Factors Affecting the Formation of an M-like Intermediate in the Photocycle of 13-*cis*-Bacteriorhodopsin†

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ABSTRACT: The light-induced proton pumping activity of bacteriorhodopsin (bR) is based on the photocycle of its light-adapted *all-trans*-retinal protein pigment. The photocycle of the 13-*cis* pigment lacks the M intermediate (which carries a deprotonated retinal Schiff base, characteristic of the *all-trans* photocycle) and is not associated with proton release and uptake. Aiming at establishing the reasons for the lack of light-induced Schiff base deprotonation and proton pumping in 13-*cis*-bR, we carried out pulsed-laser and continuous excitation experiments with artificial 13-*cis*-bR pigments derived from 13-demethylretinal, 13-demethyl-14-fluoro-bR, and 13-demethyl-12,14-difluoro-bR. Pulsed-laser photolysis shows that both M formation and proton pumping are restored in 13-*cis*-13-demethyl-bR by raising the pH to 8.5–9. M formation, but not proton pumping, is restored at neutral pH by 14-fluorine substitution. Continuous-illumination experiments lead, in all cases, to the generation of extremely long-lived (minutes to hours) M photoproducts. We show that such species are due to secondary photoreactions of late intermediates of the primary photolysis. Feasible mechanisms accounting for Schiff base deprotonation in the *all-trans* photocycle, but not in that of 13-*cis*-bR, are considered. Our findings favor a mechanism which attibutes the lack of light-induced Schiff base deprotonation of 13-*cis*-bR to an insufficient change in the relative pK_a of the donor (Schiff base) and acceptor (probably Asp-85) groups and/or to a high activation barrier for the proton transfer. The required change in relative pK_as may be achieved either by deprotonation of a protein moiety (YH) with $pK_a \approx 8.5$ or by fluorine substitution at position 14. Similarly, both YH titration and 14-fluorine substitution may reduce the barrier for proton transfer by affecting H-bonding interactions in the vicinity of the Schiff base linkage. The lack of proton release and uptake in the photocycle of 13-*cis*-13-demethyl-14-fluoro-bR, despite the presence of an M intermediate, is discussed. It appears that Schiff base deprotonation does not essentially imply proton release and uptake. Our conclusions bear on the molecular mechanism of the photocycle and of proton pumping in *all-trans*-bR.

Bacteriorhodopsin (bR)[1] is a 26 000 molecular weight pigment in the purple membrane of *Halobacterium salinarium* [Oesterhelt & Stoeckenius 1974; see Birge (1990), Mathies et al. (1991), Oesterhelt (1992), Ebrey (1993), and Lanyi (1993) for recent reviews on the structure and function of bR]. bR appears in two forms, light-adapted (LA) and dark-adapted (DA), absorbing at 568 nm (bR568) and 558 nm (bR558), respectively. The light-adapted form consists of an *all-trans*-retinal chromophore bound to the lysine 216 residue via a protonated Schiff base (PSB), whereas the dark-adapted form, obtained by thermal equilibration of bR(LA) in the dark, consists of (according to the most recent estimate) a 2:1 mixture of the 13-*cis* and *all-trans* isomers (Scherrer et al., 1989). Following light absorption by the retinal chromophore, bR568 experiences a series of structural changes in both chromophore and protein. These changes give rise to a photocycle characterized by a sequence of spectroscopically distinguishable (ground-state) intermediates designated J, K, L, M, N, and O. The L to M transition is associated with deprotonation of the Schiff base and protonation of D85 located on the extracellular side of the Schiff base (Braiman et al., 1988; Butt et al., 1989; Stern et al., 1989; Metz et al., 1992). Approximately on the same time scale, a proton is released to the outside by a still-unidentified protein moiety denoted as HX (Zimanyi et al., 1992; Ebrey, 1993). Subsequently, a proton is taken up from the cytoplasmic side, regenerating the bR568 pigment. Accumulated evidence indicates, though not strictly proves, that the formation of the M intermediate is a prerequisite for initiating the proton pumping activity of bR. For example, Longstaff and Rando (1987) have shown that methylation of Lys-216 eliminates both light-induced M formation and proton pumping activity.

The 13-*cis* form of bR (bR548) has its own photocycle, characterized by primary events similar to those of the *all-trans* chromophore. However, at neutral pH, it lacks the M intermediate (Kalisky et al., 1977; Hofrichter et al., 1989) and does not induce a proton pump. On the basis of their sequential appearance, the three detected intermediates of the 13-*cis* photocycle are denoted as J^c (picoseconds), K^c (microseconds), and L^c_{610} (milliseconds). (Note that both J^c and K^c are kinetically and spectroscopically similar to J and K in the *all-trans* photocycle, while L^c_{610} is red-shifted and longer-lived compared to L.) It was recently shown that at high pH (>8.5–9) the 13-*cis* photocycle does form the M intermediate and also exhibits a proton pumping activity

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[1] Abbreviations: bR, bacteriorhodopsin; DA, dark-adapted; DDW, double-distilled water; DIBAH, diisobutylaluminum hydride; LA, light-adapted; PSB, protonated Schiff base.

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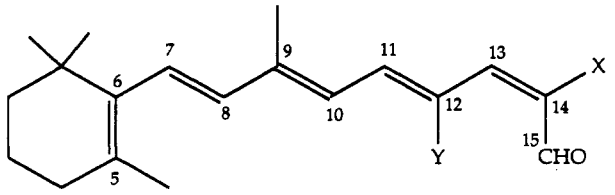
Chart 1



1 X = F Y = H

2 X = F Y = F

3 X = H Y = H

(Drachev et al., 1988; Kaulen et al., 1990; Drachev et al., 1993). The effect was attributed to the titration of a protein moiety YH, tentatively identified as a tyrosine or a carboxyl residue. Accordingly, YH was suggested to serve as an acceptor to the Schiff base proton when present in its deprotonated Y^- form (pH > 8.5–9) but to block Schiff base deprotonation when protonated.

Since deprotonation of the Schiff base appears to be the key step in the proton pump mechanism of *all-trans*-bR, the reasons for the occurrence or absence of an M intermediate in the photocycle of 13-*cis*-bR are of primary importance for understanding the molecular events which govern proton translocation. In this work, we approach the problem by studying artificial (13-*cis*) bR pigments in which intrinsic changes in the pK_a of the Schiff base are induced by using synthetic chromophores with fluorine substituents (Sheves et al., 1986; Steinberg et al., 1993). The results bear on the reasons for the "deficiency" of the photocycle of 13-*cis*-bR manifested by the lack of PSB deprotonation at neutral pH. We propose that the "deficiency" is due to an insufficient change in the relative pK_a of the donor (PSB) and acceptor (possibly Asp-85) and/or to a high barrier for proton transfer between the two groups rather than to the absence of a second acceptor moiety in an appropriate (Y^-) form. The M-generation pattern of the *all-trans* photocycle can be restored in the photocycle of 13-*cis*-bR either by titration of a protein (YH) residue or by fluorination of C14. The results are obviously relevant to the mechanisms of M generation and proton pumping in the photocycle of *all-trans*-bR.

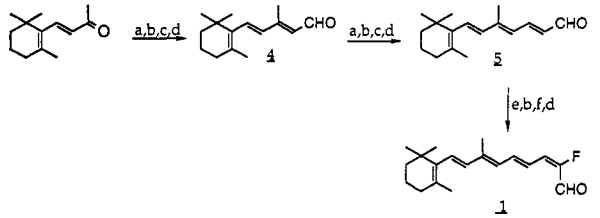
MATERIALS AND METHODS

Materials. Purple membranes from *Halobacterium salinarium* were isolated as described previously (Oesterhelt & Stoeckenius, 1974). Dark adaptation of the pigments was carried out by incubation in the dark at 25 °C for 3 h.

Light adaptation and continuous-illumination experiments were carried out by pigment irradiation with a 520-nm cutoff filter for 1 min at 25 °C. pH was adjusted using appropriate buffers (50 mM salt concentration).

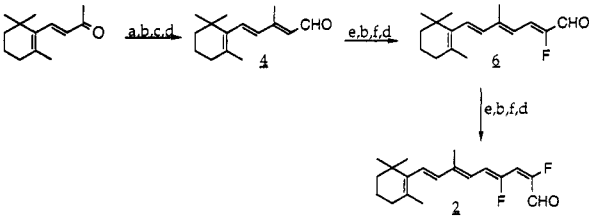
Synthesis of Chromophores: (*A*) *13-cis-13-Demethyl-14-fluororetinal* **1**. Chromophore **1** (see Chart 1) was synthesized according to Scheme 1. β-Ionone was condensed with the sodium salt of $(EtO)_2POCH_2CN$, followed by reduction with DIBAH in hexane (–78 °C, 1 h) and hydrolysis with silica in the presence of ether and a few drops of water to give, after isomer separation by flash chromatography (18% ether in hexane), aldehyde **4**. A similar sequence of reactions yielded aldehyde **5**, which was further condensed with the lithium salt of $(EtO)_2POCHFCO_2Et$ (0 °C, 1 h), followed by reduction with DIBAH (hexane, –78 °C, 1 h) and oxidation with active MnO_2 at 25 °C for 12 h to give, after isomer separation by flash chromatography (15% ether in hexane), aldehyde **1**.

