Articles

Unbleachable Rhodopsin with an 11-*cis*-Locked Eight-Membered Ring Retinal: The Visual Transduction Process†

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ABSTRACT: Visual transduction occurs through photorhodopsin, the primary photoproduct of rhodopsin, which relaxes to bathorhodopsin and a series of other intermediates until it reaches the metarhodopsin II stage, upon which the enzymatic cascade leading to vision is activated. Despite advances in areas related to visual transduction, the triggering process itself, a key problem in the chemistry of rhodopsin, has remained unsolved. In order to clarify the extent of involvement of the chromophoric excited state versus the 11-*cis* to *trans* isomerization, and as an extension of past studies with 11-*cis*-locked seven-membered ring rhodopsin (Rh7), 11-*cis* eight- and nine-membered ring retinal analogs, ret8 and ret9, respectively, have been synthesized. The bulkiness of the tetramethylene bridge in ret8 led to numerous unexpected obstacles in attempts to reconstitute a ret8-containing rhodopsin (Rh8) embedded in lipid bilayer membranes. These obstacles were solved by using methylated rhodopsin which gave MeRh8 containing 11-*cis*-ret8 as its chromophore. MeRh8 exhibited UV–vis and CD spectra very similar to those of native rhodopsin (Rh); furthermore, the quantum efficiency of photorhodopsin formation was comparable to that of Rh. Flash photolytic studies of Rh8 and other ring analogs [Mizukami, T., Kandori, H., Shichida, Y., Chen, A.-H., Derguini, F., Caldwell, C. G., Bigge, C. F., Nakanishi, K., & Yoshizawa, T. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4072–4076] coupled with the present enzymatic studies with MeRh8 and a series of dihydro-rhodopsins have led to the conclusion that (i) charge translocation in the excited state does occur; however, (ii) full *cis–trans* isomerization around 11-ene involving the entire polyene moiety is required for efficient transduction to occur. Repeated attempts to incorporate ret9 into opsin have as yet not been successful.

The visual pigment rhodopsins are one of the most fully investigated group of G-protein-coupled membrane-bound proteins. The best studied bovine rhodopsin (Rh), λ_{max} 500 nm, is a single polypeptide consisting of 348 amino acids folded into seven membrane-spanning α-helices; its chromophore, the 11-*cis* isomer **2** of *all-trans*-retinal **1**, is bound to Lys-296 via a protonated Schiff base (SBH^+) **3** (see Table I). Absorption of a photon by the chromophore leads to 11-*cis* to *all-trans* isomerization which initiates a series of conformational changes in Rh. The primary photoproduct of Rh excitation is now considered to be photorhodopsin, λ_{max} 580 nm, discovered by Shichida et al. (1984); it is formed in less than 13 fs (Schoenlein et al., 1991) and is considered to have a highly distorted 11-*trans* double bond. Photo-Rh thermally decays to bathorhodopsin, λ_{max} 543 nm,[1] which also has a distorted *trans*-ene but less distorted than in photo-Rh. After thermal relaxation through several further intermediates, Rh is converted into the key intermediate meta-Rh II, λ_{max} 380 nm (0 °C), an unprotonated Schiff base. At this stage, meta II binds to transducin, a member of the G-protein superfamily, thus activating the enzymatic cascade leading to visual transduction. The steps of single photon absorption → transducin activation → phosphodiesterase (PDE) activation results in hydrolysis of 10^5 molecules of cGMP to GMP, upon which meta II is phosphorylated by rhodopsin kinase and becomes inactivated (Stryer, 1991; Hargrave & McDowell, 1992; Khorana, 1992; Nathans, 1992). The OS (opsin shift) in Table I stands for the difference, expressed in cm^{-1}, between butyl amine protonated Schiff base (SBH^+) **4**, 440 nm (22 727 cm^{-1}) (measured in MeOH) and pigment λ_{max}, i.e., the OS for Rh **3** (Table 1) λ_{max} 500 nm (20 000 cm^{-1}) is ca. 2700 (Honig et al., 1979; Nakanishi et al., 1980). The OS gives a measure of the influence of the binding site on the maxima of pigments.

It is established that *cis–trans* isomerization occurs during the process of visual excitation (Wald, 1968). However, the more subtle aspects involving the triggering process of transduction have remained unsolved. A theoretical "sudden polarization" model involving excited state polarization of the polyene system has been proposed for the mechanism of visual triggering (Salem & Brukmann, 1975; Salem, 1979). Recent model exact calculations have also shown that a large charge separation, indeed, occurs in the excited state of substituted polyenes (Albert & Ramasesha, 1990). The excited state charge redistribution models postulate that, during isomerization, as the distortion angle around the 11-ene approaches 90°, a highly polar excited state appears and that this triggers the conformational changes in rhodopsin leading to transducin activation. In fact, determinations of dipole moments in the excited states of *all-trans*-retinal SBH^+ (Mathies & Stryer, 1976), bacteriorhodopsin (Birge & Zhang, 1990), and pK_as of 11-*cis* and *all-trans*-retinal SBH^+ in the ground and excited states (Schaffer et al., 1975) do demonstrate an increased polarity in the excited state. However, the extent to which

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[1] The conventional 543 nm value for bathorhodopsin is from measurements at −140 °C (Yoshizawa & Wald, 1963). The 569 nm value is the recently estimated maximum from flash photolysis (Mizukami et al., 1993).

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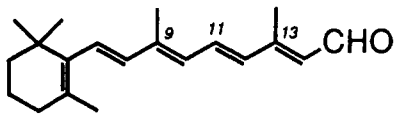
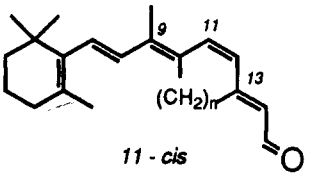
Table 1: Retinal Analog Structures and Spectroscopic Data

