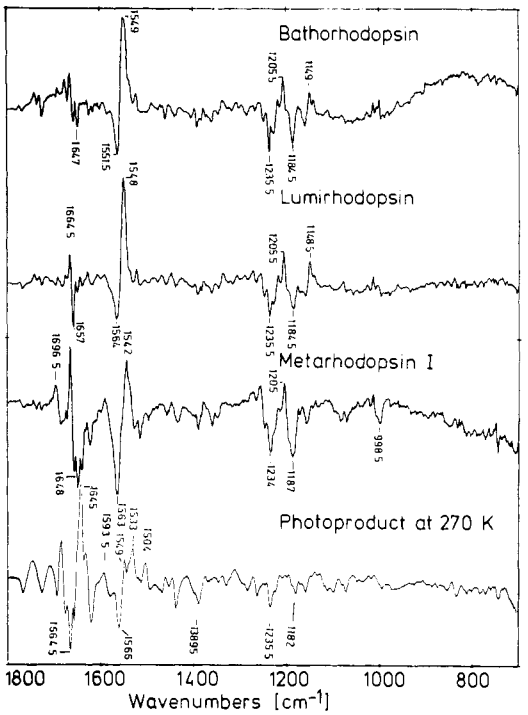


FIGURE 3: Infrared difference spectra of the phototransitions of 9-demethylrhodopsin. From the top: 9-H-bathorhodopsin, 9-H-lumirhodopsin, 9-H-metarhodopsin I, and the photoproduct obtained at 270 K.

2; the corresponding spectra of 9-H-rhodopsin are shown in Figure 3. The rhodopsin–bathorhodopsin and rhodopsin–lumirhodopsin difference spectra of 9-H-rhodopsin are almost identical with small differences in the 1600–1700 $cm^{-1}$ region. They resemble the rhodopsin–lumirhodopsin difference spectrum of unmodified rhodopsin much more than the rhodopsin–bathorhodopsin difference spectrum. Especially the protein bands around 1730 $cm^{-1}$ are present both in the spectra of 9-H-rhodopsin and in the normal rhodopsin–lumirhodopsin spectrum, but they cannot be seen in the rhodopsin–bathorhodopsin spectrum of unmodified rhodopsin. Also, as previously reported (Eyring et al., 1982), the strong hydrogen out of plane vibrations (HOOP) of bathorhodopsin (921 $cm^{-1}$) are not present in the difference spectrum of 9-H-rhodopsin.

The difference spectra at 243 K are shown in Figures 2 and 3. The strong alterations in the region of the carbonyl stretching vibration of the unmodified species are not observed for 9-H-rhodopsin. Again, this part of the difference spectrum of the modified species resembles more the rhodopsin–lumirhodopsin spectra. The ethylenic mode at 1542.5 $cm^{-1}$ of 9-H-metarhodopsin I is downshifted relative to the lumirhodopsin band at 1548 $cm^{-1}$. A similar downshift is observed for the unmodified pigment.

Since it is assumed that carboxylic groups interact with the chromophore, the spectral range of the corresponding bands deserves special interest. In Figure 4, the region of the carbonyl stretching vibration of protonated carboxylic acids (1800–1700 $cm^{-1}$) is shown for the different phototransitions of unmodified rhodopsin. In comparison, the spectra of 9-H-rhodospin are shown in Figure 5. The traces on the left of both figures correspond to measurements in $H_2O$ and those on the right to measurements in $^2H_2O$. The spectra of the rhodopsin–bathorhodopsin and the rhodopsin–lumirhodopsin transitions of unmodified rhodopsin have been reported previously (Bagley et al., 1985; Ganter et al., 1988) and are only shown for comparison. The same spectral range has been investigated by de Grip et al. (1985) for the rhodopsin–me-

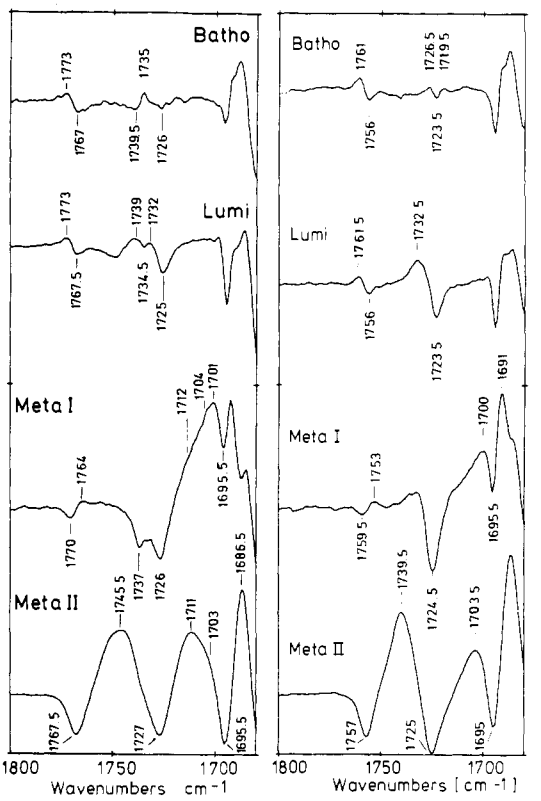


FIGURE 4: Infrared difference spectra in the region of the carbonyl stretching frequency of unmodified rhodopsin. Left-hand traces, measurements in $H_2O$; right-hand traces, measurements in $^2H_2O$. From the top: bathorhodopsin, lumirhodopsin, metarhodopsin I, and metarhodopsin II.