Scheme 1[a]



[a] (a) $(MeO)_2POCH_2CN$/NaH, THF, 0 °C. (b) DIBAH, hexane, –78 °C. (c) Silica/ether, water. (d) Separation of isomers. (e) $(EtO)_2POCHFCO_2Et$/LDA, THF. (f) MnO_2/methylene chloride.

Scheme 2[a]



[a] (a) $(MeO)_2POCH_2CN$/NaH, THF, 0 °C. (b) DIBAH, hexane, –78 °C. (c) Silica/ether, water. (d) Separation of isomers. (e) $(EtO)_2POCHFCO_2Et$/LDA, THF. (f) MnO_2/methylene chloride.

^1H NMR ($CDCl_3$) (13-*cis*) δ 1.03 (s, 6), 1.72 (s, 3, 5-CH_3), 2.00 (s, 3, 9-CH_3), 6.15 (d, 8-H, J = 16.3 Hz), 6.20 (d, 10-H, J = 11.5 Hz), 6.37 (d, 7-H, J = 16.3 Hz), 6.75 (d of d, 13-H, J = 11.5 and 18 Hz), 6.96 (dd, 11-H, J = 11.5 and 16 Hz), 6.97 (dd, 12-H, J = 11.5 and 16 Hz), 9.82 (d, 15-H, J = 14 Hz).

^1H NMR ($CDCl_3$) (*all-trans*) δ 1.04 (s, 1-CH_3, 6), 1.73 (s, 5-CH_3) 2.09 (s, 9-CH_3), 6.21 (d, 8-H, J = 16.4 Hz), 6.29 (d, 10-H, J = 12 Hz), 6.50 (d, 7-H, J = 16.5 Hz), 6.56 (dd, 13-H, J = 11.8 and 29.4 Hz), 7.07 (dd, 12-H, J = 11.7 and 14.9 Hz), 7.54 (dd, 11-H, J = 11.9 and 14.9 Hz), 9.21 (d, 15-H, J = 18.5 Hz).

(*B*) *13-cis-13-Demethyl-12,14-difluororetinal* **2**. The synthesis was carried out as described in Scheme 2. Aldehyde **4** was condensed with the lithium salt of $(EtO)_2POCHFCO_2Et$ (0 °C, 1 h), followed by reduction with DIBAH (hexane, –78 °C, 1 h) and oxidation with active MnO_2 at 25 °C for 12 h to give, after isomer separation by flash chromatography (15% ether in hexane), aldehyde **6**. The latter was converted to aldehyde **2** using a similar sequence of reactions.

^1H NMR ($CDCl_3$) (13-*cis*) δ 1.04 (s, 6), 1.73 (s, 3, 5-H_3), 1.99 (s, 3, 9-H_3), 6.22 (d, 8-H, J = 16 Hz), 6.26 (dd, 11-H, J = 12 and 30.5 Hz), 6.40 (d, 7-H, J = 16 Hz), 6.41 (d, 10-H, J = 12 Hz), 6.57 (dd, 13-H, J = 18 and 34 Hz), 9.96 (d, 15-H, J = 20.5 Hz).

^1H NMR ($CDCl_3$) (*all-trans*) δ 1.04 (s, 6), 1.73 (s, 3, 5-CH_3), 1.99 (s, 3, 5-CH_3), 6.19 (dd, 13-H, J = 23.5 and 30.5 Hz), 6.23 (d, 8-H, J = 16 Hz), 6.40 (d, 10-H, J = 12 Hz), 6.41 (d, 7-H, J = 16 Hz), 6.47 (dd, 11-H, J = 12 and 32 Hz), 9.23 (d, 15-H, J = 16 Hz).

The isomer structure was determined using the nuclear Overhauser effect (NOE). Irradiation of 15-H led to an increase of 10% in the 13-H signal in the *trans* isomer, but had no effect in the *cis*. Irradiation of 11-H led to an increase of the 13-H signal by 17% in both isomers.

Preparation of Artificial Pigments. Artificial pigments were prepared by reconstituting the apomembrane (suspension in water) with the 13-*cis* isomers of the retinal analogs. The preparation of apomembrane and reconstitution procedures have been described previously (Tokunaga & Ebrey, 1978). The pigments derived from chromophores **1** and **2** were

irradiated (following incubation) with a $\lambda > 390$ nm cutoff filter to obtain further formation of pigment.

Isomer Analysis. The isomer composition of the pigment was determined by HPLC analysis. The chromophore was extracted from the pigment suspension using a 1:1 ethanol–hexane mixture according to Scherrer et al. (1989). The hexane layer was separated and immediately analyzed by HPLC using a silica 60 column and eluted with 5% ethyl acetate in hexane.

Pulsed Laser Photolysis. Pulsed laser photolysis studies of membrane suspensions were carried out using an Nd–YAG (frequency doubled with 15 mJ/cm^2 intensity of pulse) or a N_2 dye laser system with 0.1 mJ/mm^2 intensity. The fraction of the cycling pigment was ca. 20% and 5%, respectively. Laser-induced transient absorbance changes were recorded using a pulsed or continuous Xe lamp, a photomultiplier, and a Tektronix 2440 digitizer. Data were averaged and analyzed using a PC. Low monitoring light intensities were obtained by placing an interference filter between the Xe lamp and the sample. Continuous-illumination experiments were performed using an Xe source and a Hewlett-Packard Co. (Palo Alto, CA) 8450A diode array spectrophotometer. Contributions of bR_{570} contamination in the artificial pigments were taken into account by carrying out blank photochemical experiments with an apomembrane preparation under identical conditions. For proton release and uptake measurements, the pH indicator pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid and trisodium salt; purchased from Eastman-Kodak) was used in the suspensions exposed to pulsed laser experiments without further purification.

Low-Temperature Studies. Aqueous membrane suspensions were mixed with glycerol, yielding a final 66% concentration of glycerol. The sample was cooled in a glass cryostat with quartz windows. Irradiation was carried out with a 100-W bulb with a 520-nm cutoff filter. Difference spectra were taken before and after irradiation. The absorption spectra were measured using the HP spectrophotometer.

RESULTS

(A) Artificial 13-cis-bR Pigments Derived from Fluorinated Chromophores. One possible explanation to account for the lack of the M intermediate following light absorption by the 13-*cis* pigment is associated with insufficient alteration of the relative pK_a of the (Schiff base) donor and of the acceptor groups to cause proton transfer (see Discussion below). To explore this possibility, we looked for artificial pigments that would predominantly adopt the 13-*cis* configuration, in both light- and dark-adapted forms, while being characterized by intrinsically reduced protonated Schiff base pK_a values.

It has been demonstrated that the artificial pigment derived from 13-demethylretinal consists of 85% 13-*cis* isomer in both light- and dark-adapted forms (Gartner et al., 1983). Recently, we have shown that substitution of the retinal chromophore with a fluorine group at its 14 position reduces the pK_a value of the protonated Schiff base in solution by ca. 2.7 units (Steinberg et al., 1993). Thus, with the purpose of combining both effects (i.e., isomer composition and pK_a change), we synthesized 13-cis-13-demethyl-14-fluororetinal (chromophore **1**) and 13-cis-13-demethyl-12,14-difluororetinal (chromophore **2**). Incubation of chromophores **1** and **2** [λ_{max} = 384 and 392 nm (in EtOH), respectively] with the apomembrane at pH = 6.8 for 2 h formed artificial pigments I and II, absorbing at 594 and 608 nm, respectively (Figure 1), at ca. 30% yield relative to the amount of native bR obtained following incubation with the *all-trans*-retinal. Further pigment formation could be induced (increasing the pigment yield to ca. 50%) by irradiating the incubated suspension with a $\lambda > 390$ nm cutoff filter. This effect is probably due to irradiation of a species absorbing at ca. 480 nm, which was obtained along with pigments I and II following incubation of chromophores **1** and **2** with the apomembrane.

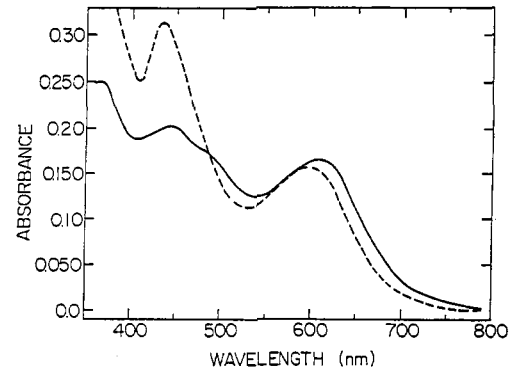


FIGURE 1: Absorption spectra of pigments I (---) and II (—). The absorption at ca. 430 nm corresponds to chromophore excess.