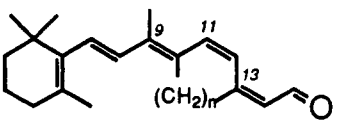


1 *All - trans retinal*



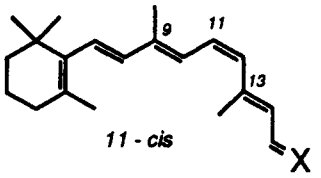
11 - cis

5a n = 1 **8a** n = 4
6a n = 2 **9a** n = 5
7a n = 3



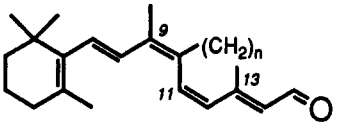
11, 13 - di-cis

5b n = 1 **7b** n = 3
6b n = 2 **8b** n = 4



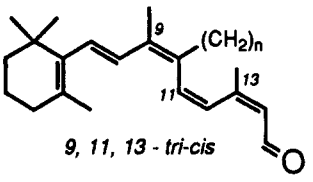
11 - cis

2 X = O
3 X = N⁺H-Opsin
4 X = N⁺H-*n*-Butyl



9, 11 - di-cis

5c n = 1 **7c** n = 3
6c n = 2 **8c** n = 4



9, 11, 13 - tri-cis

6d n = 2 **8d** n = 4
7d n = 3

	5a	5b	5c	6a	6b	6c	6d	7a	7b	7c	7d	8a	8b	8c	8d	9a
$\lambda_{\alpha\text{-band}}$ (nm)[a]	405	392	402	374	369	366	360	376	371	371	366	346	337	350	335	245 (s), 330 (s)
$\lambda_{\beta\text{-band}}$ (nm)[a]												276	286	275	278	281
A_α/A_β[a]												0.76	1.21	0.57	0.78	
SB (nm)[b]	353			368	362	360	354	355	351	351	346	285, 335	318			285
SBH^+	464			449			451	447	436	441	430	315, 435				324
pigment (nm)[c]	495			510			494	496[e]	488	489	483	502				no pigment
OS (cm^{-1})[d]	1350			2660			1930	2210	2440	2230	2550	3070				

[a] Absorption maxima of α-and β-bands measured in ethanol (**5**), hexane (**6**, **8**, **9**), or methanol (**7**) and the corresponding A value ratios. [b] λ_{max} of the *n*-butylamine Schiff base measured in methanol and that of the protonated form with a methanolic solution of HCl. [c] λ_{max} of rhodopsin analogs measured in digitonin (**5**) and nonylglucoside (**6**, **7**, **8**). [d] Opsin shift, defined as the difference (expressed in cm^{-1}) between the λ_{max} of the SBH^+ of the chromophore and that of the corresponding pigment. [e] Remeasured value. **5a–c**, Ito et al. (1982), Fukada et al. (1984); **6a–d**, De Grip et al. (1990); **7a–d**, Akita et al. (1980). Except for literature data, all absorption spectra were recorded on a Perkin-Elmer Lambda 4B UV–vis spectrophotometer.

the excited state polarization is actually involved in the visual transduction process remained unknown.

Since Wald's pioneering work (Hubbard & Wald, 1952), rhodopsin analog studies have been invaluable in clarifying various aspects of the protein–chromophore interactions in both the ground state and/or during bleaching (Derguini & Nakanishi, 1986; Ganter et al., 1989; Liu et al., 1984; Renk & Crouch, 1989; Einterz et al., 1990; Randall et al., 1990; Ottolenghi & Sheves, 1989). Among these, a series of retinal analogs, **5**–**9** (ret5–ret9) (Table 1), in which the 11-ene is locked into its 11-*cis* configuration through methylene chains bridging across C-10 and C-13 have played critical roles in investigating the involvement of *cis–trans* isomerization in the formation of bathorhodopsin and subsequent rhodopsin activation (Akita et al., 1980; Fukada et al., 1984; Nakanishi, 1985; De Grip et al., 1990; Bhattacharya et al., 1992; Mizukami et al., 1993).

The first member of this series, 11-*cis*-ret7 **7a**, readily forms a pigment (Rh7, 496 nm) (Akita et al., 1980), which was totally stable to light and did not lead to visual transduction, neither *in vitro* nor *in vivo* (Zankel et al., 1990; Crouch et al., 1984). 11-*cis*-ret5 **5a** forms a pigment (Rh5, 495 nm) only in digitonin solution at a very slow rate (Fukada et al., 1984); again Rh5 is nonfunctional.

Attempts to make a light- and thermally-stable Rh analog to facilitate rhodopsin crystallization led to studies on Rh6 by two groups (De Grip et al., 1990; Bhattacharya et al., 1992). Similar to native Rh, it exhibited two maxima at 340 and 513 nm; however, unlike native Rh, the 513-nm maximum is not bleached upon irradiation but instead undergoes a blue-shift to 494 nm upon prolonged illumination.

We turned our attention to the next higher retinal analogs **8** and **9** locked by eight- and nine-membered rings (Caldwell et al., 1993) hoping that the more flexible rings might lead to the room temperature isolation of an intermediate corresponding to batho-Rh containing a *trans*-11-ene within its eight-membered ring (Nakanishi, 1985; Balogh-Nair & Nakanishi, 1990). Furthermore, enzymatic activity studies with Rh8 and Rh9 would help clarify the very subtle point, i.e., the interrelation between *cis–trans* isomerization and the excited state charge redistribution, and its bearing on visual transduction.

Insurmountable difficulties were encountered in preparing Rh8 pigment. This was mainly caused by two factors: the instability of ret8 during incubation, and formation of a species absorbing at 425 nm which was found to be totally unrelated to rhodopsin. The problem was finally solved by using methylated rhodopsin (MeRh) (Calhoon et al., 1985; Van der Steen et al., 1989) in which the exposed free amino groups of rhodopsin and lipid were methylated while leaving the crucial terminal amino group of Lys-296 within the α-helix unmethylated.

In the following we discuss these difficulties, the successful preparation of MeRh8 and its spectral data, the nature of the contaminant absorbing at 425 nm, the results of assays with the two enzyme systems, phosphodiesterase and rhodopsin kinase, and the differences in properties of rhodopsins containing 11-*cis*-locked retinals of different ring sizes.

EXPERIMENTAL PROCEDURES

Materials. Digitonin and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from

Aldrich. Concanavalin A-Sepharose 4B, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), methyl α-D-mannopyranoside (methyl-α-mannoside), protease inhibitors, L-α-phosphatidylethanolamine (PE) from bovine brain, L-α-phosphatidylcholine (PC) from frozen egg yolk, *n*-nonyl β-D-glucopyranoside (nonylglucose), and 3-[(3-cholamidopropyl)dimethylamino]-2-hydroxy-1-propanesulfonate (CHAPSO) were purchased from Sigma Chemical Co. Bovine retinas were purchased from W. Lawson Co. (Lincoln, NE); frozen retinas were shipped on dry ice and fresh retinas on ice within 24 h.

