Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4209–4213, June 1986
Biochemistry

Deprotonation of the Schiff base of rhodopsin is obligate in the activation of the G protein

(methylation)

COLIN LONGSTAFF, ROGER D. CALHOON, AND ROBERT R. RANDO*

Department of Pharmacology, Harvard Medical School, Boston, MA 02115

Communicated by William von Eggers Doering, February 19, 1986

ABSTRACT **Photolysis of rhodopsin leads to the formation of an activated intermediate that activates a G protein, thus beginning the visual cascade. This activated form of rhodopsin appears coincident in time with the spectroscopically defined intermediate, metarhodopsin II. Metarhodopsin I, the precursor of metarhodopsin II, contains a protonated Schiff base, whereas metarhodopsin II does not. The question of whether the deprotonation of the protonated Schiff base is obligate in the formation of activated rhodopsin was addressed by monomethylating the active-site lysine of permethylated rhodopsin and determining whether this pigment can activate the G protein upon photolysis. The photolysis of the new pigment, which absorbs at 520 nm, led to the formation of a relatively stable metarhodopsin I-like intermediate with a λ_{max} of ≈485 nm, with no apparent formation of either metarhodopsin II- or metarhodopsin III-like intermediates. The only probe available to detect formation of the active form of rhodopsin is G protein activation. Photolysis of the pigment in the presence of the G protein did not lead to measurable activation of the GTPase activity of the latter. These studies establish a functional link between Schiff base deprotonation and activation of the G protein. It is concluded that proton transfer from the protonated Schiff base of rhodopsin is obligate for the initiation of visual transduction.**

Vision begins with the absorption of a photon of light by rhodopsin, a holoprotein containing 11-*cis*-retinal bound to lysine-296 by a protonated Schiff base linkage as the chromophore (1). The absorbed photon leads to the isomerization of the chromophore to its all-*trans* congener, which eventually results in its hydrolysis to form opsin and all-*trans*-retinal in a process called bleaching (2). An intermediate of rhodopsin on the way to bleaching, apparently the spectroscopically defined metarhodopsin II, is responsible for the activation of the G protein as revealed by its GTPase activity (3, 4). G protein activation in turn leads to the activation of a phosphodiesterase specific for cGMP (5, 6), which is thought to be the internal transmitter in rod outer segments (7). The function of cGMP is to act as an agonist, maintaining the plasma membrane-bound sodium channels in an open state (7). Activation of the G protein is the only biochemical step known to be directly affected by the bleaching of rhodopsin.

Since metarhodopsin II spectroscopically labels that rhodopsin conformer capable of beginning this cascade of events, it is of some interest to define the molecular mechanisms by which it arises and decays. Of particular interest here is the spectroscopic change (478 nm → 380 nm) that accompanies the metarhodopsin I to metarhodopsin II transformation (8). The usual interpretation here is that this change signals the deprotonation of the Schiff base and this interpretation is solidly backed up by resonance Raman studies (9). In this interpretation, proton transfer from the Schiff base is necessary in the metarhodopsin I to metarhodopsin II conversion. We wish to ask whether this proton transfer is obligate in the formation of activated rhodopsin and further to determine whether metarhodopsin II and the activated form of rhodopsin are structurally linked. That is, even if deprotonation of the Schiff base were to occur in the formation of activated rhodopsin, is it obligate or a by-product of the conversion? For the purpose of answering this question, we have prepared active-site monomethylated rhodopsin (10).