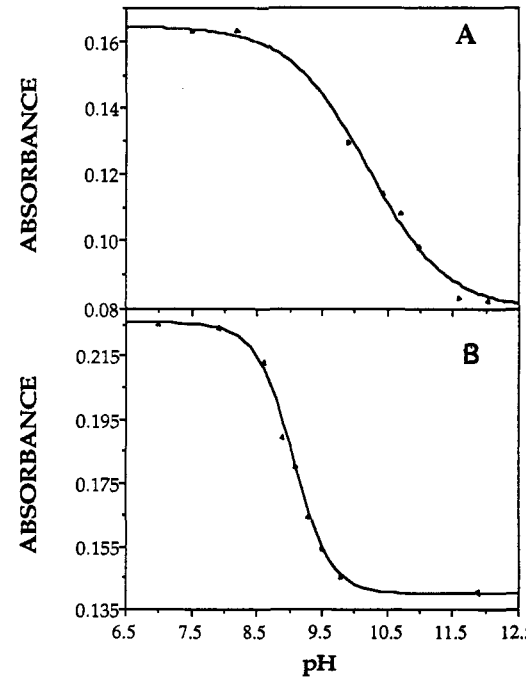


FIGURE 2: Titration curves of pigments I (A) and II (B), showing the absorption of the protonated forms at 594 nm for I (A) and 608 nm for II (B).

The pK_a values of the protonated Schiff base derived from **1** and **2** (in a 1:1 MeOH–water solution) were found to decrease to 4.7 and 4.0, respectively, relative to the 7.2 value of the retinal protonated Schiff base. Analogous pK_a reductions were observed with the corresponding artificial pigments I and II, whose protonated Schiff bases were titrated with pK_as of 10.2 ± 0.1 and 9 ± 0.1 (50 mM salt), respectively (Figure 2). This is consistent with the general observation of additivity of the pK_a changes induced intrinsically in the retinal by electron-withdrawing substituents to those changes induced by protein–retinal interactions in the binding site (Sheves et al., 1986). Chromophore extractions and isomer analysis by HPLC indicated that the pigments had a ca. 80:20 ratio of 13-*cis* and *all-trans* isomers, respectively, in both light- and dark-adapted forms (Figure 3). In the case of the 14-fluoro-13-demethyl pigment, a small amount (<10%) of three other unidentified isomers was detected. The isomer composition of pigments I and II is analogous to that previously found for the 13-demethyl-bR (Gartner et al., 1983), indicating that

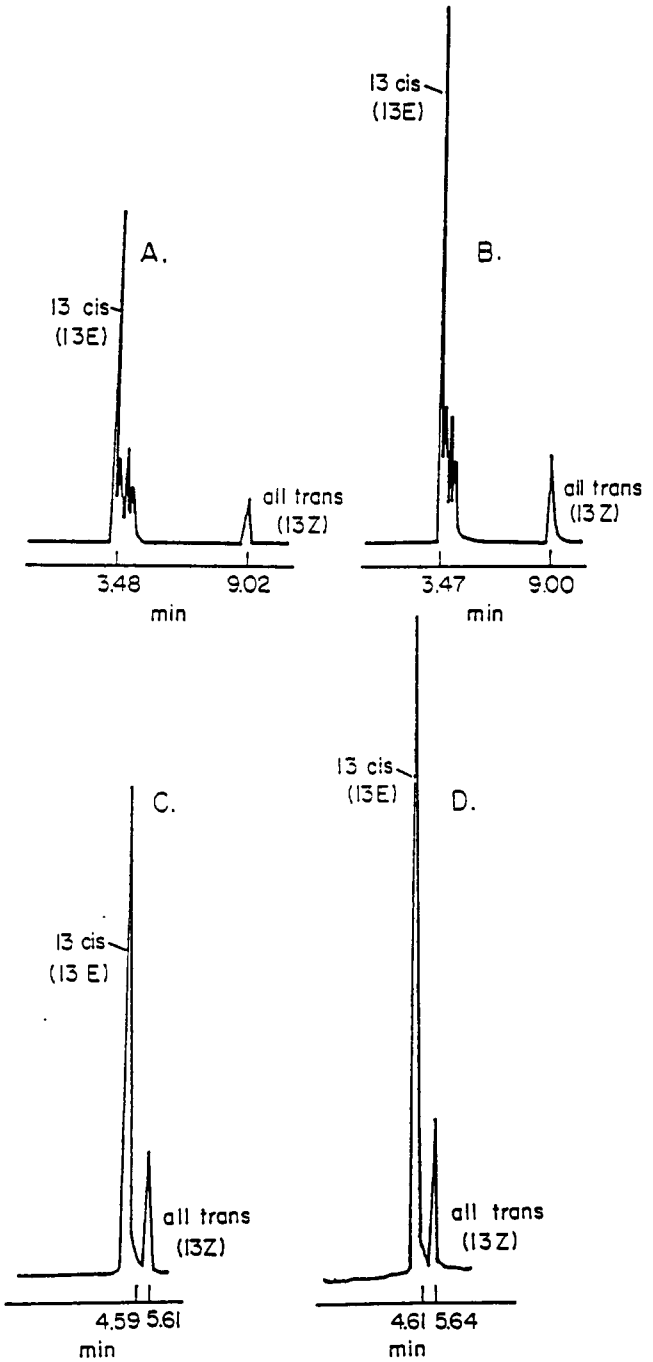


FIGURE 3: Characteristic HPLC chromatographs associated with pigments I (A, B) and II (C, D). (A, C) Chromatographs of extracts from dark-adapted pigments. (B, D) Extracts from pigments that were illuminated for 2 min, followed by relaxation for 5 min in the dark.

14- and 12,14-fluorine substitution alters the PSB pK_a but does not substantially affect the isomer composition of the pigment.

(B) Pulsed Photolysis of Pigment I and of 13-Demethyl-bR. Figure 4 shows characteristic traces following 532-nm pulsed laser excitation of pigment I at pH = 7. The primary difference spectrum (Figure 5), obtained within the ~50-ns time resolution of the system, shows slight bleaching in the region of the main absorption band of the pigment and an increased absorption above 575 nm due to the formation of a red-shifted intermediate. As indicated by the decay at 630 nm and the corresponding growing-in at 410 nm (Figure 4A,B), the red-shifted species is converted into a blue-shifted intermediate, M^c, which is spectroscopically analogous to the M intermediate of the *all-trans*-bR photocycle. The relative

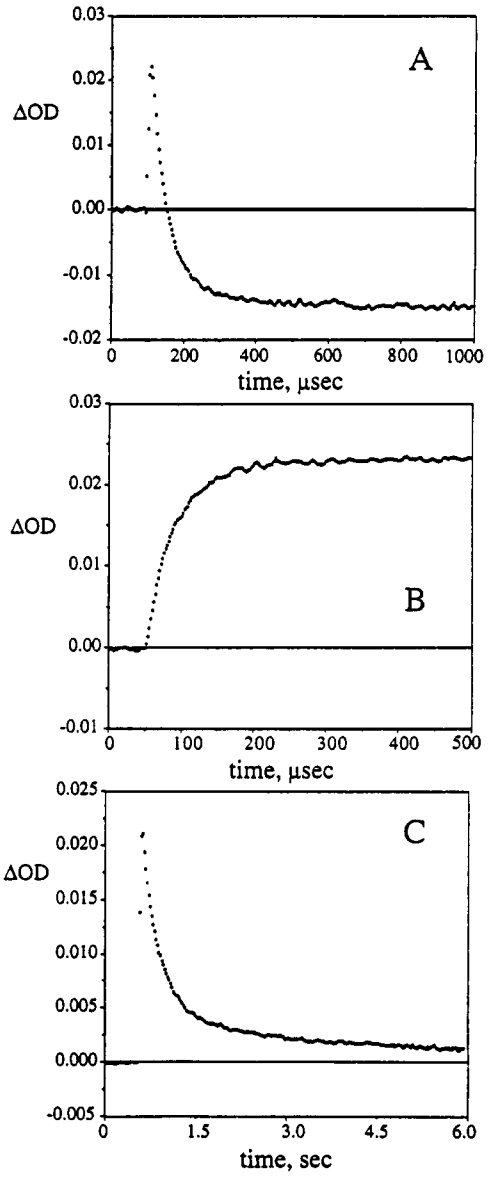


FIGURE 4: Characteristic traces showing transient absorbance changes (ΔOD) in the (532 nm, Nd–YAG) photolysis of 13-demethyl-14-fluoro-bR recorded (A) at 630 nm and (B and C) at 420 nm (average of 32 accumulated pulses). To assure completion of the photocycle after each pulse, a slow repetition rate of one pulse every 13 s was employed.