Preparation of Bovine Rod Outer Segments and Extraction of Enzymes. Isolation of rod outer segments was carried out at 4 °C under dim red light (>680 nm) following a standard procedure (Papermaster, 1982) with minor modifications. Briefly, sucrose solutions were prepared with isotonic buffer (buffer A: 10 mM Tris, pH 8.0, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol) which allows water-soluble enzymes such as transducin, PDE, and kinase to adhere to the membrane during isolation of ROS. Retinas (100) were gently shaken for 1 min in 80 mL of 35% (w/v) sucrose solution and then centrifuged for 10 min in a Sorvall SS-34 rotor at 5000 rpm. The pellets were resuspended in 35% sucrose solution (80 mL) and similarly centrifuged. The two supernatants were pooled and diluted to 26% sucrose with buffer A. After centrifugation in a Sorvall SS-34 rotor at 15 000 rpm for 30 min, the crude ROS pellets were resuspended gently with a pipet in 26% sucrose and loaded onto a 26–35% discontinuous gradient. Centrifugation in a Beckman swing bucket rotor (SW 28) at 23 000 rpm for 45 min resulted in a thin layer of purified ROS at the interface of the discontinuous gradient. It was collected with a syringe fitted with a flat tipped needle and then washed twice with buffer A. Enzymes (transducin, PDE, kinase) were extracted twice with 25 mL of hypotonic buffer (buffer B: 10 mM Tris, pH 8.0, 2 mM EDTA, 2mM dithiothreitol, 2 mg of each soybean trypsin inhibitor, aprotinin, benzamidine, leupeptin, and pepstatin A per 100 mL of buffer); after 2 h at 0 °C the mixture was centrifugated, and the supernatant was pooled out and concentrated by means of an Amicon Centriprep-30 concentrator to 1–2 mL. After being mixed with an equal volume of glycerol, the enzyme extract, from frozen or fresh retinas, could be stored up to 2 weeks at −20 °C without loss of activity.

The apoprotein (opsin) was obtained from purified ROS after resuspension in buffer C (67 mM Na_2HPO_4/NaH_2PO_4, pH 7.0) containing 100 mM hydroxylamine (final concentration of 0.5 OD ROS/mL) and bleaching under room light for about 2 h, at ice-bath temperature. Excess hydroxylamine was removed by washing with buffer C. The ROS opsin was resuspended in 10 mM HEPES buffer (pH 7.0) or solubilized in 10 mM CHAPSO/10 mM HEPES (pH 7.0) for binding. The bleaching of reconstituted rhodopsin vesicles and methylated ROS were carried out similarly.

11-cis-Locked Eight-Membered Ring Retinal Analogs. The synthesis of 11-*cis*-locked eight-membered ring retinal analogs (ret8) has been reported recently (Caldwell et al., 1993). Since these retinal analogs undergo rapid isomerization even when kept in solution at −78 °C under argon, all ret8 chromophores had to be purified by HPLC prior to use.

Preparation and Purification of Rh8. ROS with an A_{278}/A_{498} ratio less then 2.5 was used for binding; they were further washed, prior to bleaching, with 8 M urea (Shichi & Somers, 1978) to strip any remaining enzymes from the ROS. Addition of a 10-fold OD excess of 11-*cis*-ret8 to opsin in 10 mM CHAPSO/10 mM HEPES (pH 7.0) formed two pigments absorbing at 425 nm (P425) and 502 nm (P502). Incubation was first carried out at room temperature for 3–5 h and then continued at 4 °C overnight. These conditions were necessary to minimize decomposition of P502, which occurs upon prolonged incubation at room temperature. Purification of crude Rh8 was performed by chromatography on Con A-Sepharose column (De Grip, 1982) at 4 °C under room light, with a detection wavelength of 280 nm. Excess 11-*cis*-ret8 and the chromophore released from the bleachable P425 contaminant were first eluted with 2% digitonin in buffer D (20 mM PIPES, 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 6.0 mM EDTA, 1 mM dithiothreitol, pH 6.5), followed by P502 eluted with 0.1 M methyl-α-mannoside in 2% digitonin. The A_{278}/A_{502} ratio of Rh8 thus obtained was ca. 4.3. Alternatively, 20 mM nonylglucose was used instead of digitonin, when reconstitution of Rh8 vesicles was necessary. CHAPSO failed to wash rhodopsin off the column.

Preparation and Purification of Methylated Rh8 (MeRh8). Methylation of ROS was carried out according to literature procedures (Calhoon & Rando, 1985; Van der Steen et al., 1989). Under these conditions all exposed amino groups, namely, nonactive lysine residues on rhodopsin, lipid amino groups contained in the disk membrane, and the lysine amino groups on the surface of any remaining water soluble enzymes such as transducin, PDE, and kinase, are dimethylated. The resulting methylated ROS are composed of methylated rhodopsin in vesicles consisting of phospholipids modified during the methylation procedure. After bleaching and washing with buffer C, regeneration with 11-*cis*-ret8 was carried out either in 10 mM HEPES (pH 7.0) or in 10 mM CHAPSO/10 mM HEPES (pH 7.0), using 5-fold OD excess chromophore in ethanolic solution, limiting ethanol to less than 3% by volume. Incubation proceeded under argon for 3–5 h at room temperature and then at 4 °C overnight. Chromatographic purification of MeRh8 was carried out, in the dark, following the general procedure described for the purification of Rh8. The A_{278}/A_{502} ratio of purified MeRh8 was 2.6. When MeRh8 was used for CD measurements, elution was carried out with 2% digitonin in 0.1 M K_2HPO_4/KH_2PO_4, pH 7.0.

Measurement of CD Spectrum of MeRh8. Pure MeRh8 of highest homogeneity in digitonin was chosen for CD measurements to minimize the interference of the protein absorbance at 280 nm on the β-band of the pigment CD curve (Figure 7b). CD spectra were measured on a Jasco J-720 spectropolarimeter with a 1-cm cell from 270 to 570 nm. After one measurement, the cell was sealed and the pigment was thermally bleached at room temperature in order to record the baseline and minimize possible contributions from the protein and/or impurities from digitonin.

Low Temperature UV–Vis Measurement. Pure MeRh8 in 2% digitonin from Con A-Sepharose column was concentrated by centrifugation (Centriprep-30, Aminco) to about 6 OD/mL. A 1/1 (v/v) mixture of cold glycerol and concentrated MeRh8 was added to the cell of the low temperature UV–vis set-up (Perkin Elmer IF 320) to make a ca. 1-mm thick film. Low temperature was achieved using a model SC compressor from CTI-Cryogenics regulated by a model 4025 cryogenic thermometer/controller. The samples were irradiated with a Sylvania tungsten halogen lamp using an interference filter of 460 ± 10 nm cut off for Rh and 480 ± 10 nm for MeRh8.

Phosphodiesterase Assay of MeRh and MeRh8. PDE assays (Liebman & Evanczsuk, 1982) on MeRh and MeRh8 were carried out using the same Me-opsin (bleached until chromophore is absent in UV spectra, i.e., no absorbance at

498 nm), which constituted the negative control. Photoactivated Rh binds to transducin which in turn activates PDE. PDE hydrolyses cGMP leading to the release of a proton which is continuously monitored in real time as a decrease in pH. The difference in pH values before and after irradiation is plotted on the vertical axis as ΔpH. In complete darkness, the pigment is added to isotonic buffer (10 mM Tris, 60 mM KCl, 30 mM NaCl, 3 mM Dextrose, 2 mM $MgCl_2$, 1 mM DTT, pH 8) to a final concentration of 6 μM and a final volume of 1 mL after addition of all components. An aliquot of the crude enzyme extract is added to approximate the natural ratio. After incubation for 10 min, GTP is added to a final concentration of 500 μM followed by cGMP (2 mM final concentration) with an initial pH of 7.9. The dark activity is monitored continuously for ~1 min by use of a pH electrode (Fisher) coupled through a pH meter (Orion 811) to a servo chart recorder (Chessell 321). The mixture is exposed to a suitably attenuated flash of light (xenon flash Metz-Mecablitz 45 CL), and then the light activity is followed for ~2 min. The relative percentages of light activities shown in Figure 6 are calculated as follows: [(light activity of pigment–dark activity of pigment)/(light activity of MeRh–dark activity of MeRh)] × 100%. The error ranges in the percent activity are derived from four runs.