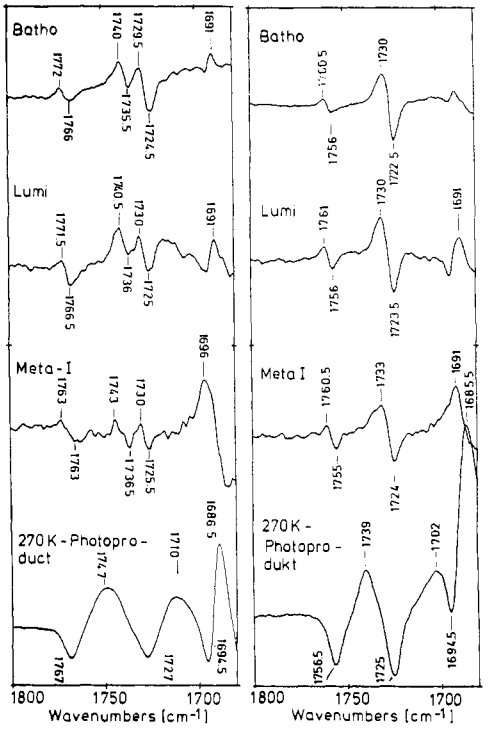


FIGURE 5: Infrared difference spectra in the region of the carbonyl stretching frequency of 9-demethylrhodopsin. Left-hand traces, measurements in $H_2O$; right-hand traces, measurements in $^2H_2O$. From the top: 9-H-bathorhodopsin, 9-H-lumirhodopsin, 9-H-metarhodopsin I, and the photoproduct obtained at 270 K.

tarhodopsin I and rhodopsin–metarhodopsin II transitions.

In the rhodopsin–metarhodopsin I difference spectra of unmodified rhodopsin (Figure 4), a difference band appears at 1770/1764 $cm^{-1}$. On $H/^2H$ exchange, the difference band

Table I: C=N Stretching Frequencies ($cm^{-1}$) and Isotopic Shifts of Rhodopsin, 9-H-Rhodopsin, and the Photoproducts[a]

| | rhodopsin | | 9-H-rhodopsin | |
|---|---|---|---|---|
| | $H_2O$ | $^2H_2O$ | $H_2O$ | $^2H_2O$ |
| rhodopsin | 1659 | 1623 | 1660 | 1627 |
| batho | 1659 | 1623 | 1660 | 1636 |
| lumi | 1635 | 1631 | 1660 | 1638 |
| meta I | 1648 | 1630 | 1660 | 1636 |
| 270 K photoprod | | | 1646 | |

[a] With the exception of metarhodopsin I, the values for rhodopsin are from Ganter et al. (1988).

is shifted down to 1759.5/1753 $cm^{-1}$. Two negative bands are observed at 1737 and 1726 $cm^{-1}$. The former is sensitive to $H/^2H$ exchange and shifts down to 1724.5 $cm^{-1}$. Thereby, the intensity of the latter which does not show an isotopic shift is increased. While in the difference spectra of the earlier photoproducts only small absorption changes are observed around 1700 $cm^{-1}$, in the rhodopsin–metarhodopsin I difference spectrum new large bands appear. A broad, asymmetric band with a peak at 1701 $cm^{-1}$ and a narrower one at 1692.5 $cm^{-1}$ can be attributed to metarhodopsin I. In $^2H_2O$, the intensity of the broad band is drastically reduced, the remaining line being only slightly shifted to 1700 $cm^{-1}$. Concomitant, an intensity increase is observed around 1690 $cm^{-1}$.

In the rhodopsin–metarhodopsin II difference spectra (Figure 4), a rhodopsin line occurs at 1767.5 $cm^{-1}$ and a larger broad metarhodopsin II line at 1745.5 $cm^{-1}$. Both are shifted in $^2H_2O$ to 1757 and 1739.5 $cm^{-1}$, respectively. Further rhodopsin lines are located at 1727 and 1695.5 $cm^{-1}$ in $H_2O$ and at 1725 and 1695 $cm^{-1}$ in $^2H_2O$. An asymmetric metarhodopsin II band arises in $H_2O$ at 1711 $cm^{-1}$ and in $^2H_2O$ at 1703.5 $cm^{-1}$. The band at 1686.5 $cm^{-1}$ is present in metarhodopsin II both for $H_2O$ and for $^2H_2O$.