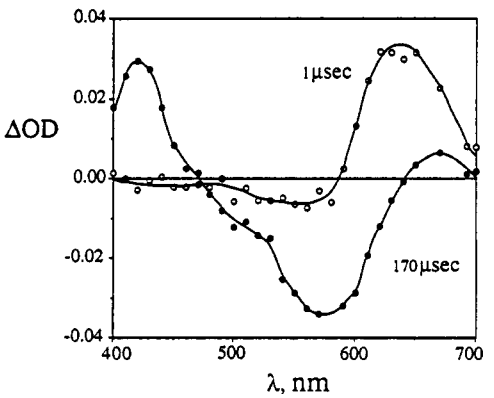


FIGURE 5: Transient absorbance changes recorded 1 and 170 μs after pulsed laser excitation (579-nm dye laser line) of 13-demethyl-14-fluoro-bR. Sixty-four accumulations with a 10-s delay between consecutive pulses were gathered.

yield of M^c was found to be comparable to that of M in the photocycle of bR [characterized by a quantum yield = 0.7 (Govindjee et al., 1990; Tittor & Oesterhelt, 1990)] excluding

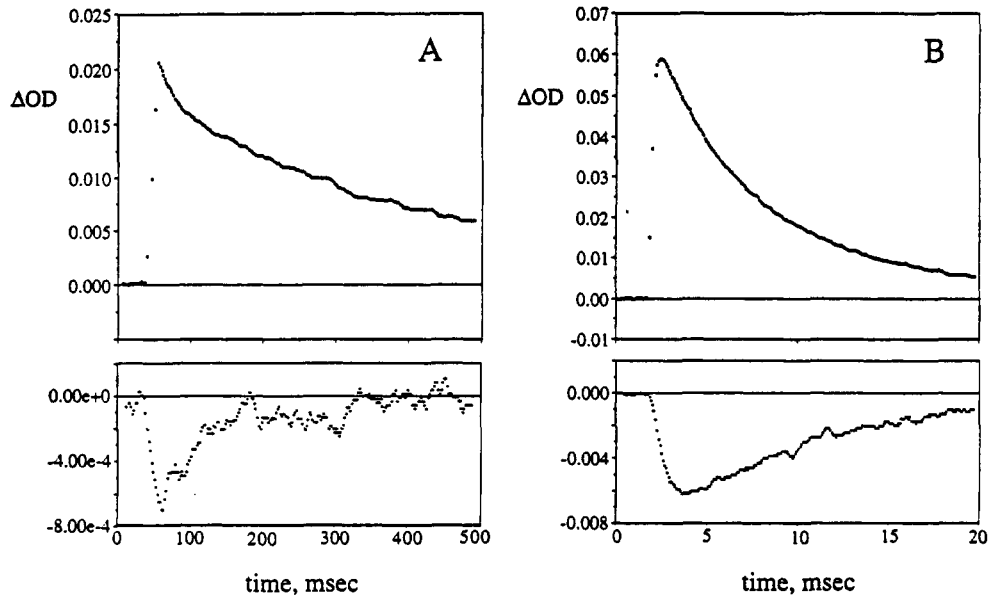


FIGURE 6: Transient absorbance changes induced (0.1 M NaCl, pH = 7, unbuffered) in 13-demethyl-14-fluoro-bR (A) and in light-adapted bR reference (B). Top: Absorbance changes at 420 nm. Bottom: Net pyranine absorbance change at 456 nm obtained in the presence of 10 μM pyranine after subtracting the effect induced in analogous solution in the absence of pyranine.

the possibility that the observed M intermediate of pigment I is due to the ~20% contribution of the *trans* isomer. (If M had originated from *all-trans*, it would have implied a quantum yield of >1.) The rise in absorbance at 420 nm is followed by a slow decay (Figure 4C), which is clearly nonexponential. Two distinct decay stages are observed with approximate lifetimes of τ_1 = 100 ms (~80%) and τ_2 = 5 s (~20%). Experiments similar to the above were carried out at several pH values in the 3.5 < pH < 8.1 range. The basic patterns of the photocycle are maintained over the above pH range, though a drop of approximately a factor of 2 was observed, for both yield and lifetime of the slow component, upon decreasing the pH from ~7 to ~3.5.

In the attempt to look at the proton pump activity associated with the photocycle of pigment I, we performed experiments in the presence of the pH dye indicator pyranine (Grzesiek & Dencher 1986). Detection wavelengths were at 420 nm (M absorption maximum) and 456 nm, where the dye exhibits a pH-dependent absorbance change. The absorbance at 456 nm, attributable to protonation of the dye following proton release during the photocycle of pigment I, was compared to the analogous change in native bR suspensions at the same pH, salt concentration, and pyranine concentration. Characteristic results are shown in Figure 6. The relatively low signal/noise ratio in the dye signals (corrected for the reference signals in the absence of the dye or in the presence of a buffer which eliminates the light-induced pH change) are due to the limits imposed on the pyranine concentration by the total absorbance and scattering of the solution at 456 nm. It may be concluded that, within the limits of our experimental accuracy, a small but detectable proton translocation (Figure 6A, bottom) is present in the suspensions of pigment I. Scaled to the absorbance of M at 420 nm, the transient change in pH after 100–150 ms amounts to 20% ± 10% of that measured (Figure 6B) in the case of light-adapted bR. We note that a fast initial decay component in the upper trace of Figure 6A is attributed to contamination of the sample by reconstituted *all-trans*-retinal. Thus, the initial pyranine signal in the lower trace includes some contribution of H^+ release and uptake by *all-trans*-bR. However, the latter effect is over after ~150 ms so that the pyranine effect after this time should be attributed exclusively to pigment I, probably to its 20% *trans* fraction.

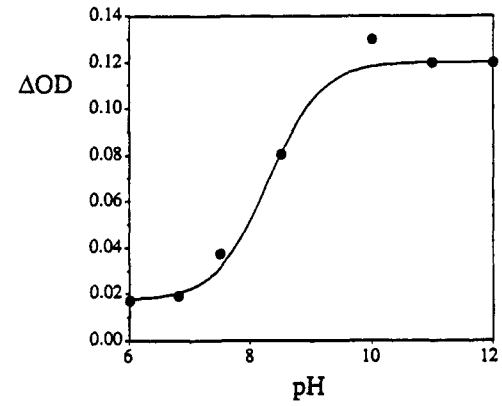


FIGURE 7: Effect of pH on the initial absorbance change at 420 nm (data points), due to an M-like intermediate induced by 532-nm Nd–YAG, laser excitation of 13-demethyl-bR. Thirty-two accumulations with 13 s between pulses were gathered. The continuous curve is a calculated titration curve with pK_a = 8.3.

It has been shown that the transient flash excitation phenomena characteristic of pigment III (13-demethyl-bR) at neutral pH show relatively small changes in absorbance at 420 nm due to M formation (Gartner et al., 1988). However, we find that the maximum (initial) absorbance change at 420 nm increases with increasing pH, leveling off at pH > 10 (Figure 7). As shown in Figure 8, the high-pH patterns show a (~10-μs) growing-in of an M-like intermediate from a red-absorbing species. A subsequent M decay component ($\tau_1 \approx 20$ ms, not shown) is accompanied by an absorbance increase at 630 nm. These processes are followed by a slower (τ_2 = 600 ms) regeneration of the original absorption.

Proton translocation experiments with 13-demethyl-bR are shown in Figure 9. At pH 7.0, a dye absorbance change is observed which (relative to the M absorbance at 420 nm) is comparable to that of native bR. The light-induced changes in the absorbance of the pyranine dye at pH 8.3, which is far from its 7.2 pK_a value, are relatively small [Figure 9C,D and Zimanyi et al. (1993)], imposing a low signal/noise ratio in these experiments. It appears, however, that proton translocation does take place with 13-demethyl-bR at pH 8.3, with an efficiency comparable to that at pH 7 (scaled to the amount of M absorbance at 420 nm).

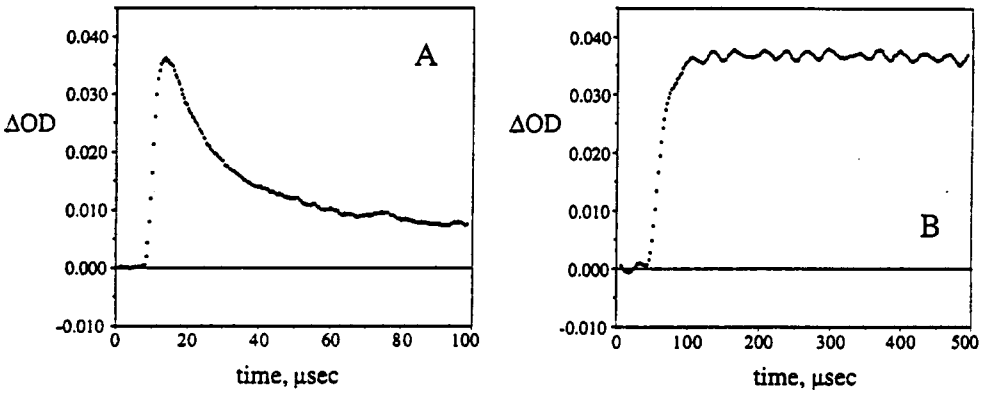


FIGURE 8: Decay of a red-absorbing species at 630 nm (A) and growing-in of an M-like intermediate at 420 nm (B) in the pulsed photolysis of 13-dm-bR at pH = 10. Dye laser excitation was at 536 nm; 32 accumulations with 13-s intervals were gathered.