Rhodopsin Kinase Assays. The kinase assay used was a modified literature procedure (Bhattacharya et al., 1992). Pigment or opsin (100 μL, 1.5 OD/mL), 40 μL of 10 mM Tris-HCl buffer (pH 7.0), 40 μL of 8 mM ATP/40 mCi-[γ-^{32}P]ATP/10 mM $MgCl_2$/10 mM HEPES (pH 7.0), and 20 μL of enzyme extract were mixed in a 1.5-mL Eppendorf tube. The sample was incubated in the dark at 23 °C for 15 min followed by irradiation with daylight for 15 min and then taken back to the dark and allowed to incubate for 10 additional min. Trichloroacetic acid [100 μL of a 10% (v/v) solution] was added to the mixture to quench any enzymatic reaction. A control sample was run in parallel, without irradiation. The mixture was centrifuged at 1400 rpm for 2 min, and the supernatent was discarded. After being washed with 2 × 0.5 mL of water, the pellet was dissolved in 200 μL of buffer/detergent (67 mM solution of Tris-HCl, 10% glycerol, 10% SDS, 5.3% β-mercaptoethanol, and 0.1% bromophenol blue, pH 6.8) and kept at 0 to −20 °C for 10 h before it was applied to electrophoresis gel.

Electrophoresis. The detergent solution (100 μL) was loaded to 12% polyacrylamide stacking gel (100 V), and the electrophoresis was run in 2 h. After being stained with Coomassie blue, the gel was dried and then exposed to a Kodak X-ray film.

RESULTS

Binding of 11-Cis-Locked Eight-Membered Ring Retinal Analogs with Opsin. Four isomers of ret8 have been synthesized, namely, 11-*cis*- **8a**, 11,13-*dicis*- **8b**, 9,11-*dicis*- **8c**, and 9,11,13-*tricis*-ret8 **8d**. Unlike 11-*cis*-retinal, which exhibits a major UV absorption band at 365 nm (α-band, ε 26 360 in hexane) and a minor band at 250 nm (β-band or "*cis*-band") with an A_α/A_β ratio of 1.85, all ret8 isomers exhibit two major bands near 340 and 280 nm with the α-band markedly shifted to the blue and the β-band to the red, with A_α/A_β values ranging from 0.57 to 1.21 (Table 1), suggesting a less planar conformation.

Incubation of 11-*cis*-ret8 with bovine opsin, either suspended in buffer or solubilized in octyl glucoside, yielded only a single pigment absorbing at 425 nm (P425) under various conditions; no pigment with higher λ_{max} was observed. However, an additional pigment absorbing at 502 nm (P502) was formed when fresh opsin in 10 mM CHAPSO/10 mM HEPES (pH 7.0) was used (Nakanishi, 1985; Balogh-Nair & Nakanishi, 1990; Chen, 1990). As shown in Figure 1a, at 37 °C, P502 reached its maximum in 30 min and then decreased gradually with time. After 14 h, P502 disappeared, while P425 remained. In contrast, the three other 11-*cis*-ret8 isomers formed only a single pigment, each between 410 and 430 nm, throughout the incubation period (Figure 1b–d). Unlike Rh5 and Rh6, Rh8 (P502) was completely stable to light. Namely, irradiation of P502 with >510-nm light at ice-bath temperature for 3 h caused neither bleaching nor changes in the λ_{max} and absorbance values of the pigment. On the contrary, P425 and the pigments regenerated from the *di*- and *tricis*-ret8 bleach and partially regrow in the dark. Incubation of 11-*cis*-ret8 with opsin in CHAPSO at 22 °C led to similar results. As shown in Figure 2a the λ_{max} of the chromophore around 350 nm shifts with time, reflecting isomerization of the 11-*cis*-ret8 and/or decomposition.

In contrast, 11-*cis*-ret8 **8a** was smoothly converted to MeRh8 when added to methylated opsin at room temperature in CHAPSO (Figure 2b). The decrease in the 290- and 370-nm

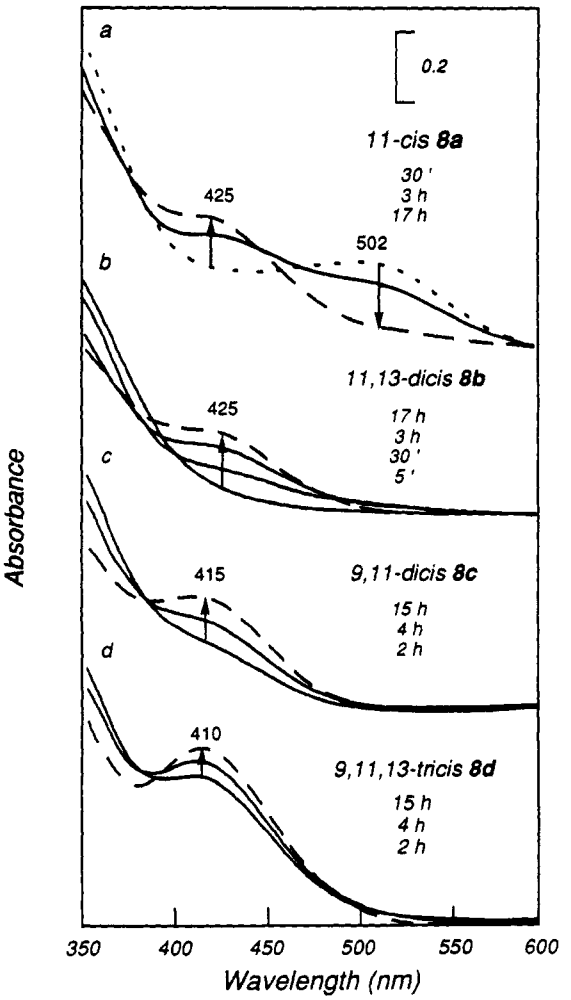


FIGURE 1: Interaction of ret8 **8a–d** with opsin in CHAPSO at 37 °C: incubation was carried out by addition of the chromophore to 1 mL of opsin solubilized in 10 mM CHAPSO/10 mM HEPES (pH 7.0; 0.5 OD opsin/mL). (a) After addition of 1 OD 11-*cis* **8a**, a peak around 500 nm began to grow immediately, reached its maximum in 30 min, and then decreased; P425 grew gradually and reached its maximun after 14 h. (b) Incubation of 1 OD 11,13-*dicis* **8b** with 1 mL of opsin for 17 h resulted only in the formation of P425. (c) Incubation of 2 OD 9,11-*dicis* **8c** or 2 OD 9,11,13-*tricis* **8d** (d) with 1 mL of opsin for 15 h led to the sole formation of P415.