As for normal rhodopsin, in the difference spectra of 9-H-rhodopsin difference bands are present at 1772/1766.5 $cm^{-1}$ (1760.5/1756 $cm^{-1}$ in $^2H_2O$) both for the rhodopsin–bathorhodopsin and for the rhodopsin–lumirhodopsin transitions (Figure 5). In contrast to the spectra of unmodified rhodopsin, the lines at 1740.5, 1736, 1730, and 1725 $cm^{-1}$ of the rhodopsin–lumirhodopsin difference spectrum are already present in the rhodopsin–bathorhodopsin difference spectrum of 9-H-rhodopsin.

In the spectra of the rhodopsin–metarhodopsin I transition of 9-H-rhodopsin (Figure 5), the difference band caused by the carboxylic group located in a hydrophobic environment does not correspond to a downshift but still exhibits the upshift pattern as observed for the earlier transitions. In addition, the difference bands at 1736/1743 $cm^{-1}$ and 1725.5/1730 $cm^{-1}$ are still observed. Upon deuteration, the first difference band is shifted to 1724/1731 $cm^{-1}$ while the second remains unaffected. A further 9-H-metarhodopsin I line is observed at 1696 $cm^{-1}$ in $H_2O$ and at 1691 $cm^{-1}$ in $^2H_2O$. However, the difference spectra of 9-H-rhodopsin obtained at 270 K (Figure 5) are very similar to those of unmodified rhodopsin in this spectral region.

The C=N stretching vibrations of protonated Schiff bases absorb in the 1700–1600 $cm^{-1}$ region. For rhodopsin and bathorhodopsin, they are located at 1659 $cm^{-1}$ (1623 $cm^{-1}$ in $^2H_2O$) (Palings et al., 1987; Ganter et al., 1988) and for lumirhodopsin at 1635.5 $cm^{-1}$ (1631.5 $cm^{-1}$ in $^2H_2O$) (Ganter et al., 1988). The C=N stretching frequency of metarhodopsin I has been determined to 1648 $cm^{-1}$ by the method previously described (Ganter et al., 1988) (data not shown): by subtracting a difference spectrum of unmodified rhodopsin from a spectrum of rhodopsin regenerated with retinal labeled

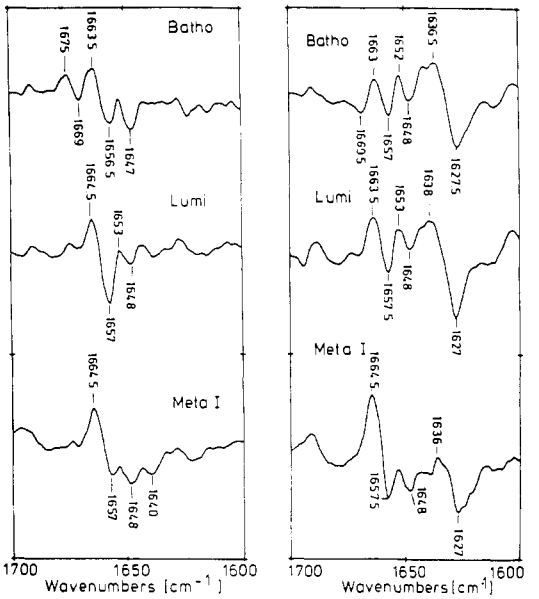


FIGURE 6: Infrared difference spectra in the region of the Schiff base stretching frequency for 9-demethylrhodopsin. Left-hand traces, measurements in $H_2O$; right-hand traces, measurements in $^2H_2O$. From the top: 9-H-bathorhodopsin, 9-H-lumirhodopsin, and 9-H-metarhodopsin I.

with $^{13}C$ at $C_{15}$, the C=N stretching vibrations are revealed. $^{13}C$ labeling shifts this band to 1627 $cm^{-1}$ and deuteration of the Schiff base to 1628 $cm^{-1}$. The frequencies and isotopic shifts upon deuteration are summarized in Table I.

In Figure 6, the difference spectra of 9-H-rhodopsin are shown (left hand, measurements in $H_2O$; right hand, measurements in $^2H_2O$). In the rhodopsin–bathorhodopsin difference spectra, $^2H_2O$ causes a new 9-H-rhodopsin line at 1627.5 $cm^{-1}$ and a new bathorhodopsin line at 1636.5 $cm^{-1}$. In addition, the positive band at 1675 $cm^{-1}$ disappears. In the rhodopsin–lumirhodopsin difference spectra, only two larger lines are observed, but on $H/^2H$ exchange, they remain unaffected. A new 9-H-rhodopsin line appears again at 1627 $cm^{-1}$ and a new 9-H-lumirhodopsin line at 1638 $cm^{-1}$. Similarly, in the rhodopsin–metarhodopsin I difference spectra, $H/^2H$ exchange shifts the 9-H-rhodopsin band to 1627 $cm^{-1}$ and the 9-H-metarhodopsin I band to 1636 $cm^{-1}$.