This material was prepared by reductively methylating rhodopsin to completion, a procedure in which all 10 non-active-site lysine residues are dimethylated (10). This permethylated rhodopsin (PM-Rh) is fully active with regard to its ability to activate the G protein after exposure to light. Photolysis of this pigment, followed by reductive methylation of the active-site lysine, leads to the formation of permethylated active-site lysine monomethylated opsin, which was purified from the mixture using *o*-phthalaldehyde/mercaptoethanol (OPA) to specifically react with the free active-site lysine containing opsin (10) (Scheme I). The purified protein forms a Schiff base with 11-*cis*-retinal, producing an active-site methylated, non-active-site permethylated rhodopsin referred to as AMPM-Rh, which absorbs at 520 nm (Scheme I). Since the active-site lysine proton has been replaced by a methyl group, we can determine whether a proton loss at the protonated Schiff base of rhodopsin is obligate in the activation of the G protein (GTPase) and in the formation of a metarhodopsin II-like intermediate. It is demonstrated here that the photolysis of AMPM-Rh in the presence of the G protein does not lead to the activation of the latter. The spectroscopic changes accompanying the absorption of light by AMPM-Rh are consistent with a process in which a long-lived metarhodopsin I-like intermediate is formed that slowly hydrolyzes to the modified opsin molecule and all-*trans*-retinal without forming metarhodopsin II- or III-like intermediates. It is concluded that deprotonation of the protonated Schiff base of rhodopsin is obligate in visual transduction. These studies establish a link between the spectroscopically defined and biochemically defined mechanisms of visual transduction.

MATERIALS AND METHODS

Materials. Bovine retinas were obtained from Hormel. Pipes and pyridine borane were obtained from Aldrich. Dodecylmaltoside was obtained from Behring (La Jolla, CA). *o*-Phthalaldehyde, 2-mercaptoethanol, and hydroxyalkoxy-

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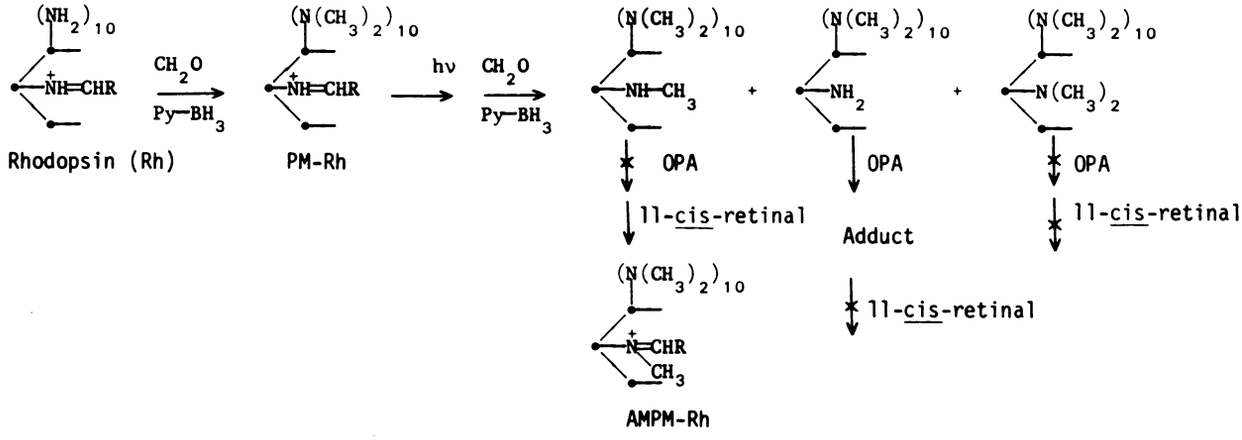
Abbreviations: PM-Rh, permethylated rhodopsin; AMPM-Rh, active-site lysine monomethylated, permethylated rhodopsin; OPA, *o*-phthalaldehyde/mercaptoethanol.

*To whom reprint requests should be addressed.



Scheme I

propyldextran were from Sigma. Hydroxylapatite, DNA grade, was obtained from Bio-Rad. Minicon concentrators were obtained from Amicon.

Preparation and Purification of Proteins. Rod outer segments and solubilized rhodopsin were prepared by standard methods as described (11). Detailed procedures for the preparation of methylated rhodopsins have been published (10). All rhodopsin modifications were carried out in a buffer consisting of 10 mM Pipes, pH 6.5/100 mM NaCl/6 mM dodecylmaltoside. Methylations were performed with formaldehyde and pyridine borane (10). Treatment of the methylated opsins with OPA was carried out as described (10).