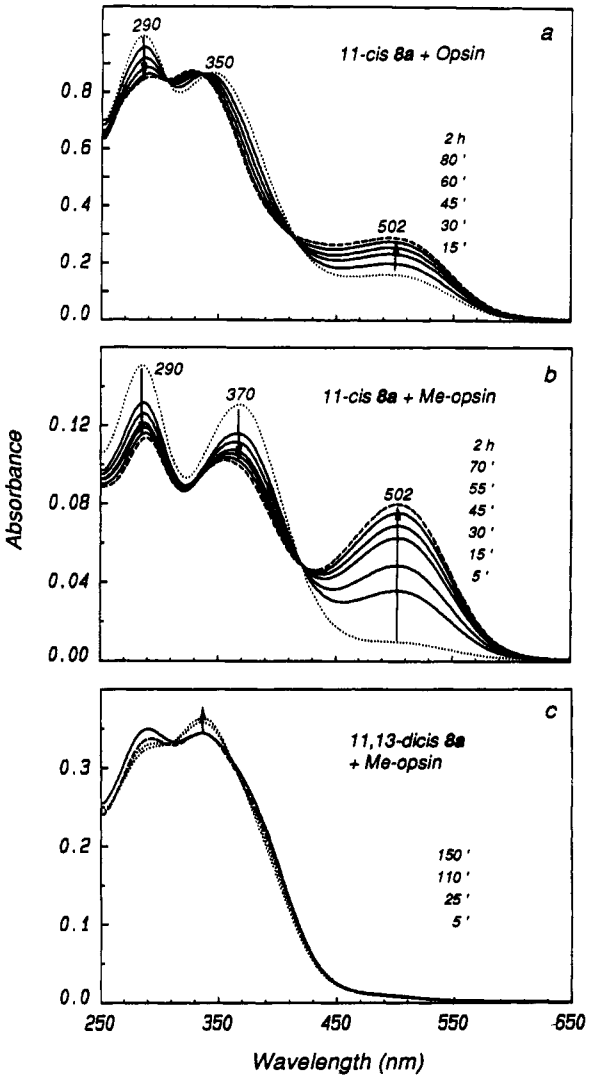


FIGURE 2: Binding of 11-*cis* **8a** and 11,13-*dicis* **8b** with opsin or Me-opsin in CHAPSO at 22 °C: opsin was solubilized in 10 mM CHAPSO/10 mM HEPES (pH 7.0). (a) 0.86 OD 11-*cis* **8a** was added to 1 mL of opsin (0.71 OD/mL), and a broad band developed around 500 nm accompanied with a decrease in absorbance and a shift in the λ_{max} of the chromophore, reflecting both consumption and isomerizition of the chromophore. (b) Addition of 0.14 OD 11-*cis* **8a** to 1 mL of a 0.56 OD/mL solution of Me-opsin led to the sole formation of P502, accompanied by decrease in absorption bands of the chromophore; a clear isosbestic point is seen. (c) Incubation of 0.38 OD 11,13-*dicis* **8b** with 1 mL of Me-opsin (0.39 OD/mL) for 2.5 h resulted in the formation of neither P502 nor P425.

peaks of the chromophore resulted in the formation of a peak at 502 nm (MeRh8 or P502) and a clear isosbestic point. P502 reached its maximum within 2 h and then began to deteriorate slowly as it did during the incubation of 11-*cis*-ret8 with natural opsin in CHAPSO, but P425 was not detected over the entire incubation period (curves not shown). These results strongly suggest that P425 and the species absorbing at 410–430 nm were side products, and that their formation was the result of interactions between the 11-*cis*-ret8 analogs and random amino groups, i.e., amino groups from phosphatidylethanolamine and phosphatidylserine in the disk membranes, lysine residues on the opsin and on the surface of water soluble enzymes such as transducin, PDE, and kinase, etc. In support of this hypothesis, Figure 2c shows that incubation of 11,13-*dicis*-ret8 **8b** with methylated opsin in CHAPSO generates neither P502 nor P425, while the same experiment with unmethylated opsin led to P425 (Figure 1b).

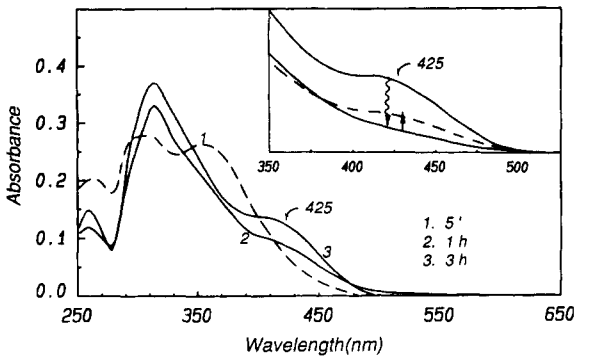


FIGURE 3: Interaction of 11-*cis* **8a** with random amino groups. 11-*cis* **8a** (0.29 OD) was added to 0.88 OD of rhodopsin solubilized in 1 mL of 10 mM CHAPSO/10 mM HEPES (pH 7.0). P425 formed within 3 h (curves 2 and 3), bleached when irradiated with 410 ● 10 nm light, and regrew after 60 min in the dark (inset). The same rhodopsin solution was used as reference.

Additional evidence supporting the above hypothesis was the formation within 3 h of P425 during the incubation of 11-*cis*-ret8 with an enzyme extract from crude ROS. In this case also, P425 bleached completely under irradiation with >420-nm light for 10 min and then underwent regrowth after standing in the dark for 100 min. Similarly, incubation of 11-*cis*-ret8 **8a** with unbleached ROS in CHAPSO led to formation within 3 h of a species (Figure 3) which was light-sensitive P425 (Figure 3 insert). The same side product was formed when 11-*cis*-ret8 was added to reconstituted opsin in phosphatidylethanolamine vesicles.

From these experiments we conclude that (i) only 11-*cis*-ret8 forms a rhodopsin pigment P502 and (ii) the geometry of the other ret8 analogs does not allow them to enter the binding site, thus leading to formation of the side product P425.