The presence of strong bands in the spectrum of the 270 K intermediate, which are caused by the protein, shows that major structural changes occur (Figure 3, lower trace). These bands are caused by amide I vibrations of the protein peptide chain and, depending on the structure, can also exhibit an isotope shift upon deuteration. Thus, deuteration of the medium does not allow an unequivocal identification of the C=N stretching vibration (Siebert et al., 1983). Therefore, we applied the method of regenerating rhodopsin with 9-H-retinal labeled with deuterium at $C_{15}$ (Ganter et al., 1988). Subtracting the difference spectra of labeled and unlabeled 9-H-rhodopsin reveals the C=N stretching vibrations. The result is presented in Figure 7. The upper two traces correspond to the two difference spectra; the lower trace shows the subtraction (labeled minus unlabeled). The subtraction constant was chosen to compensate for the strong protein bands above 1670 $cm^{-1}$. The subtraction reveals two small positive bands and one larger negative band. The otherwise flat base line demonstrates that the subtraction is reliable, although the isotopic purity of the retinal was only 75%. The spectral feature is explained by a C=N stretching vibration of 9-H-rhodopsin at 1660 $cm^{-1}$, which is shifted upon labeling to 1646 $cm^{-1}$. At this position, the unshifted band of the photoproduct

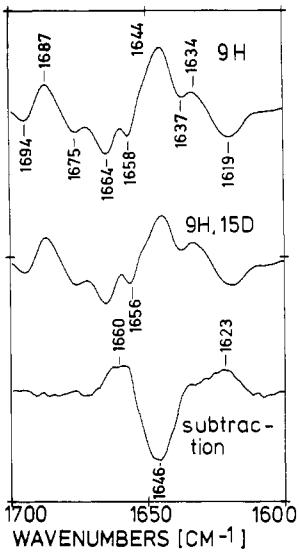


FIGURE 7: Infrared difference spectra of the 270 K photoproduct in the region of the Schiff base stretching frequency. Upper trace, unmodified 9-H-rhodopsin; middle trace, 9-H-rhodopsin deuterated at $C_{15}$ of the 9-H-retinal; lower trace, subtraction of the upper spectrum from the middle spectrum.

Table II: Comparison of G-Protein Activation by Illuminated 9-H-Rhodopsin and Rhodopsin[a]

| irradiation procedure | modified rhodopsin | [$^{35}$S]GTPγS binding ($\times 10^{-3}$ pmol/s) | rel activation |
|---|---|---|---|
| none | native Rh | −0.04 ± 1.4 | |
| | reconstituted | −0.52 ± 0.8 | |
| | 9-H-Rh | −0.05 ± 0.7 | |
| orange light (10 s) | native Rh | 58.54 ± 7.8 | |
| | 9-H-Rh | 4.90 ± 13 | 8 |
| orange light (1 min) | native Rh | 58.72 ± 7.3 | |
| | reconstituted | 43.82 ± 6.0 | 75 |
| | 9-H-Rh | 4.35 ± 1.8 | 7 |
| white light (1 min) | native Rh | 14.47 ± 3.8 | |
| | reconstituted | 11.69 ± 0.9 | 81 |
| | 9-H-Rh | 3.20 ± 0.9 | 22 |

[a] Assays were performed as described under Materials and Methods. Results are mean values of three different experiments assayed in triplicate ± SEM. Relative activation of G-protein is expressed as percentage of the activation by native rhodopsin. Native rhodopsin, purified rhodopsin solubilized with dodecyl maltoside; reconstituted rhodopsin, rhodopsin in dodecyl maltoside treated as 9-H-rhodopsin but regenerated with normal 11-*cis*-retinal.

superimposes, causing the larger negative band. The shifted band of the photoproduct corresponds to the positive band around 1623 cm$^{-1}$.

*G-Protein Activation.* The extent of activation of retinal G-protein by photolyzed rhodopsin or 9-H-rhodopsin was studied by measuring the rate of exchange of GDP for GTPγS. The extent of regeneration of opsin with 9-H-retinal in dodecyl maltoside was similar to that observed for opsin in membranes. Also the bleaching pattern of 9-H-rhodopsin in dodecyl maltoside was essentially identical with that described above for 9-H-rhodopsin in membranes. Most important, no metarhodopsin II like species could be detected (Figure 1B).