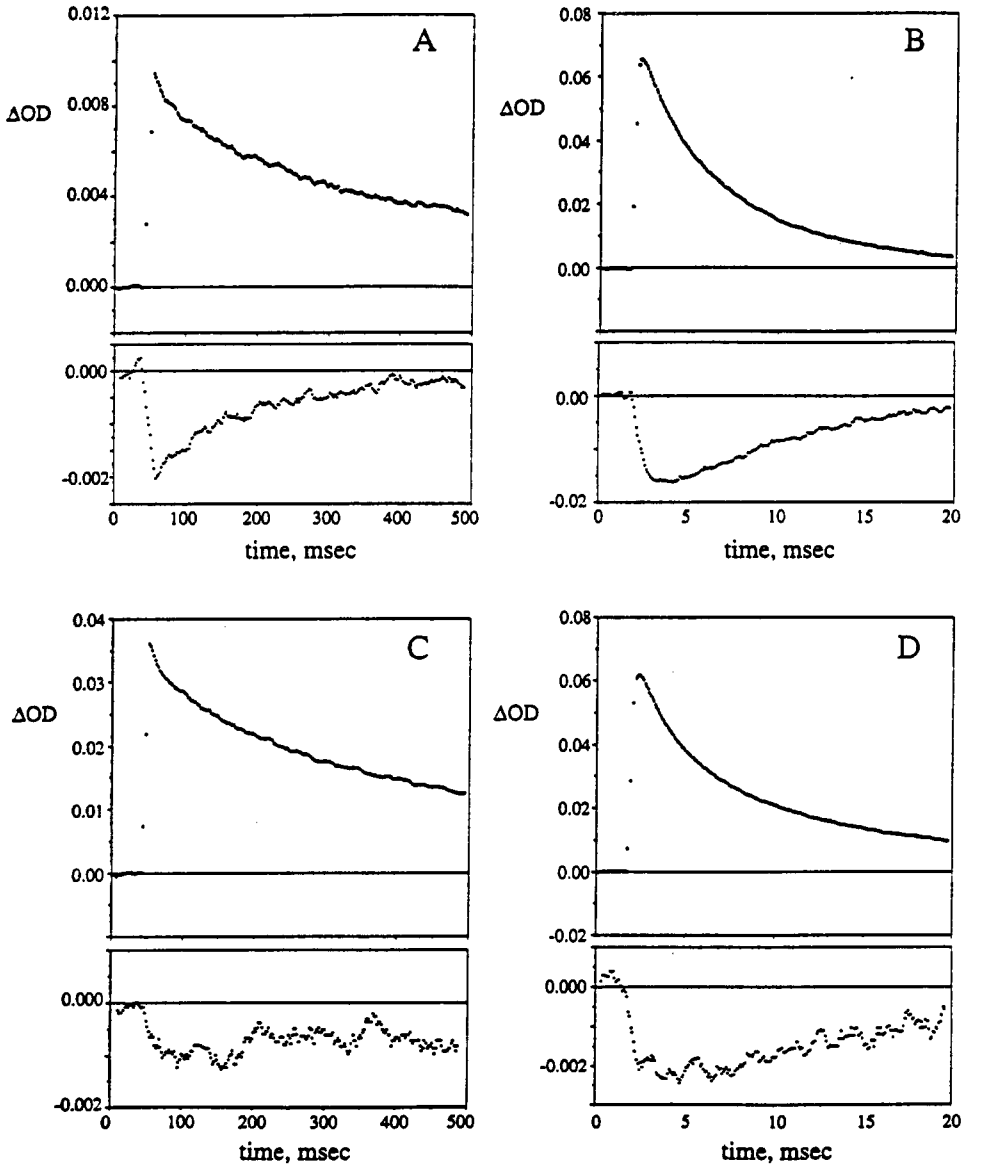


FIGURE 9: Proton pumping activity of 13-demethyl-bR and (reference light-adapted bR. Left: 13-demethyl-bR at pH 7 (A) and pH 8.3 (C). Right: Light-adapted bR at pH 7 (B) and pH 8.3 (D). Top traces are absorbance changes at 420 nm (M decay). Lower traces are net pyranine effects obtained as described in Figure 6. Pyranine concentration was 50 μM in the presence of 0.1 NaCl. pH values were adjusted using NaOH. Sixty-four (A, C, D) or 32 (B) accumulations with 1-s intervals were gathered.

(*C*) *Continuous-Illumination Experiments at 25 °C.* Continuous irradiation of the 14-fluoro-13-demethyl pigment I (pH 7, 25 °C) with a 550-nm cutoff filter indicated that more than 90% of the pigment (absorbing at 598 nm) is converted to an M-like species absorbing at 420 nm (Figure 10). Its decay in the dark, to a species characterized by a 598-nm absorption, is biphasic, with the largest fraction decaying with τ = 5 s and the rest with τ = 3 min (Figure 10C). The slight decrease in the amount of pigment at a later stage is probably due to partial pigment decomposition. Very similar phenomena are observed following irradiation of pigment II derived from 12,14-difluoro-13-demethylretinal (Figure 11). We found that the relative amount of the long-lived M component increases upon increasing the illumination

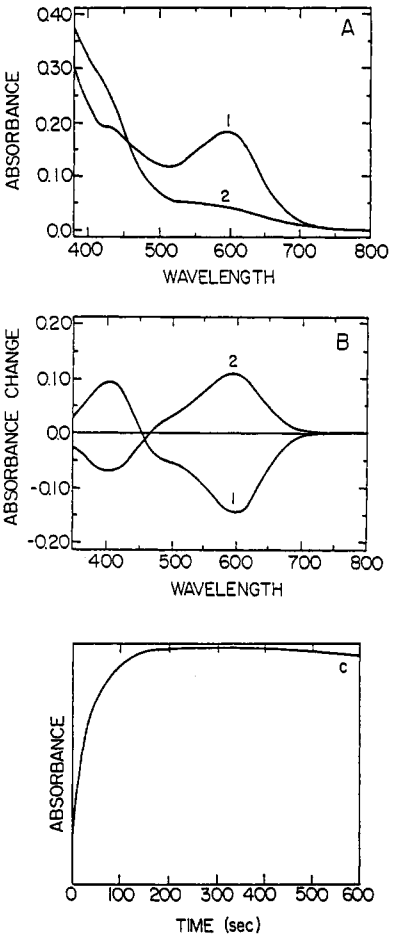


FIGURE 10: Formation of M-like intermediate of pigment I by steady-state illumination. (A) Absorption of pigment I, before (1) and after (2) irradiation to steady state with a 550-nm cutoff filter. (B) Difference spectrum following illumination (1) and the difference spectrum of thermal decay (2). (C) Re-formation of pigment absorption monitored at 590 nm, following steady-state illumination of pigment I.

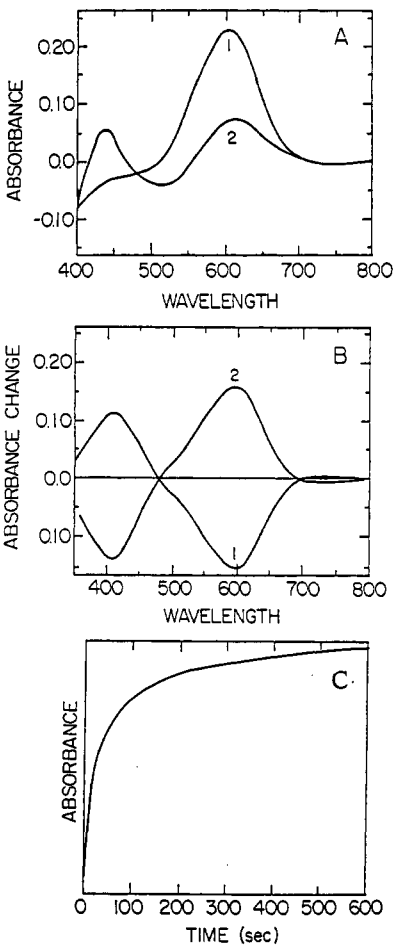


FIGURE 11: Steady-state illumination of pigment II. (A) Absorption of pigment II, before (1) and after (2) illumination with a 550-nm cutoff filter. (B) Difference spectrum before and after illumination (1) and the difference spectrum of thermal decay (2). (C) Re-formation of pigment absorption following steady-state illumination monitored at 600 nm.

time. We identify the shorter decay component with the second (τ_2) phase of the M^c decay observed in the pulsed laser experiment. The increased accumulation of the τ_3 = 3 min component (which is not observed with pulsed laser excitation) with exposure time suggests that we are dealing with a secondary M^c species (denoted M_p^c) generated by the back photoreaction of a later intermediate of the primary photocycle. The effect is reminiscent of the back photoreaction of the N intermediate in the photocycle of native bR (Kouyama et al., 1988; Fukuda & Kouyama, 1992; Brown et al., 1993).

To determine the chromophore isomer configuration in the above M-like species (at pH 7, 25 °C), induced by steady-state irradiation of pigments I and II, we carried out chromophore extractions comparing the isomer ratio immediately (i.e., within 1–2 s) following irradiation, with that measured after a subsequent 10-min delay in the dark. The results presented in Figure 12 clearly indicate that irradiation, which generates M^c (τ_2) and M_p^c (τ_3), increases the amount of the *all-trans* isomer. The original isomer composition is restored as the M^c and M_p^c species decay. Since we are unable to separate the contributions of M^c and M_p^c, we conclude that the M^c/M_p^c (τ_2 and τ_3) mixture consists of an *all-trans* isomer.