Purification of the modified proteins varied depending on whether they were to be used for spectral/bleaching studies or for GTPase activation assays. For spectral studies, AMPM-Rh was purified by hydroxylapatite column chromatography. Fig. 1 shows a spectrum of the purified AMPM-Rh along with a profile from the hydroxylapatite column.

GTPase Activation Studies. Samples of bleached AMPM-Rh and PM-Rh reacted identically with OPA as described (10). Both samples were passed through a small column (0.5 ml) of hydroxyalkoxypropyldextran type I or type III. In this way, excess 11-*cis*-retinal (60–80% of that added for regeneration) was removed, but this treatment had no effect on the composition of regenerated and nonregenerated proteins in each sample. Protein samples were occasionally concentrated up to 5 times using a Minicon concentrator. A hydroxylapatite purified sample was also prepared and tested for its ability to activate the G protein (Table 1). These samples were added to lipid to form vesicles as described by Calhoon and Rando (11). The GTPase activation assays were performed by standard methods as described (11).

Spectral Studies. All spectra were recorded with a Perkin–Elmer λ 3B UV/Vis spectrophotometer. Protein samples were bleached for 10 sec in an ice water bath using a source of intense light (600 W) filtered through 15 cm of water, 4 cm of glass, and an orange cut-off filter (Corning 3-68, cut-off at 540 nm). After the bleach, the sample cuvette was transferred quickly (<30 sec) to the spectrophotometer and then a spectrum was recorded within 1–1.5 min from the start of the bleach. Spectra were recorded at various times thereafter over a period of up to 6 days, during which time the sample was maintained at 4°C in the dark.

RESULTS

Spectroscopic Studies on Methylated Rhodopsins. Prior to investigating the bleaching behavior of AMPM-Rh, the photochemistry of PM-Rh itself was studied to compare it to rhodopsin. When this pigment was exposed to orange light for 10 sec at 4°C, the spectral changes displayed in Fig. 2*A* were observed. Immediately upon bleaching, the 500-nm absorption of the chromophore was replaced with one absorbing at ≈380 nm. The formation of this peak absorbing at 380 nm is exactly what would be expected at these temperatures and clearly represents the formation of a metarhodopsin II-like intermediate. The precursors of metarhodopsin II decay too rapidly to be observed at 4°C (8). When the sample was allowed to remain at 4°C for 3 hr, a new peak developing at ≈460 nm was observed (Fig. 2*A*). This is again exactly what would be expected by analogy with published studies on bleaching of unmodified rhodopsin, with the 460-nm peak revealing the formation of a metarhodopsin III-like state (8). This peak slowly decays with a concomitant increase in absorption at 380 nm, as would be expected for the decay of metarhodopsin III (Fig. 2*B*). Furthermore, in our hands, rhodopsin showed identical bleaching behavior to its permethylated form PM-Rh. These results are in marked contrast to those observed with the purified AMPM-Rh (Fig. 3). In this case, a 10-sec flash of orange light at 4°C led to an immediate shift in spectrum from 520 nm to ≈485 nm. This 485-nm peak is where a metarhodopsin I-type intermediate might be expected to absorb light (8). With time, at 4°C, this peak decays to a peak with a λ_{max} of ≈380 nm, which we ascribe to the slow hydrolysis of the metarhodopsin I-like intermediate to form all-*trans*-retinal and AMPM-opsin. The immediate addition of 11-*cis*-retinal to the photolyzed AMPM-Rh results in the increase in absorption at ≈500 nm, a result consistent with the notion that the 380-nm absorption observed is due to all-*trans*-retinal and not a bound chromophore intermediate. These results in aggregate are entirely consistent with the idea that the active-site methylated rhodopsin cannot achieve a metarhodopsin II-like state and is "frozen" at a metarhodopsin I-like state, which is slowly hydrolyzed to form all-*trans*-retinal and the modified opsin.