As shown in Figure 2b, P425 formation is avoided by using methylated opsin. Incubation of 11-*cis*-ret8 (5-fold OD excess) with bleached methylated ROS led to 0.183 OD of a single pigment, MeRh8 absorbing at 502 nm. Similarly, addition of 11-*cis*-retinal (5-fold OD excess) to the same opsin preparation yielded 0.258 OD of MeRh. However, addition of 11-*cis*-retinal to MeRH8 preparation resulted in less than 5% rhodopsin regeneration. From these yields and from the ϵ value of 40 600 for rhodopsin, the extinction coefficient of MeRh8 was estimated as being 30 400; similarly, an A_{278}/A_{502} ratio of 2.1 was estimated for MeRh8 based on the 1.6 value, reported for pure bovine rhodopsin (Hargrave, 1982). Formation of MeRh8 proceeds through a pseudo-first-order reaction. The bimolecular reaction constant of 13.4 M^{-1} s^{-1} was calculated by dividing the pseudo-first-order reaction constant by the concentration of chromophore. The reaction constants corresponding to Rh, Rh7, and Rh6 regeneration in 10 mM CHAPSO were estimated to be 850, 140, and 81 M^{-1} s^{-1}, respectively. In the latter experiments, a 5-fold OD excess of opsin instead of chromophore was used to slow down the rate of regeneration in order to carry out UV–vis measurements, and thus the bimolecular reaction constants were calculated by dividing the pseudo-first-order reaction constants by the concentration of opsin. As far as the formation rate is concerned, 10 mM CHAPSO was a much better system than 2% digitonin; in the latter, the bimolecular reaction constant of rhodopsin was only 43.3 M^{-1} s^{-1} (Wald & Brown, 1956; Cusanovich, 1982).

Stability of Rh8 to Heat and NH_2OH. Compared to Rh, Rh6, or Rh7, Rh8 is a much less stable pigment. It decomposed gradually in 20 mM nonylglucose at 22 °C, with a half-life

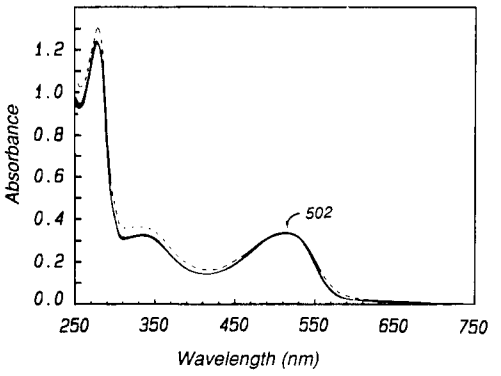


FIGURE 4: Low-temperature studies of MeRh8. Irradiation of MeRh8 with 480 ± 10 nm light for 1 h at 68, 100, 140, and 200 K successively resulted in no change in the UV–vis spectrum (one single solid curve), while at 240 K (dashed curve) the spectrum broadens due to temperature effect on the spectrum width.

of only 14 h. However, at 4 °C there was no visible decomposition up to 40 h, and a 1:1 (v/v) mixture of Rh8 in glycerol was stable at −20 °C for 6 months. The thermal lability of Rh8 accounts for its gradual decomposition after 30 min incubation at 37 °C (Figure 1a). Rh8 was also unstable toward hydroxylamine; when 1 M NH_2OH (pH 7.0) was added to Rh8 in 20 mM nonylglucose to a final concentration of 100 mM, about half of the pigment decomposed within 2 h in the dark at 4 °C.

Photochemistry of Rh8 and/or MeRh8. Low-temperature UV–vis measurements were carried out on MeRh8, since methylation causes no interference with the biophysical and biological properties of rhodopsin (Govindjee et al., 1988). UV–visible spectra were taken, at 68, 100, 140, 200, and 240 K, before and after irradiation of MeRh8 with 480 ± 10-nm light for 1 h. As shown in Figure 4, at temperatures ranging from 68 to 200 K, the spectrum does not change; when the temperature reaches 240 K, the entire spectrum (dotted curve) slightly broadens on both sides of the α-band, reflecting a common effect of temperature on the absorption band width. As a control, irradiation of rhodopsin for only 20 min at 68 K resulted in an equilibrium between rhodopsin, isorhodopsin, and bathorhodopsin, which thermally decayed to meta I at 240 K, meta II near 270 K, and meta III at 300 K (data not shown). In contrast to flash photolysis experiments which showed the formation of photo-Rh8 (585 nm) and batho-Rh8 (577 nm) species (Mizukami et al., 1993), the low-temperature studies could not trap any photointermediates of MeRh8.

CD Spectra of MeRh8 (P502) and P425. As shown in Figure 7b, MeRh8 exhibits a CD spectrum similar to that of Rh; namely, two positive CE bands are present, the α- and β-bands at 484 and 348 nm, respectively; in Rh they are at 490 and 336 nm. P425 did not show any CD band (data not shown).

Enzymatic Assays. Figure 5a shows a typical experimental curve for MeRh, MeRh8, and Me-opsin PDE assays. The curves for MeRh8 fall within the range of the negative control, and, therefore, it is concluded that MeRh8 cannot activate the visual transduction cascade. This result was corroborated by the rhodopsin kinase assays (Figure 6), which show clear 40-kDa rhodopsin bands for Rh (E*) and MeRh (I*) resulting from phosphorylation with $^{32}PO_4$ upon irradiation, whereas MeRh8 (G) and the control Me-opsin (J) remained inert.

Parallel PDE (Figure 5b) and kinase assays (Figure 6) were also performed under the same conditions with rhodopsins containing dihydroretinals (Arnaboldi et al., 1979; Koutalos et al., 1989; Takahashi et al., 1990). The general trends