The total amount of M-like species found following irradiation of both pigments I and II to steady state is pH-dependent. The amount of accumulated M^c and M_p^c species decreases at low pH showing transiitons characterized by an apparent pK_a of 4.7 ± 0.2 for pigment I and of 5.7 ± 0.2 for

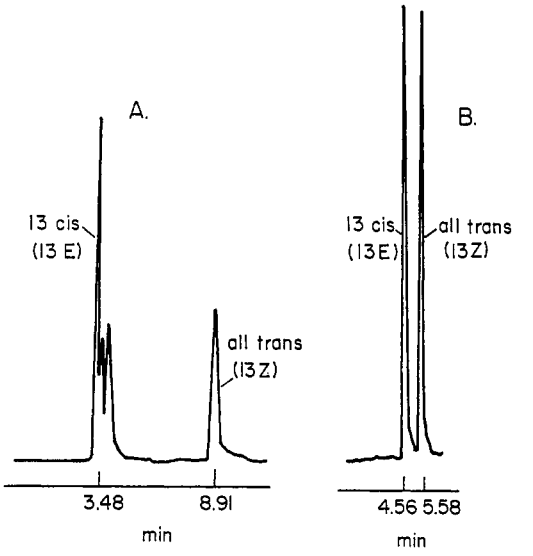


FIGURE 12: HPLC chromatography of irradiated pigments I (A) and II (B). The chromophores were extracted immediately following irradiation to steady state at pH 6.8.

pigment II (Figure 13). These observations are accounted for by the previously reported pH effects on both yield and lifetime of the τ_2 component. A decrease in τ_2 and in the amount of the corresponding component, M^c (τ_2), will decrease the yield of the slow M_p^c (τ_3) species generated via the secondary photochemical route.

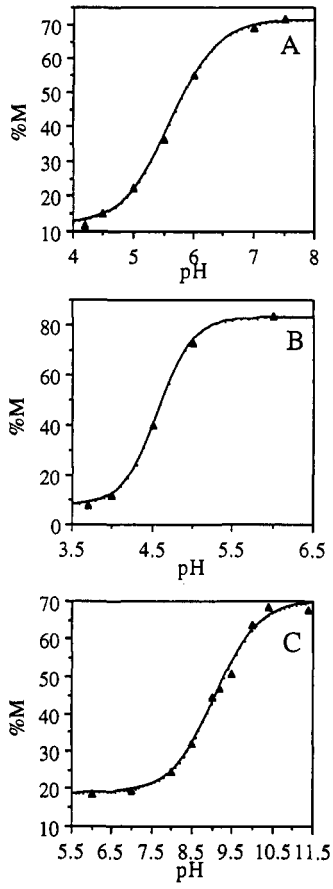


FIGURE 13: pH dependence of detected amount of M-like intermediate of pigment II (A) and of pigments I (B) and III (C) following illumination to steady state with a 550-nm cutoff filter at 25 °C. The absorption changes are measured at 608, 594, and 562 nm for panels A, B, and C, respectively.

We found that increasing the pH markedly affects the stability of the secondary (slow) M_p^c component. Thus, above pH 7, a fraction of a stable M-like species (M_{ps}^c) accumulates, replacing the $\tau_3 = 3$ min decay. At 21 °C, the lifetime of this species (τ_4) is well above 12 h. M_{ps}^c may be reverted to the pigment following irradiation with a 400-nm interference filter. At pH = 8.7 the amount of this stable M-like intermediate reaches ~90% of the total amount of M-like species observed at pH = 7.

In an attempt to ascertain whether the unusual stability of the (secondary) high-pH M-like intermediate of pigments I and II is associated with any specific retinal isomer, we carried out pigment extractions and HPLC analysis. It was found that, in the dark-adapted form, the amount of *trans* isomer at pH = 8.5 increased relative to the amount at pH = 7, yielding a 13-*cis*:*trans* ratio of 2:1. Following illumination to steady state, which produces the stable high-pH M-like species, HPLC analysis revealed that a new unidentified isomer (X) was produced. The isomer ratio was 1:4:3 13-cis:X:*all-trans* (Figure 14). It therefore appears that the high stability of M_{ps}^c is associated with a photoreversible retinal isomer, which is neither 13-*cis* nor *all-trans*.

For comparison, we have also studied the steady-state illumination effects on the artificial pigment III derived from 13-demethylretinal **3**. The pigment is characterized by 85% 13-*cis*-isomer in both dark- and light-adapted forms (Gartner et al., 1983). Steady-state irradiation of the pigment at pH = 7 results in the conversion of ca. 15% of the pigment to an M-like intermediate with a lifetime of ca. 2 s. pH elevation dramatically increased the amount of the M-like intermediate formed in keeping with a transition characterized by a

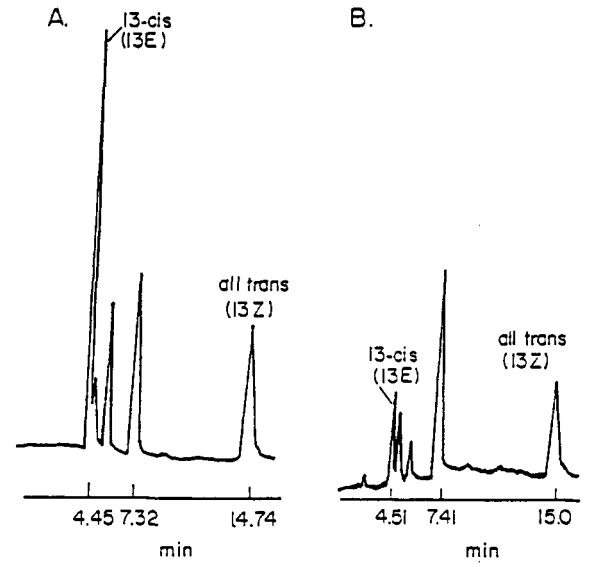


FIGURE 14: HPLC chromatography of pigment I chromophore extracted before (A) and after (B) illumination to steady state with a 550-nm cutoff filter at pH 8.5, 25 °C.

pK_a of 9.1 ± 0.2 (Figure 13C). Similarly to the photoproduct, M_{ps}^c, formed following irradiation of pigments I and II, the high-pH M species of pigment III is relatively highly stable, showing a biphasic decay with lifetimes of $\tau_3 \approx 20$ s and $\tau_4 \approx 5$ min at pH 9. Isomer characterization analysis carried out by chromophore extraction and HPLC analysis indicates that, in variance with pigment I, pigment III consists of 85% 13-*cis* isomer not only at pH 7 but also at pH 4 and 10. The titration curve shown in Figure 13C is similar to that obtained for the total (initial) amount of M-like intermediate as determined in the flash photolysis of pigment III (Figure 7). Experiments with varying illumination times show that, analogously to the long-lived M species of pigments I and II, both high-pH (τ_3 and τ_4) M components of III are due to the back photoreaction of a long-lived intermediate of the primary photocyle (probably with the 630-nm species equilibrated with the τ_2 M component, observed in the flash experiments). We, therefore, conclude that the high-pH effect observed in the continuous-illumination experiments of Figure 13C is due to that associated with the primary M yield (Figure 7). Increasing the amount of primary M^c at high pH leads to an increase in the (secondary) M_p^c photoproducts of the red-absorbing species equilibrated with the primary M^c. In this respect, the behavior of pigment III is analogous to the low-pH effects of pigments I and II shown in Figure 13A,B.

(*D*) *Continuous Illumination at Low Temperatures.* Irradiation of pigment I (derived from 14-fluoro-13-demethyl retinal) at −180 °C with a 520-nm cutoff filter produced a red-shifted intermediate absorbing around 630 nm, which we denote as K_{630}^c, characterized by a broad band which might represent two species. The latter is capable or reforming pigment I upon irradiation with a 660-nm cutoff filter.

In contrast to the *all-trans*-bR photocycle characterized by a K intermediate which decays to the blue-shifted L species ($\lambda_{max} = 550$ nm) at ca. −140 °C, the K_{630}^c intermediate of pigment I shows complex decay patterns. The various reactions occurring over a wide range of temperatures are shown in Figure 15. Between −140 and −120 °C, part of K_{630}^c decays into a species absorbing around 560 nm. Raising the temperature to −100 °C leads to a further decay into a mixture consisting of an M-like species absorbing at 420 nm, a (partially) recovered absorption around 580 nm, and a residual absorption around 630 nm. Between −80 and −40 °C, the rest of the K_{630}^c intermediate transforms mainly into