G Protein Activation by the Modified Rhodopsins. Currently the only way to reveal whether or not the active conformer of rhodopsin is being formed is by studying the ability of the modified pigment to activate the G protein. Bleached PM-Rh by itself activates the G protein as well as rhodopsin (Table 1). Permethylated active-site methylated opsin was treated with OPA (Fig. 4) and purified according to the procedure outlined in *Materials and Methods*. Treatment with OPA causes the derivitization of only the unmethylated lysine containing opsin (Scheme I). This occurs because OPA can only react irreversibly with primary amino groups (12, 13). Regeneration studies on OPA-treated protein verify this assumption because regeneration ability was only lost to the extent that there was unmethylated active-site lysine present (Fig. 4). The OPA is used to ensure that virtually no unmodified active-site lysine containing rhodopsin remains. However, given the steepness of the dose–response curve for

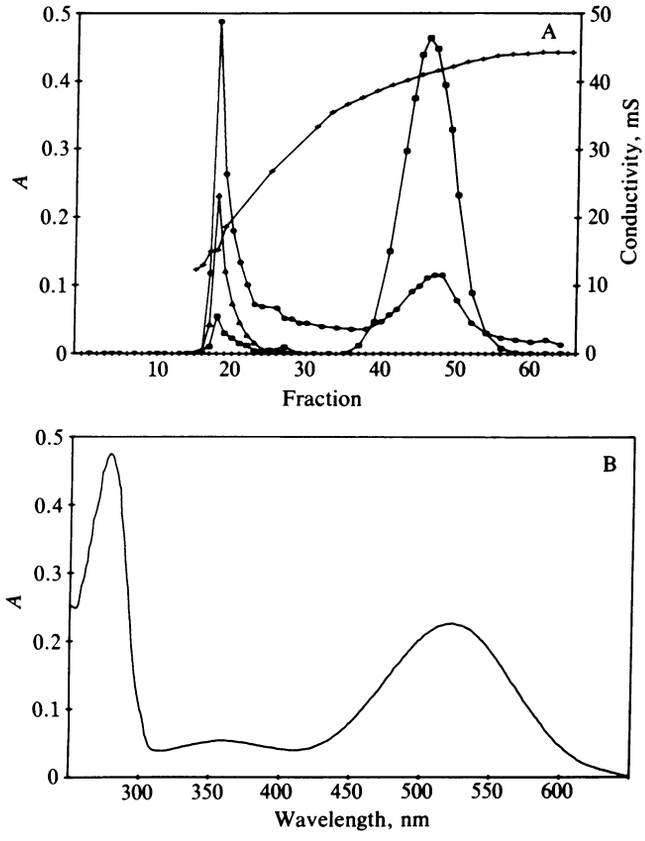


FIG. 1. Purification and spectrum of AMPM-Rh. (*A*) The column was preequilibrated with 20 mM K phosphate buffer (pH 6.5) containing 6 mM dodecylmaltoside and 100 mM NaCl (buffer A), and the regenerated modified protein sample (9 ml), consisting of 140 nmol of protein including ≈37 nmol of regenerated AMPM-Rh, was made 20 mM in K phosphate and 100 mM in NaCl before being loaded onto the column. Elution of bound protein was effected by using a gradient formed by mixing buffer A (50 ml) with buffer B consisting of 0.5 M K phosphate (pH 6.5) containing 100 mM NaCl and 6 mM dodecylmaltoside. The flow rate was ≈15 ml/hr and fractions of 2.2 ml were collected. Regenerated rhodopsin (▲, A_{520}) elutes early in the gradient, before nonregenerable protein (●, A_{278}) and excess 11-*cis*-retinal (■, A_{370}). (*B*) The spectrum of purified AMPM-Rh eluted in the peak fraction (no. 18) of the regenerated protein peak from the column. The ratio of A_{278}/A_{520} of protein in fraction 18 was 2.1 as compared with 6.8 for material applied to the column. This protein sample was also 1.5-fold more concentrated (measured by A_{520} absorbance) compared with applied material.