The results of the GTP exchange assays are presented in Table II. The experiments had to be performed in detergent rather than in lipid vesicles due to the instability of 9-H-rhodopsin. The duration of time required for the incorporation of 9-H-rhodopsin into vesicles is such that the unstable rhodopsin would have dissociated thermally. Rhodopsin or 9-H-rhodopsin in dodecyl maltoside was irradiated with intense light for the indicated times and then assayed. Activation of G-protein by 9-H-rhodopsin was less than 10% of the activation

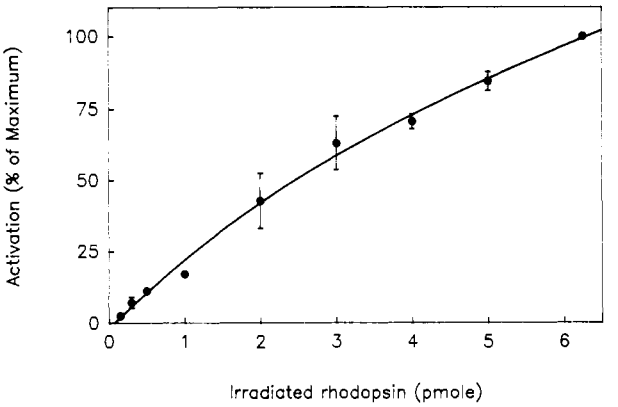


FIGURE 8: Effect of partial irradiation of rhodopsin on G-protein activation. Rhodopsin in dodecyl maltoside was irradiated and mixed with varying percentages of unirradiated rhodopsin, also in dodecyl maltoside. Total rhodopsin content was maintained constant at 25 nM. G-Protein activity was calculated as the rate of exchange of GDP for [$^{35}$S]GTPγS. G-Protein activation relative to the activation obtained with irradiation of 100% of the rhodopsin was calculated as follows: [(G-protein activity with given percent irradiation of rhodopsin) − (G-protein activity with unirradiated rhodopsin)]/[(G-protein activity with 100% irradiated rhodopsin) − (G-protein activity with unirradiated rhodopsin)] × 100. Results are mean values of three different experiments assayed in triplicate ± SEM.

achieved with native rhodopsin. Values obtained when assays were performed under dim red light with unirradiated rhodopsin or 9-H-rhodopsin were negligible. For comparison, also data of "reconstituted" rhodopsin, i.e., opsin regenerated with 11-*cis*-retinal and processed in the same way as 9-H-rhodopsin, are presented. It might be argued that light with wavelengths longer than 540 nm might not be effective for bleaching 9-H-rhodopsin having an absorption maximum at 465 nm. Therefore, the illumination period was extended to 1 min, but no increase in activation was observed. Subsequently, 9-H-rhodopsin was bleached with white light for 1 min; still, no increase in activation could be detected. The decrease in activation, when native rhodopsin or reconstituted rhodopsin was bleached with white light, is due to the formation of rhodopsin and isorhodopsin by back-reaction from metarhodopsin I. This causes the apparent increase in relative activation of 9-H-rhodopsin (relative to the activation of rhodopsin bleached with white light). Virtually the same results were obtained when the GTPase activity of G-protein was measured rather than the exchange activity (data not shown).

It was of interest to determine the relationship between the proportion of rhodopsin that is photochemically activated and the G-protein activation in the same assay system used. In the experiments shown in Figure 8, known amounts of irradiated rhodopsin were mixed with unirradiated rhodopsin and incubated with G-protein and [$^{35}$S]GTPγS under the assay conditions described under Materials and Methods. The total amount of rhodopsin was kept constant. The dose–response curve shows that a relative activation of 8% is equivalent to the photoactivation of appromately 0.4 pmol of native rhodopsin, that is, 6% of the rhodopsin present in the incubation.

## Discussion

*UV–Vis Investigations.* The low-temperature experiments show that for 9-H-bathorhodopsin a red-shift is observed, which is reduced in 9-H-lumirhodopsin and 9-H-metarhodopsin I. The absorption maxima are blue-shifted relative to those of the corresponding intermediates of unmodified rhodopsin. These unusual blue-shifts of the absorption maximum of 9-H-rhodopsin and its photoproducts must be caused

by the missing methyl group. However, these shifts are not observed in the model compounds (comparison of protonated Schiff bases of 9-H-retinal and unmodified retinal). This indicates that the protein causes no major opsin shift for the 9-demethyl species. It is possible that the steric interaction of the methyl group with the protein controls the proper position of the retinal with respect to the negative counterion and to the second point charge (Honig et al., 1979). In addition, the missing methyl group might cause a different conformation of the bound retinal. At 270 K, a photoproduct is obtained, which absorbs at 465 nm, indicating that the Schiff base linkage is still protonated (see below). Thus, this photoproduct cannot be identified as a metarhodopsin II species. In addition, as indicated by the laser flash photolysis experiments, the red-shifted intermediate which is formed in times shorter than 50 $\mu$s does not decay within 2 s.

*Infrared Spectra.* The C=N stretching vibration of unmodified metarhodopsin I is located at 1648 $cm^{-1}$. The coupling with the N–H bending mode, as evidenced by the deuteration-induced downshift to 1629 $cm^{-1}$, is somewhat less than that observed for rhodopsin, isorhodopsin, and bathorhodopsin but much larger than for lumirhodopsin. Therefore, lumirhodopsin exhibits a unique arrangement of the Schiff base within the protein which does not allow for a strong coupling of the N–H bending mode with the C=N stretching mode (Table I).