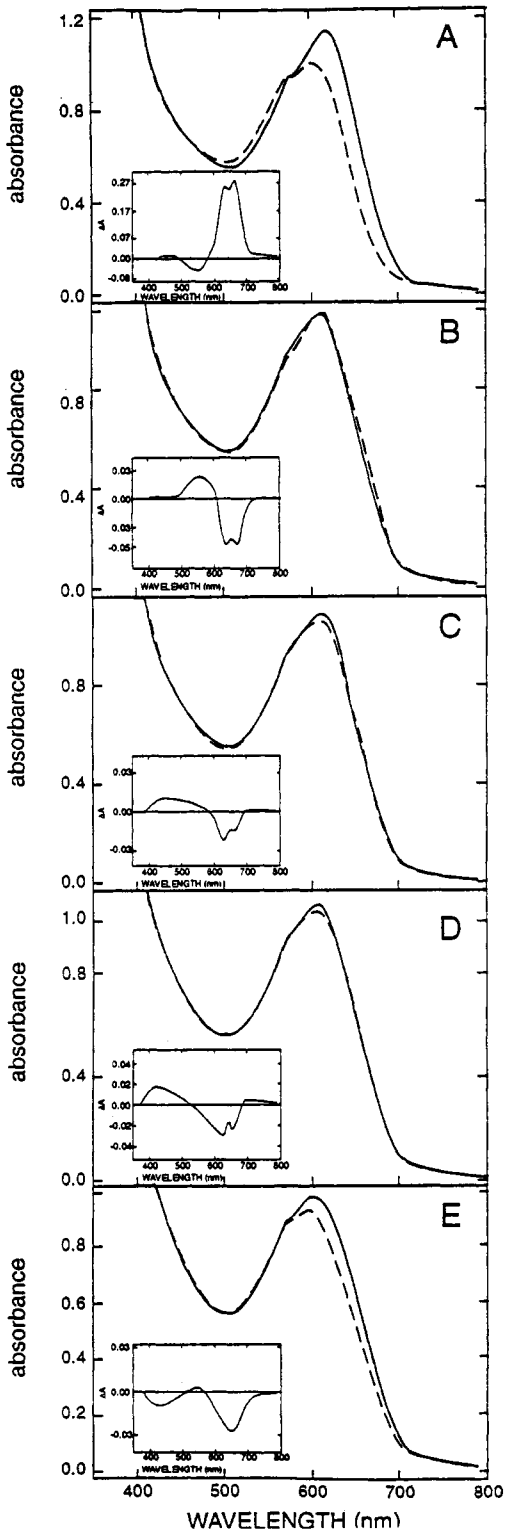


FIGURE 15: Photochemical and thermal reactions of pigment I at low temperatures. (A) Absorption spectra at −180 °C before (---) and after (—) irradiation to steady state with 550-nm cutoff filter. Inset: Difference spectrum between the photoproduct and the pigment. (B) Thermal decay of the irradiated mixture at −120 °C. Absorption spectrum of the mixture after 1 min at −120 °C (---) and after 30 min (—) at the same temperature. Inset: Difference spectrum between 30-min and 1-min spectra. (C) Thermal decay at −100 °C. The irradiated mixture described in (B) was warmed to −100 °C (—) and another spectrum was taken after 30 min (---). Inset: Difference spectrum between 30-min and 1-min spectra at −100 °C. (D) Thermal decay at −80 °C. Absorption spectra were taken after 1 min (—) and 30 min (---) at −80 °C. Inset: Difference spectrum between 30-min and 1-min spectra. (E) Absorption spectra after warming the irradiated mixture to −40 °C after 1 min (—) and 30 min (---) at this temperature. Inset: Difference spectrum between 30-min and 1-min spectra.

an M-like intermediate. Above −40 °C, the M-like intermediate decays to the original pigment along with teh residual 630-nm band. We have not attempted to investigate the exact nature of these complex patterns, including the identification of the residual high-temperature 630-nm band. It is evident, however, that the experiments are in keeping with the room-temperature data showing that a red-shifted intermediate is a precursor of M. Moreover, no long-lived M-like component is observed following low-temperature irradiation and warming. This is consistent with attributing the long-lived component to accumulation via secondary back photoreactions.

DISCUSSION

(A) Mechanism of M Generation in the Photocycle of 13-cis-bR. It is well established that at neutral pH the photocycle of 13-*cis*-bR does not include any M-like intermediate in which the Schiff base has undergone deprotonation (Kalisky et al., 1977). This also applies to the 85% 13-*cis* component of the 13-demethyl artificial pigment (Gartner et al., 1983, 1988). Nevertheless, as shown by Kaulen et al. (1990), M generation does take place in 13-*cis*-bR when the pH is raised to 8.5. The effect was attributed to the titration of a protein residue, denoted here as YH, exhibiting an apparent pK_a of ~9.5. Our present study shows that an essentially identical pH effect takes place with 13-*cis*-13-demethyl-bR. However, we show that deprotonation of the Schiff base in the 13-*cis* photocycle, leading to M formation, may also be induced (at neutral pH) by 14-C fluorine substitution. Although the isomer configuration of the M^c intermediate is not yet fully established, our results show that there is an increment in the *all-trans* component following M formation in keeping with 13-*cis* → *all-trans* isomerization. However, since the mixture contains some M^p species (M which probably originated from back photoreaction of N), we cannot definitely clarify the nature of the M isomer. The data with pigment I, showing that M^c is generated from a red-shifted species, are consistent with two alternative schemes, (1) and (2), in which either K^c or L^c_{610} is the precursor of M^c:

$$bR^c(I) \xrightarrow{h\nu} K^c \xrightarrow{k_1} L^c_{610} \xrightarrow{k_2} M^c \rightleftharpoons N^c \longrightarrow bR^c(I) \quad (1)$$

(k_3)

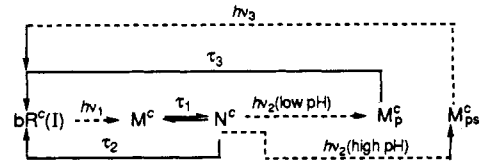
(with $k_2 >> k_1, k_3$)

$$bR^c(I) \xrightarrow{h\nu} K^c \xrightarrow{k_2} M^c \rightleftharpoons N^c \longrightarrow bR^c(I) \quad (2)$$

(k_1, L^c_{610})

(with $k_2 >> k_1$)

The presence of the N^c intermediate is being invoked in view of the biphasicity of the M^c decay, in analogy with the characteristic nonexponential decay of M in the photocycle of *all-trans*-bR (Hess & Kuschmitz, 1977; Ort & Parson, 1978; Ohno et al., 1981; Scherrer & Stoeckenius, 1985; Groma & Dancshazy, 1986; Varo & Lanyi, 1991). The formation of the N^c intermediate in equilibrium with M component is further supported by the back photoreaction induced by steady-state illumination. The equilibrated pair $M^c \rightleftharpoons N^c$ has a sufficiently long lifetime (τ_2) to undergo secondary photolysis, producing the long-lived (τ_3) and probably the stable, high-pH, M-like intermediates, according to the following scheme:



(3)

A blue-shifted L_{550}-like speices, which is associated with the *trans* photocycle, was not detected in the photocycle of the 13-*cis* artificial pigments at 25 °C. However, its presence as a short-lived intermediate between L^c_{610} and M cannot be completely excluded; primarily since at low temperatures a partial decay of K^c_{630} to a 580-nm absorbing species was observed. At this time we could not unambiguously identify this intermediate, which may carry analogies to L_{550}.

Essentially the same model applies to pigment III if it is assumed that the various rate constants are pH-dependent, namely, $k_3 \gg k_2$ (1) or $k_1 \gg k_2$ (2) at pH < 7 and $k_3 \ll k_2$ (1) or $k_1 \ll k_2$ (2) at pH > 10. On the molecular level, the titrationlike curves of the relative M amount in the photocycles of both 13-*cis*-bR (Kaulen et al. 1990) and 13-cis 13-demethyl-bR (Figures 7 and 15) may be interpreted in terms of three approaches:

(1) A protein moiety serves as the proton acceptor to the Schiff base proton only when present in its deprotonated (Y^-) form (high pH). This approach was invoked by Kaulen et al. (1990) and Drachev et al. (1993), who suggested that the alkaline transition forms Y^-, which serves as a proton acceptor and permits Schiff base deprotonation in the 13-*cis* photocycle.

(2) The lack of M generation in the 13-*cis* photocycle, rather than being due to the absence of an appropriate proton acceptor, is attributed to an insufficient (light-induced) change in the ΔpK_a and/or to a high barrier for proton transfer between the Schiff base (H^+ donor) and the acceptor. Identification of the latter is still unavailable. A plausible suggestion is that the same protein moiety, i.e., Asp85, acts as the proton acceptor from the Schiff base in both *all-trans* and 13-*cis* photocycles.

(3) The curves represent direct titrations of the Schiff base which is equilibrated with the external water medium. Accordingly, its pK_a value has been reduced from its "dark" value of 13.2 (Druckmann et al., 1982; Sheves et al., 1986) to about 8.7 in the M stage of the photocycle.

The present observations, namely, that pigment I exhibits M formation over the whole pH range between 3.5 and 8.5, is inconsistent with mechanisms 1 and 3. Thus, the pK_a of the Schiff base of pigment I is reduced by only 2.5 units with respect to the native pigment. Assuming that the same ΔpK is maintained during the photocycle, one would expect, according to mechanism 3, that SB deprotonation in the photocycle of pigment I will take place 2.5 pK units below the pK of the titration curve of Figures 7 and 13C, namely, around pH 6. The fact that M is observed at pH values as low as 3.5 is inconsistent with this mechanism. As to mechanism 1, if the $pK_a = 8.7$ and the titration curve is associated with a YH group which functions as a proton acceptor, there is no reason for losing the pH dependence in the 14-fluoro chromophore.