Table 1. Activation of the G protein by active-site modified rhodopsins

Modification of rhodopsin	% GTPase activation relative to native Rh	% Rh bleach required for equivalent GTPase activation*
Unmodified at the active-site		
PM-Rh	105	100
Active-site modifications		
Preparation 1		
a. AMPM-Rh†	2	<0.5
b. AMPM-Rh‡	2	<0.5
Preparation 2		
a. AMPM-Rh	17	1.5
10 min irradiation at 30°C	7	0.5
20 min irradiation at 30°C	4	<0.5
b. AMPM-Rh	14	1.2
20 min irradiation at 30°C	3	<0.5
c. OPA-treated PM opsin	10	0.8
Preparation 3		
a. AMPM-Rh§	3	<0.5
20 min irradiation at 30°C	4	<0.5
b. AMPM-Rh¶	4	<0.5
c. OPA-treated PM opsin	6	0.5
Preparation 4		
a. AMPM-Rh	5	<0.5
b. OPA-treated PM opsin	2	<0.5
Combined Assays		
a. AMPM-Rh (16)‖	3.0 ± 1.3	<0.5
b. OPA-treated PM opsin (6)‖	4.8 ± 2.2	<0.5

Each sample contained an excess of unregenerated protein over regenerated AMPM-Rh. The total protein was used to calculate the amount of lipid to be added in making vesicles. The values for each preparation are given as follows: micromolar concentration of AMPM-Rh (assuming ε_{520} = 40,000 $M^{-1}\cdot cm^{-1}$), micromolar concentration of total protein (assuming ε_{280} = 64,000 $M^{-1}\cdot cm^{-1}$), and volume of sample in ml. Preparation 1a, 3.7, 10.3, 1.0; preparation 1b, 2.3, 15.4, 3.0; preparation 2, 2.4, 15, 2.25; preparation 3a, 3.3, 40.9, 1.5; preparation 3b, 2.2, 25.9, 1.5; preparation 4, 2.0, 16.1, 1.0.
*The curve relating the response of GTPase activation to partial rhodopsin bleach was generated by a standard protocol as described (11). The curve for the G protein preparation used in the above experiments was virtually identical to the dose–response curve already described (11).
†Protein purified on hydroxylapatite.
‡Protein purified on hydroxyalkoxypropyldextran.
§Pool of concentrated fractions.
¶Pool of dilute fractions.
‖Number of assays.

G protein activation in the presence of photolyzed rhodopsin, even a percent or so of OPA unmodified material would be problematic (11). To control for this possibility, a bleached PM-Rh sample was treated with OPA identically to that of the active-site methylated sample. These two preparations were purified independently but identically, and the ability of these rhodopsins, when incorporated into phosphatidylcholine-based vesicles, to activate the G protein was examined. In Table 1, results from four different modified rhodopsin preparations are reported. The samples were photolyzed and incubated with G protein. In all cases, the active-site methylated rhodopsin showed no activity when compared to controls (Table 1). The small, statistically insignificant activity of the AMPM-Rh could be completely accounted for by the listed amounts of PM-Rh remaining in the sample. This, in turn, is accounted for by the OPA reaction not quite going to completion but, nevertheless, being exceedingly effective, as expected from the results shown in Fig. 4. Another point to note in Table 1 is that preparations that initially showed GTPase activation lost this ability after extended periods of incubation at 30°C in the light, showing that this activity was due to a PM-Rh contaminant rather than a slowly forming biochemically competent form of AMPM-Rh.

DISCUSSION

Detailed accounts of the spectroscopically defined intermediates occurring after light absorption by rhodopsin have been published (8). The first intermediate, bathorhodopsin, occurs within 6 psec of the absorption of a photon (14). By this time, double-bond isomerization has already largely occurred (15) with the storage of ≈35 kcal of energy (16). A protonated Schiff base is presumed to be critical in this process. After the formation of bathorhodopsin, spontaneous conformational changes occur resulting in the formation of lumirhodopsin and then metarhodopsin I (8). The Schiff base is still protonated at this stage (9). The metarhodopsin I to metarhodopsin II transition is of great interest because this latter intermediate(s) labels the biochemically competent one