In the rhodopsin–bathorhodopsin, rhodopsin–lumirhodopsin, and rhodopsin–metarhodopsin I spectra, the C=N stretching vibrations of the 9-demethyl pigment cannot be assigned in $H_2O$ (Figure 6). Probably, they absorb at the same frequency and cancel each other in the difference spectra. That position, however, can be determined to 1660 $cm^{-1}$ from Figure 7, where the spectra of the 270 K photoproduct obtained from unlabeled and labeled (15-deuterio-9-H-retinal) 9-H-rhodopsin are compared. The high position of the band of the photoproduct (1646 $cm^{-1}$) clearly demonstrates that the Schiff base must be protonated, in agreement with the data from UV–vis measurements. The ethylenic stretching vibration further supports this conclusion. From Figure 3, it can be assigned to the band at 1549 $cm^{-1}$, which shifts down a few wavenumbers in the spectrum of the 15-deuterio pigment (data not shown). Thus, the C=C vibration of the 270 K photoproduct is downshifted as compared to the initial state, corresponding to the red-shifted absorption maximum observed in the UV–vis measurements.

The C=N stretches of the deuterated Schiff bases of 9-H-rhodopsin, 9-H-bathorhodopsin, 9-H-lumirhodopsin, and 9-H-metarhodopsin I are located at 1627, 1636.5, 1638, and 1636 $cm^{-1}$, respectively. Since the isotopic shift is larger for 9-H-rhodopsin as compared to the photoproducts, the surrounding of the Schiff base changes somewhat with the first step of the photoreaction but then remains the same in 9-H-lumirhodopsin and 9-H-metarhodopsin I. Despite these small differences, the C=N stretches of 9-H-rhodopsin and of its photoproducts are comparable to each other and to those of unmodified rhodopsin, isorhodopsin, and bathorhodopsin. The same holds true for the isotopic shifts, indicating similar environments. This is in striking contrast to the photoproducts of unmodified rhodopsin. Here, a large change has been observed with the formation of lumirhodopsin, which is only partially restored with the formation of metarhodopsin I (Table I) (Ganter et al., 1988).

In the rhodopsin–bathorhodopsin difference spectrum of unmodified rhodopsin, the difference band at 1767/1773 $cm^{-1}$ (Figure 4) is most likely caused by a protonated aspartic or glutamic acid side chain (Bagley et al., 1985; Siebert et al., 1983). In lumirhodopsin, the position of this band is unchanged (Ganter et al., 1988). Due to the similar position, the difference band at 1770/1764 $cm^{-1}$ in the rhodopsin–metarhodopsin I difference spectrum can probably be assigned to the same protonated carboxyl group. In contrast to the earlier transitions, for which an upshift of a few wavenumbers is observed for the photoproduct, the frequency of the metarhodopsin I line is decreased. In the rhodopsin–metarhodopsin II difference spectra, a rhodopsin line appears at 1767.5 $cm^{-1}$ and a metarhodopsin II line at 1745.5 $cm^{-1}$. This indicates either that the carbonyl band of this group is further downshifted or that this carboxylic acid becomes deprotonated and another one protonated. However, in view of the spectral changes observed for the earlier intermediates, the first explanation seems more likely. Thus, as compared to rhodopsin, this group is less hydrogen-bonded in bathorhodopsin and lumirhodopsin but is increasingly hydrogen-bonded in metarhodopsins I and II.

In the difference spectra for the rhodopsin–lumirhodopsin transition (Ganter et al., 1988), we assigned the lines at 1734.5 and 1739 $cm^{-1}$ to the carbonyl stretching vibration of an internal carboxyl group. In the rhodopsin–bathorhodopsin difference spectra, only weak bands can be detected in this region. As compared to the rhodopsin–lumirhodopsin difference spectrum, in the rhodopsin–metarhodopsin I difference spectrum (Figure 4) the intensity of the rhodopsin line at 1737 $cm^{-1}$ is increased. The negative band at 1736 $cm^{-1}$ and the positive band at 1701 $cm^{-1}$, both of which are shifted by $^2H_2O$, can be explained either by a large change in hydrogen bonding of a single carboxylic acid or by a deprotonation–protonation reaction of two different carboxylic groups (see below). De Grip et al. (1985) did not describe the 1737 $cm^{-1}$ rhodopsin line in their rhodopsin–metarhodopsin I spectra. However, in contrast to our results, a shoulder at about 1737 $cm^{-1}$ seems to be present even in their rhodopsin–metarhodopsin II difference spectra. Because they measured metarhodopsin I at 263 K, a temperature at which metarhodopsin II is already formed, we assume that the band is partially masked by the positive metarhodopsin II band. In addition, a line at 1735 $cm^{-1}$ is present in their metarhodopsin II spectra of rhodopsin reconstituted in ether PC lipids. Because of the strong dependency of the metarhodopsin I/metarhodopsin II equilibrium on the fluidity of the surrounding lipids (Motoyama et al., 1985), we suggest that this line is due to a larger proportion of metarhodopsin I. In our metarhodopsin II spectra (Figure 4), the 1737 $cm^{-1}$ line is not observed. Therefore, the corresponding carboxylic group becomes reprotonated.