We are thus left with mechanism 2, which accounts for the differences in the SB deprotonation mechanism in the *all-trans* and 13-*cis* photocycles in terms of different changes in the relative pK_a values of the SB donor and acceptor groups. Thus, in the ground state, the pK_a of the protonated Schiff base of both *all-trans*-retinal and 13-*cis* isomers is unusually high and that of Asp-85 is relatively low. It was recently suggested (Gat & Sheves, 1993) that the ion pair is stabilized due to a specific angle adopted by the protonated Schiff base linkage and the carboxylate group, which allows bound water molecules to bridge the two groups and stabilize the ion pair. The ion pair can be further stabilized by other protein residues that participate in the hydrogen-bonding interaction. In the case of *all-trans*-bR, light absorption induces retinal isomerization, perturbing the ion pair structure. This results in proton transfer from the protonated Schiff base to the acceptor group. The fact that at neutral pH no deprotonation takes place in the photocycle of 13-*cis*-bR (despite the relatively long lifetime of the L^c_{610} intermediate) may be due to an improper relative pK_a values of the protonated Schiff base linkage and of the acceptor group, which are insuffiicent for inducing proton transfer. The observation that M formation is induced by 14-fluoro and 12,14-difluoro substitution shows that this "deficiency" of the 13-*cis* photocycle may be corrected by reducing the intrinsic pK_a value of the protonated Schiff base of bR(13-*cis*) (and thus of its K^c or L^c_{610} intermediates) by less than 2.5 pK units. The intrinsic pH change may not be the only effect of C_{14} fluorine substitution. Changes in H-bonding and steric effects may also play a role in controlling the relative pK_a of the Schiff base and of the acceptor groups. Despite the high feasibility of these arguments, we must note that the absence of M formation in the photocycle of 13-*cis*-bR at neutral pH may not be due exclusively to an insufficient ΔpK_a between the two groups. An alternative explanation may be associated with a high barrier for proton transfer from the Schiff base to the acceptor group. In such a case, the effect of 14-fluoro substitution in generating the M intermediate may be due to a reduction of the H^+ transfer barrier via appropriate steric or H-bonding interactions involving protein residues and/or water molecules.

The titrationlike curves representing the yield of SB deprotonation in the 13-*cis* photocycle are accounted for in terms of mechanism 2 by assuming that at high pH a protein residue (YH) is deprotonated, inducing M formation due to either elevation of the pK_a of the acceptor or reduction of the pK_a of the protonated Schiff base via mechanisms that are still unclear. Additional phenomena showing titrationlike transitions between pH ~8 and ~10 are the rate of light to dark adaption, i.e., *trans* to 13-*cis* isomerization (Ohno et al., 1977; Warshel & Ottolenghi, 1979), and the yield of light-induced proton release (Kono et al., 1993). It is conceivable that the first possibility is applicable since introducing an additional negative charge in the binding site will destabilize the negative charge on a plausible proton acceptor residue. In this respect, we note that it was suggested that deprotonation of Arg-82 at high pH may affect the pK_a of Asp-85 and M formation (Balashov et al., 1993).

The nature of the YH group should be considered in terms of previous work indicating pH-induced changes in bR between pH ~8 and ~10. Primarily, the kinetics of M formation in the light-adapted bR photocycle are affected by pH; M formation is faster at high pH (Ort & Parson, 1978, Kalisky et al., 1981; Rosenbach et al., 1982; Hanamoto et al., 1984; Liu, 1990) due to the appearance of a biphasic rise time of 6 and 0.4 μs (Liu, 1990), which replace the slower processes at low pH. The pK_a of this transition is 9.6 in the presence of 0.1 M KH_2PO_4 (Hanamoto et al., 1984). Later it was shown that the absorption of bR is slightly shifted (by ~1.5 nm) wiht a pK_a of ~9. This transition correlates with the appearance of the fast component of M formation with an absorption increase at 238 and 297 nm, which are caused by deprotonation of a tyrosine residue and a red shift of the absorption of a tryptophan residue (Balashov et al., 1991). It is possible that in all these cases the same group (YH) is responsible for the pH-induced effects, coinciding with that responsible for modifying the pK_a values of the donor–acceptor pair in the 13-*cis* cycle inducing M^c formation. It is interesting to note that the effect of inducing the formation of the stable high-pH M form of pigment I (M^c_{ps}) is observed in the same pH range, namely, between pH ~8 and ~9. It is thus possible that the effect is associated with a structural change induced by the titration of the same YH group as discussed above. The high-pH form of the pigment affects the path of the back

photoreaction of N^c, leading to an unidentified isomer of extremely high thermal stability in its deprotonated M form. Further work with appropriate bR mutants may help to ascertain whether only one YH group may is responsible for all these pH effects, possibly leading to its identification.

(*B*) *Later Stages in the 13-cis Photocycles*: *M^c Decay and Secondary (Back) Photoreactions*. We should also consider the mechanism of M decay in the photocycle of pigment I. In the pulsed laser excitation experiments, two decay components are observed. The biphasicity of the M^c decay is reminescent of the complexity observed in the kinetics of M^c decay in the *all-trans* cycle (Groma & Dancshazy, 1986; Varo & Lanyi, 1991; Eisfeld et al., 1993). The currently preferred mechanism accounts for the biphasic M decay in terms of a relatively fast equilibration between M and N, followed by a slower decay of the M ⇌ N equilibrium pair to O and bR (Otto et al., 1989; Varo & Lanyi, 1991; Ames & Mathies, 1990; Lozier et al., 1992). Assuming that most of the observed M intermediate is due to the 13-*cis* component, it appears that a similar mechanism also applies to the decay of M^c in the photocycle of the 13-*cis* pigment. Accordingly, an N-like stage was incorporated in both suggested mechanisms 1 and 2. We note that our analysis overlooks the contribution of the ~20% *all-trans* component in pigment I. As long as relevant information allowing discrimination between the two photocycles is not available, we assume that the photocycles of the two isomers are kinetically and spectroscopically similar. Similar arguments apply to the biphasic decay of M^c of pigment III at high pH.

Apart from the above considerations, an additional complexity in the M population of pigments I and II becomes evident when using continuous illumination. The steady-state experiments reveal an M^c intermediate which decays on a time scale of several seconds, which corresponds to the long-lived component (τ_2) observed with pulsed flash excitation. The question arises, however, with respect to the identification of the long-lived species ($\tau_3 \approx 5$ min) observed only in the steady-state experiments. These may in principle be due to the accumulation of low-yield residual amounts of M formed in parallel or consecutively to the major photocycle route as observed in the pulsed excitation experiments. However, as suggested above, dependence of the amount of the long-lived M on the irradiation time suggests that the latter species are generated by a secondary back photoreaction induced by red light ($\lambda > 550$ nm) which is not abosrbed by M. The most plausible explanation for these observations, in keeping with scheme (3) suggested above, is to suggest the species N^c (equilibrated with M^c) as the one undergoing the secondary photoreaction. This interpretation creates further analogy with the photocycle of *all-trans*-bR, where irradiation of N produces a long-lived M-like intermediate (Kouyama et al., 1988; Brown et al., 1993; Fukuda & Kouyama, 1992). The high efficiency of the back photoreactions in the cases of pigments I, II, and III (high pH) is due to their relatively slow photocycles compared to that of native bR.

We finally consider the question as to whether the appearnce of an M-like intermediate in the 13-*cis* photocycle is associated with a proton translocation activity. It has been claimed that a proton pumping activity is associated with the photocycle of 13-*cis*-bR only at high pH when an M-like intermediate is present (Kaulen et al., 1990; Drachev et al., 1993). Our findings with 13-demethyl-bR (see Results above), which attribute the light-induced dye protonation at pH 7 to the ~20% *trans* component (Figure 9) and the effect at pH 8.3 to both *trans* and 13-*cis* components, are in keeping with this claim. Similarly, recent photoconductivity experiments indicate that the photocycle of 13-demethyl-bR at high pH is associated with proton release (T. Marinetti et al., to be published).

It appears, however, that this conclusion cannot be extended to the photocycle of the fluorinated pigment I. Thus, as shown in Figure 6 and discussed above, pigment I shows a weak proton translocation activity, amounting to 20% ± 10% that of *all-trans*-bR (scaled to the corresponding M absorbance). Since the isomer composition of pigment I is ~20% *trans* and ~80% *cis*, it is more than likely that the observed proton release and uptake are due to the *trans* component, leaving the 13-*cis* photocycle essentially inactive with respect to proton translocation, despite the formation of the M^c intermediate. This interpretation may be supported by Figure 6A in which proton uptake seems to be faster than the decay of M^c and may thus represent a shorter lived *all-trans* photocycle. Unfortunately, in the present work, we have been unable to separate the two superimposed photocycles. The conclusion that no proton translocation takes place in the photocycle of 13-cis-13-demethyl-14-fluoro-bR was also derived from photoconductivity experiments (Marinetti et al., to be published). This would suggest that deprotonation of the Schiff base during the photocycle may be a necessary condition but is not a sufficient condition for inducing a proton pump.

CONCLUSIONS

We have shown that the lack of a deprotonated M intermediate in the photocycle of 13-*cis*-bacteriorhodopsin can be "restored" by artificial fluorianted chromophores, which decrease the pK_a of the Schiff base. The same effect may also be caused by the titration of a protein moiety with a pK_a of 8.5–9. The observations are interpreted in terms of changes in the relative pK_a of the donor (Schiff base) and acceptor (most probably Asp-85) groups and/or reducing the H^+ transfer barrier. In addition to exhibiting a deprotonated M-like species, the "restored" photocycle of 13-*cis*-bR carries further analogies to that of the *all-trans* pigment. Primarily, it shows a biphasic M^c decay which is most probably associated with an N-like intermediate (N^c). Moreover, it appears that N^c is responsible for the secondary back photoreactions which, analogously to N, yield long-lived M-like photoproducts. As to the proton pumping actiivty, it does characterize the 13-*cis* photocycle "restored" by high pH but not by 14-fluorine substitution. Thus, pigment I appears to be the first case in which M formation is not accompanied by proton release and/or uptake. This observation raises questions concerning the relations between deprotonation of the Schiff base and of the proton-releasing XH group, which will be addressed in future studies.

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