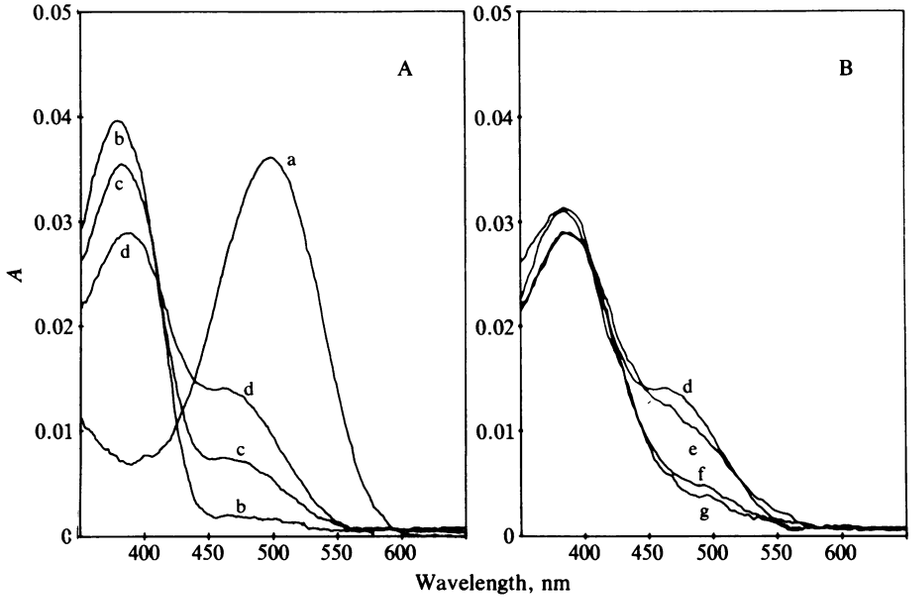


FIG. 2. The bleaching of PM-Rh. The spectra of PM-Rh were recorded before and after a short (10 sec) exposure to orange light during incubation at 4°C in the dark. (*A*) A spectrum of PM-Rh before the bleach (curve a) and spectra recorded at 1.5 min (curve b), 40 min (curve c), and 3 hr (curve d) after the bleach. (*b*) Spectra recorded after 3 hr (curve d), 1 day (curve e), 4 days (curve f), and 6 days (curve g).

(3). The metarhodopsin I to metarhodopsin II transition is accompanied by a striking spectroscopic change in which the maximal absorption shifts from 478 nm to 380 nm, which is consistent with a Schiff base deprotonation process (8). The deprotonation mechanism was proved to be correct by resonance Raman studies, which demonstrated that metarhodopsin II contained a C=N moiety (9). Subsequent to the formation of metarhodopsin II, reprotonation of the Schiff base occurs to form metarhodopsin III, with the resultant hydrolysis of the protonated Schiff base to form opsin and all-*trans*-retinal.

In this article, we have begun an attempt to ascribe the spectroscopically defined conformational alterations of rhodopsin to distinct molecular alterations in the protein backbone. An obvious place to begin this line of inquiry is at the critical metarhodopsin I to metarhodopsin II transformation. The question asked here is whether the deprotonation at the Schiff base is obligate in the activation of the G protein as it is in the usual model for the formation of metarhodopsin II. The studies reported here were, of course, critically dependent on the preparation of a highly purified active-site monomethylated rhodopsin (AMPM-Rh). Active-site monomethylated rhodopsin (AMPM-Rh) was prepared by the route shown in Scheme I (10). Since there was no way of selectively methylating the active-site lysine, the process was begun by permethylating the 10 non-active-site lysines of rhodopsin with formaldehyde and pyridine borane (10). Fortunately, this modification did not affect the bleaching of this protein and its ability to activate the G protein when compared to unmodified rhodopsin (Fig. 2 and Table 1). Bleaching of PM-Rh and its reductive methylation led to the formation of monomethylated active-site lysine-derivatized opsin in addition to the dimethylated derivative and bleached PM-Rh (Scheme I) (10). It was critical to remove virtually all of the unreacted non-active-site methylated bleached PM-Rh before proceeding with the biochemical studies. It was found that treatment with OPA, which can only react with primary amines, selectively reacted with the bleached PM-Rh to form an unregenerable fluorescent adduct as expected (Fig. 4) (10). Under conditions where there was less than a few percent bleached PM-Rh remaining, 11-*cis*-retinal was added to regenerate the new pigment AMPM-Rh. Careful hydroxylapatite chromatography of the mixture led to the formation of substantially purified AMPM-Rh with λ_{max} = 520 nm and A_{280}/A_{520} = 2.1 (Fig. 1).