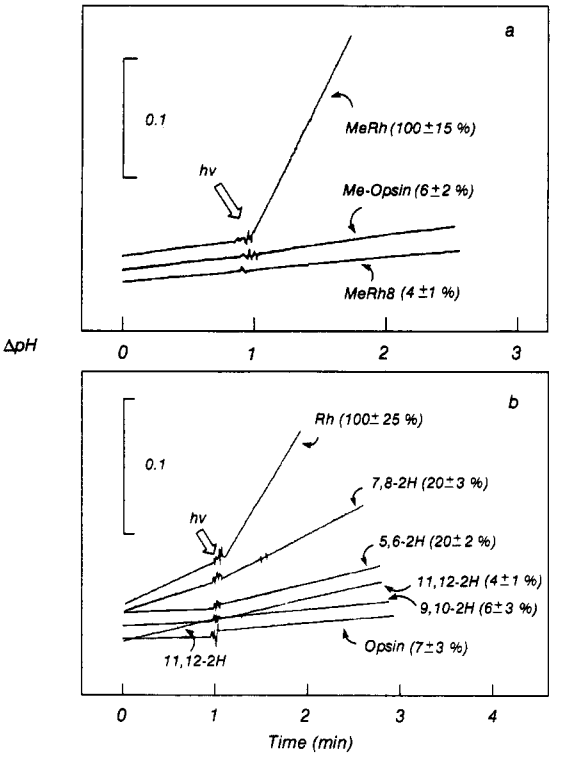


FIGURE 5: Phosphodiesterase assays. (a) PDE assay results for MeRh8 as compared to MeRh, both using the same Me-opsin, the negative control. MeRh8 activity falls within the range of the negative control and, therefore, MeRh8 cannot activate the visual transduction cascade. (b) PDE assay results for pigments reconstituted from dihydroretinals (2H). A xenon flash, with a measured spectral output corresponding to the absorption maxima of each pigment (5,6–2H, 473 nm; 7,8–2H, 428 nm; 9,10–2H, 336 nm; and 11,12–2H, 294 nm) was used for pigment activation; in addition for 11,12–2H, unfiltered 254-nm continuous irradiation did not affect the slope of the curve. A typical experiment curve for each pigment (one of three runs) is shown; the initial pH was 7.9. Pigments were generated from the same bleached opsin used for the negative control. The relative percentages of light activities are calculated as follows: [(light activity of pigment − dark activity of pigment)/(light activity of rhodopsin − dark activity of rhodopsin)] × 100%. The error ranges in the percent activity are derived from three runs. The error for Rh is the standard deviation from the average slope of the three runs.

indicate diminished activity upon saturation of the 5,6 and 7,8 double bonds; an earlier PDE assay with 7,8-dihydro pigment had also shown that it exhibited moderate activity (Calhoon & Rando, 1985). Saturation of the 9,10 and 11,12 double bonds leads to complete loss of activity.

DISCUSSION

The above results show that the various amino groups present in ROS lead to the formation of P425. Phosphatidylethanolamine and phosphatidylserine, which constitute 51–57% of the total phospholipids present in rhodopsin disk membranes (Shichi, 1983), are aligned in the lipid bilayer so that the free amino groups are directed toward the aqueous phase. When bleached ROS is suspended in buffer, these peripheral amino groups functioning as an amino wall completely prevent the entrance of ret8 **8a** (sterically far much bulkier than native 11-*cis*-retinal **2**) into the binding site; this leads to binding of ret8 **8a** with peripheral amino groups and formation of P425.

However, solubilization of bleached ROS in CHAPSO destroys the amino wall to form mixed phospholipid/CHAPSO micelles with opsins in the center. Because the native phospholipid/Rh ratio is about 62:1 (De Grip et al., 1983) in Rh disk membranes, a 1 OD opsin/CHAPSO mixture contains

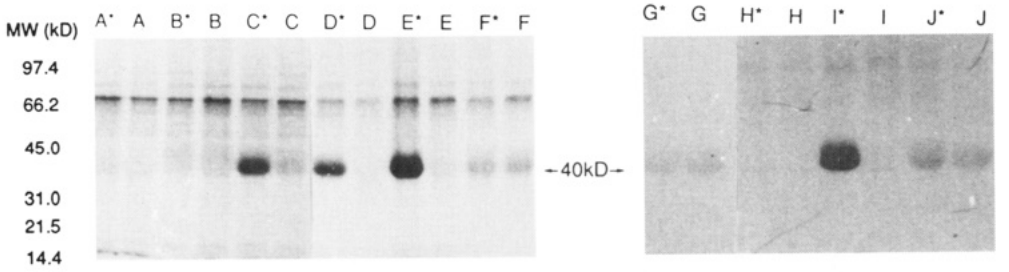


FIGURE 6: Rhodopsin kinase assays of pigments reconstituted from ret7, ret8, and 2H-retinals. Upon irradiation active pigments are phosphorylated with $^{32}PO_4$; a clear 40-kDa rhodopsin band is seen with Rh (E*), MeRh (I*), 5,6-2H-Rh (D*), and 7,8-2H-Rh (C*) while other protein analogs remained inert. Unirradiated controls are denoted by letters and irradiated samples by asterisked letters: A, 11,12-2H-Rh; B, 9,10-2H-Rh; C, 7,8-2H-Rh; D, 5,6-2H-Rh; E, Rh; F, opsin; G, MeRh8; H, MeRh7; L, MeRh; J, Me-opsin.

about 740 μM phospholipids with free amino groups. Compared to 10 mM CHAPSO molecules, phospholipids with free amino groups only comprise 7% of the lipid/CHAPSO mixed micelles; thus the probability for ret8 **8a** to enter the opsin binding site and form P502 is much greater than in suspension; however, P425 started to grow within 3 h.

Another rich source of free amino groups is provided by the lysine residues on the surface of water soluble enzymes present with ROS, i.e., PDE, transducin, kinase, etc. Thus the native PDE/Rh and tranducin/Rh molar ratios are 1~2:100 and 10:100, respectively (Pugh et al., 1986); since coexistence of these enzymes with ROS results in an increase of the A_{278}/A_{500} ratio, the higher this ratio, the lower the regeneration of P502 in 2% digitonin (Mizukami et al., 1993). The nine lysine residues on the interdiscal loops of opsin also interfere with the formation of P502. Namely, incubation of ret8 **8a** with Con A purified opsin in CHAPSO still produces a small amount of P425, however, within 73 h as compared to 3 h with crude bleached ROS (Figure 1a).

The specific nature of this "pigment" is unclear. Its λ_{max} of 425 nm is similar to the λ_{max} of 11-*cis*-ret8 SBH^+ (435 nm, Table I); however, its stability to hydroxylamine (data not shown) and its bleaching and regrowth in the presence of excess NH_2OH is not yet understood. Such blue-shifted pigments (λ_{max} 405–415 nm), stable to hydroxylamine, have been reported in the literature, when the retinal analogs failed to regenerate pigments (Liu & Asato, 1990). Our P425 results clearly show that random amino groups cause the formation of these artifacts and support the utilization of methylated opsin for difficult bindings. Indeed, the "pigment" absorbing at 410 nm formed during incubation of 11-*cis*-ret9 **9a** with natural opsin (Nakanishi, 1985; Balogh-Nair & Nakanishi, 1990) also was an artifact, since no pigment formed when Me-opsin was used. Similarly, methylated opsin was used to avoid interaction between retinoyl fluoride analogs with random amino groups during the formation of pigments containing an amide linkage between the chromophore and Lys-296 (Calhoon & Rando, 1985; Van der Steen et al., 1989).