The band at 1726 $cm^{-1}$ in the rhodopsin–lumirhodopsin difference spectra was assigned to the amide I vibration of an amino acid bound to the amino-terminal side of a proline with a twisted amide bond (Ganter et al., 1988). Since in lumirhodopsin this vibration is upshifted to 1732 $cm^{-1}$, the bond is more twisted as compared to rhodopsin. The corresponding metarhodopsin I line can tentatively be attributed to the residual band around 1700 $cm^{-1}$ observed after $H/^2H$ exchange. In any case, the absence of a band at higher frequencies indicates that the twist is largely relaxed. The bands at 1695, 1692, and 1685 $cm^{-1}$ are tentatively assigned to amide I vibrations (e.g., $\beta$-turns), which are altered with the formation of the different intermediates.

As indicated by the isotopic shifts, the rhodopsin band at 1737 $cm^{-1}$, the metarhodopsin I band at 1701 $cm^{-1}$, and the metarhodopsin II band at 1711 $cm^{-1}$ are caused by protonated carboxyl groups (Figure 4). Since the bands of the depro-

tonated groups, due to the overlap with other vibrations, cannot be determined, it is not possible to ascertain unequivocally whether protonation changes or only shift of bands occurs. Therefore, to describe the molecular events, some plausible arguments are advanced. If the rhodopsin band at 1737 $cm^{-1}$ were shifted to 1701 $cm^{-1}$ in metarhodopsin I, one would expect to observe this negative band also in the rhodopsin–metarhodopsin II difference spectrum. Since this band is missing, the molecular events in metarhodopsin I are better described by deprotonation (1737 $cm^{-1}$) and concomitant protonation (1701 $cm^{-1}$) of two carboxylic groups, respectively. The broadness and low position of the band of the latter group indicate that it is strongly hydrogen-bonded. In metarhodopsin II, the former group becomes reprotonated, and the latter group is somewhat less hydrogen-bonded (shift to 1711 $cm^{-1}$).

With respect to the molecular changes of the carboxylic groups during the photoreaction, the following model can be outlined. In rhodopsin, two groups are protonated; one of them is located in a hydrophobic part of the protein and is not hydrogen-bonded, whereas the other one is hydrogen-bonded. The third internal group is ionized and serves as the counterion for the protonated Schiff base. [In the model of Ovchinnikov (1982) and of Hargrave et al. (1983), there are only three internal carboxyl groups.] Since, with the formation of metarhodopsin I, one group becomes protonated and another one deprotonated, and since at this stage no proton is taken up from the aqueous phase, these protonation changes are in agreement with a change of the counterion. The previous counterion becomes protonated, and a new counterion is formed. Sandorfy and Vocelle (1986) suggested that the primary counterion is stabilized by a hydrogen bond from a second carboxylic group. Thus, it seems feasible that this group can be identified with the group which causes the band at 1736 $cm^{-1}$ and which becomes deprotonated with the rise of metarhodopsin I. In metarhodopsin II, all three groups are protonated; i.e., the newly formed counterion accepts the proton from the Schiff base. In addition, the environment of the group located in a hydrophobic part of the protein is changed to a hydrogen-bonding surrounding.

The carbonyl bands of 9-H-rhodopsin differ greatly in comparison to those of unmodified rhodopsin (Figure 5). In the rhodopsin–bathorhodopsin difference spectra, nearly the same bands appear as in the rhodopsin–lumirhodopsin spectra. This indicates that the two species are very similar. The lumirhodopsin line, which has been assigned to the amide I absorption of the peptide bond adjacent to a proline, is now observed at 1730 $cm^{-1}$, i.e., at lower frequency as compared to unmodified lumirhodopsin. The carbonyl stretches of 9-H-metarhodopsin I are very similar to those of the lumirhodopsin species (unmodified and 9-H). This is in contrast to what is observed for the corresponding bands of unmodified rhodopsin. Whereas the frequency of the carboxyl group at 1767 $cm^{-1}$ of unmodified rhodopsin is downshifted during the rhodopsin–metarhodopsin I transition, the band of the 9-H pigment is still upshifted, as observed for the earlier intermediates. In addition, the deprotonation of the carboxyl group, observed at 1737 $cm^{-1}$ in unmodified rhodopsin, does not occur. Only a small upshift of the band of the protonated group is visible. The peptide carbonyl at 1725.5 $cm^{-1}$ is only slightly upshifted in the 9-H pigment, but in unmodified rhodopsin, it is downshifted by at least 20 $cm^{-1}$. Finally, the protonation of the group identified with the primary counterion is not observed during this transition.