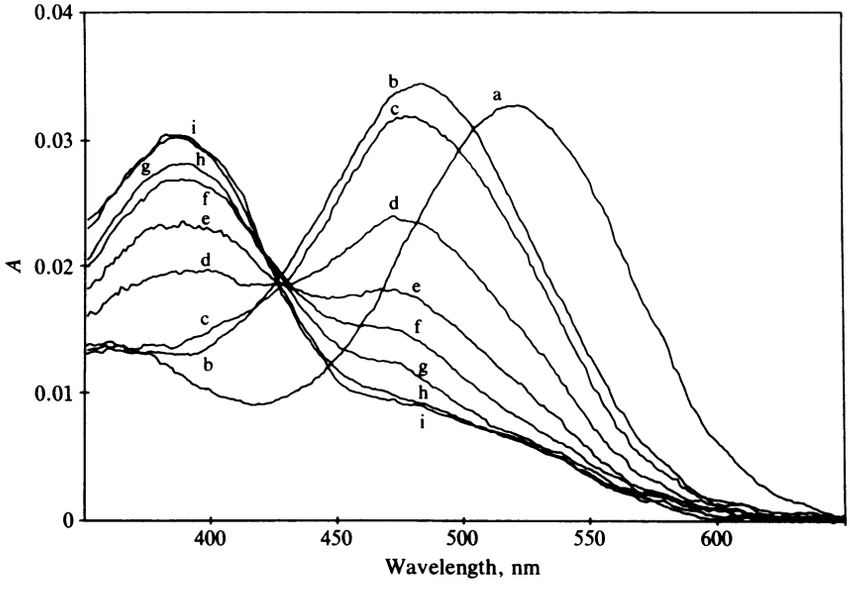


FIG. 3. The bleaching of AMPM-Rh. The spectra of AMPM-Rh were recorded before and after a short (10 sec) exposure to orange light during incubation at 4°C in the dark. Spectra are AMPM-Rh before the bleach (curve a), and spectra recorded at 1 min (curve b), 5 min (curve c), 20 min (curve d), 40 min (curve e), 1.5 hr (curve f), 4 hr (curve g), 24 hr (curve h), and 48 hr (curve i) after the bleach.