In the region between 1800 and 1700 $cm^{-1}$, the spectra of the photoproduct at 270 K are very similar to those of normal metarhodopsin II. The UV–vis and infrared data, however, demonstrate that the Schiff base is protonated. Since it was concluded that in metarhodopsin II all internal carboxyl groups are protonated, none of these can represent the negative counterion. To provide a negative charge, it could be that several external carboxyl groups co-act to protonate the Schiff base. Therefore, we tried to deprotonate the Schiff base by increasing the pH to 8. However, in this case, mainly the metarhodopsin I like intermediate is formed (data not shown), which has an internal counterion. Thus, similar to normal rhodopsin, there exists a pH-dependent equilibrium between the metarhodopsin I intermediate and the photoproduct at 270 K. Since the UV–vis spectrum does not change, this equilibrium can only be observed in the infrared. However, no definite counterion can be assigned for the 270 K photoproduct.

The visible spectra and the IR data show that 9-H-bathorhodopsin and 9-H-lumirhodopsin are almost identical. Only in the region of the amide I bands are small differences occurring. This indicates that, whereas the retinal retains the same conformation in the two photoproducts, the protein relaxes slightly in 9-H-lumirhodopsin. The photoproduct of 9-H-rhodopsin obtained at 243 K does not correspond to the metarhodopsin I species of unmodified rhodopsin as evidenced by the different behavior of the carboxylic groups and of the Schiff base, but the position of the ethylenic mode of 9-H-lumirhodopsin is a few wavenumbers higher than that of 9-H-metarhodopsin I. The same behavior is observed for the corresponding bands of the photoproducts of unmodified rhodopsin. Therefore, it appears that in the 9-demethyl species the protein still has a lumirhodopsin-like structure, while the retinal relaxes to the conformation characteristic for metarhodopsin I. The protein seems now to be disconnected from the usual reaction cascade of the retinal chromophore. At 270 K, a photoproduct is obtained which still contains a protonated Schiff base, as evidenced by the red-shifted absorption maximum, by the high C=N stretching frequency, and by the downshifted ethylenic mode. The amide I bands (Figure 3) demonstrate that the protein structure is different from metarhodopsin II, although, as in metarhodopsin II, all the internal carboxyl groups are protonated.

*G-Protein Activation.* The data presented in Table II together with the results from the dose–response curve show that the capability of illuminated 9-H-rhodopsin to activate G-protein corresponds to a 6% bleaching of unmodified rhodopsin. Since the values in the dark are nil, any positive contribution to the activation is readily discernible. Therefore, this small activation must be taken as significant. The spectroscopic data indicate that no metarhodopsin II like photoproduct has been formed. There are two explanations for the residual activation. The simplest is that a small amount of metarhodopsin II actually formed which went undetected spectroscopically. This possibility cannot be excluded since it is very difficult to disprove a 6% presence of normal metarhodopsin II. However, our data clearly demonstrate that, with high yield, a photoproduct is obtained at 270 K which represents a protonated Schiff base, but which has also some resemblance to metarhodopsin II. Then, in contrast to normal rhodopsin, two photoproducts would be produced, one with low yield, activating the G-protein, and the other high-yield photoproduct being inactive. Since no transient absorbance decrease on the 100-$\mu$s time scale could be detected, and since the activation energy of the G-protein is very high (Kohl & Hofmann, 1987), the activation by a very short-lived (<100 $\mu$s) metarhodopsin II, produced with high yield, is also unlikely. We view the second possibility as more likely: due to the limited resem-

blance to metarhodopsin II, this photoproduct activates the G-protein at a much reduced rate. It might be that the G-protein binds very weakly to this activated state or that the binding is comparable to that of activated rhodopsin, but the capability of inducing the conformational changes in the G-protein is greatly reduced. Finally, by binding of the G-protein to the 270 K photoproduct, the Schiff base could possibly become deprotonated, which again, since only 6% are involved, would be difficult to detect spectroscopically.

The 9-methyl group of the retinal is obviously of the utmost importance for the function of the visual process. By steric interaction, it seems to connect the isomerization of the retinal to the events occurring later in the protein. In this way, the information of light absorption is transmitted. The crucial step is the lumirhodopsin–metarhodopsin I transition. In this transition, besides the changes of the protonation state of two carboxylic groups, a large shift of the amide I band of the peptide bond adjacent to the amino-terminal side of a proline is observed. Thus, it seems possible that a cis–trans isomerization of this bond [as suggested by Brandl and Deber (1986)] initiates the molecular changes of the protein which trigger the enzyme cascade. In 9-H-rhodopsin, since the steric interaction is missing, this connection seems to be disrupted. As early as in 9-H-lumirhodopsin, a small deviation of the position of this amide I band is observed. This may indicate that the isomerization of the proline peptide bond does not occur. This speculation is supported by the observation that the large changes during the transition of unmodified rhodopsin to metarhodopsin I do not occur in the 9-H pigment.

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**Registry No.** 9-H-retinal, 24336-19-4; 15-deuterio-9-H-retinal, 83680-08-4; 11-*cis*-9-H-retinal, 83680-08-4; 11-*cis*-retinal, 564-87-4.

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