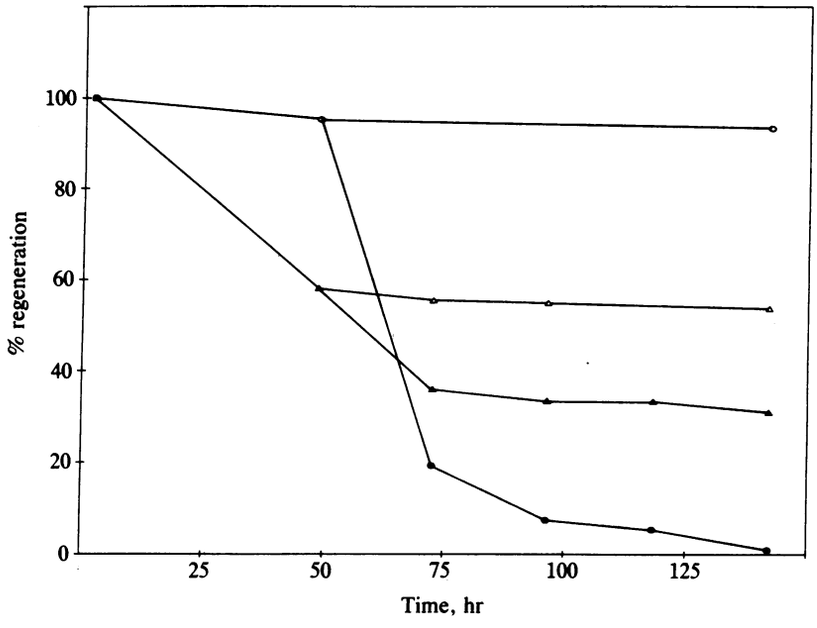


FIG. 4. The reaction of OPA with modified rhodopsins. The time course of the reaction of OPA with the active-site lysine of rhodopsin was measured at 4°C by determining the extent of regeneration with 11-*cis*-retinal. A pool of bleached PM-Rh was partially methylated (0–48 hr) at the active-site lysine and either incubated at 4°C with no further reaction (△) or reacted with OPA (0.8 mg/ml) and mercaptoethanol (15 mM) (▲) (48–142 hr). A second pool of bleached PM-Rh, which was not methylated at the active-site lysine, also reacted with OPA and lost essentially all measurable regenerability (●). Denaturation with time of bleached PM-Rh is also shown (○).

The bleaching behavior of AMPM-Rh proved to be exceptional. Rather than bleaching to form metarhodopsin II and then III, as does PM-Rh (Fig. 2), the bleaching behavior of the pigment was consistent with the formation of a relatively stable metarhodopsin I-like intermediate with a λ_{max} of ≈485 nm (Fig. 3). The λ_{max} of this intermediate is bathochromically shifted from the metarhodopsin I generated from rhodopsin, as AMPM-Rh is shifted from PM-Rh (10). It is important to stress that other than by fulfilling spectroscopic criteria, we cannot definitively demonstrate that this intermediate is indeed metarhodopsin I-like. It will be of great interest to determine the photochemical history of this intermediate and the precise kinetics of its formation. However, given that overwhelming evidence supports the view that deprotonation of the Schiff base occurs at metarhodopsin II, the latter result shown in Fig. 3 is not unexpected. Of further interest here is the fact that a metarhodopsin III-like intermediate does not appear during the photolysis of AMPM-Rh, which requires that this intermediate can only arise sequentially from metarhodopsin II.

The spectroscopic results reported for AMPM-Rh above are, as mentioned above, consistent with the formation of a long-lived metarhodopsin I-like intermediate. That this intermediate is relatively stable is of great interest. The half-life of metarhodopsin I at 20°C in the detergent dodecyldimethylamine oxide was measured to be 0.05 msec (17). The half-life of the metarhodopsin I-like state of AMPM-Rh in detergent is in the half-hour range without the formation of any detectable metarhodopsin II-like intermediate (Fig. 3). Therefore, monomethylation of the active-site lysine apparently has increased the longevity of this intermediate by $>10^6$.

From the spectroscopic results reported here and the previously published work on the biochemistry of metarhodopsin II (3), it would be predicted that photolysis of AMPM-Rh in the presence of the G protein should not lead to activation of the latter. Fortunately, this prediction could be tested, since PM-Rh was as active as unmodified rhodopsin with regard to activation of the G protein (Table 1). Indeed, photolysis of AMPM-Rh in the presence of the G protein did not lead to measurable activation of the latter as determined by its GTPase activity. Since G protein activation is the only assay for the biochemically active form of rhodopsin, it is clear that photolyzed AMPM-Rh cannot achieve this state. Thus, not only must the Schiff base of rhodopsin apparently be protonated for visual transduction to occur, the proton must also be lost. The structural and mechanistic consequences of this deprotonation remain to be determined. Our experiments confirm the view held from spectroscopic studies that the Schiff base proton is transferred during the metarhodopsin I to metarhodopsin II transition. Furthermore, they demonstrate that this proton loss is obligate in the formation of the activated biochemically competent form of rhodopsin. Thus, the experiments reported here establish a structural link between the formation of metarhodopsin II and that form of rhodopsin capable of activating the G protein.

We gratefully acknowledge the generous gift of 11-*cis*-retinal from Dr. Peter Sorter of Hoffmann–La Roche. This work was supported by U.S. Public Health Service Research Grant EY 03624 from the National Institutes of Health.

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