Despite the difficulties encountered during the binding of 11-*cis*-ret8, the binding behavior of ret8 compared to that of ret5, ret6, ret7, and ret9 have led to a better understanding of many aspects of chromophore/opsin interaction. Of the four types of ring-locked pigment analogs Rh5–Rh8, Rh8 showed several interesting differences. All four isomers (11-*cis*, 11,13-*dicis*, 9,11-*dicis*, and 9,11,13-*tricis*) of ret6 and ret7 led to pigment formation, whereas only the 11-*cis*-ret8 **8a** formed a pigment. The bulky bridging methylene chain in ret8 makes it difficult to fullfil the criteria leading to binding. It appears that, with ret8, binding occurs only when double bonds are aligned as in native Rh, since of all ret8 isomers only 11-*cis*-ret8 yielded Rh8 which also shows UV–vis and CD spectra similar to those of native Rh (Figure 7a,b). It also shows that the opsin binding site is spatially lenient at the concave side of the bent 11-*cis*-retinal (Liu et al., 1984) so that, presumably with some expansion, it can accommodate the bulging tetramethylene bridge of 11-*cis*-ret8 **8a**. The much lower thermal stability of Rh8 and its instability toward hydroxylamine, compared to Rh6 and Rh7, can probably be ascribed to the expanded binding site, which results in exposure of the SBH^+. It should be noted that, despite repeated attempts, it has so far not been possible to incorporate the bulkier 11-*cis*-ret9 **9a** into opsin or Me-opsin, either in detergent or in suspension.

Although 11-*cis*-ret8 **8a** leads to a Rh8 having UV–vis and CD spectra similar to those of native Rh (Figure 7a,b), the UV of the aldehyde differs greatly from that of 11-*cis*-retinal: two intense absorption bands at 276 and 346 nm in **8a** (in hexane) *versus* a single major maximum at 365 nm in 11-*cis* retinal. In contrast, the seven-membered ring appears to simulate the nonplanar conformation of 11-*cis*-retinal around C-12/C-13 single bond, thus leading to similar maxima for 11-*cis*-ret7 **7a** (376 nm) and native 11-*cis*-retinal **2** (370 nm in methanol). Furthermore, the CD of the corresponding pigment Rh7, with two positive Cotton effects at 491 (α-band) and 333 nm (β-band), is similar to that of native Rh (Figure 7b); the two extrema have been assigned, respectively, to the twisted 12-*s*-*trans* conformation and the ring/chain nonplanarity (Fukuda et al., 1984; Ito et al., 1985).

In the case of ret5 and ret6, the rigid planar moiety from C9 to C14 forces the opsin to change its conformation in order to accommodate this planarity. The newly rearranged binding sites might only represent a state of local minimum energy, which might convert to another substable state under certain circumstance such as irradiation. Indeed, Fukada et al. (1984) observed that upon irradiation with >560-nm light, the λ_{max} of Rh5 shifts from 495 to 466 nm. Although HPLC analysis of the chromophore extracted from this new pigment P466 shows that it is still 11-*cis*-ret5 **5a**, the CD spectrum of P466 is totally different from that of the original pigment. The original P495 pigment only displays a β-CD band at 336 nm, whereas P466 only shows a single α-CD band at 466 nm. This was attributed to the rotation of C_8–C_9 single bond upon irradiation of Rh5. We have also observed similar CD spectral changes upon irradiation of Rh6. Namely, Rh6 (λ_{max} 513 nm) exhibits a CD spectrum with a single positive CE band at 340 nm; upon prolonged illumination the λ_{max} of the new pigment shifts to 494 nm, while its CD spectrum still shows a single positive CE band, but shifted to 493 nm (Figure 7c). The origin of these changes is not clear at this stage.

Unlike Rh5 and Rh6, Rh8 was stable under prolonged illumination; however, flash photolysis experiments revealed that a primary product absorbing at 585 nm is formed within

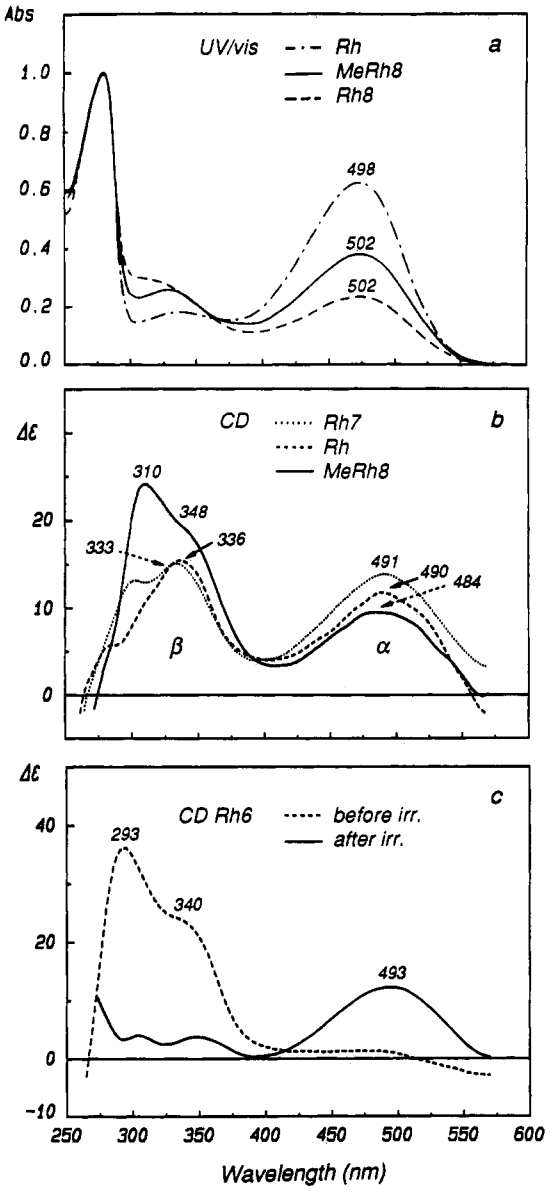


FIGURE 7: UV–vis and CD spectra of Rh and 11-*cis*-locked Rh. (a) UV–vis spectra of purified Rh, RH8, and MeRh8 (2% digitonin in 10 mM HEPES buffer, pH 7.0). The curves are normalized at 278 nm for comparison. (b) CD spectra of purified MeRh8, Rh7, and natural Rh (2% digitonin in 0.1 M KH_2PO_4/K_2HPO_4 buffer, pH 7.0). The similarities in UV and CD spectra show that the environment of the chromophores in the respective pigments are similar. (c) CD spectra of Rh6 before and after irradiation with >510-nm light for 25 min; in contrast to Rh, Rh7, and MeRH8, the CD curve of Rh6 exhibits a single broad positive CE at 340 nm; this latter shifts to 493 nm after irradiation. These data suggest that the chromophoric environment of Rh6 is quite different from that of natural Rh.

15 ps. This photoproduct corresponds to photo-Rh8, which then thermally decays in 1 ns to batho-Rh8 (577 nm). The latter, however, reverts to the starting Rh8 (502 nm) in 50 ns (Mizukami et al., 1993). Neither of these intermediates could be trapped at temperatures down to 67 K. It is not surprising that photo-Rh8 could not be trapped, because so far native photo-Rh has also not been sequestered. The fact that, unlike native batho-Rh, batho-Rh8 could not be trapped, could be attributed to ring strain; the longer absorption maximum of batho-Rh8 (577 nm) as compared to batho-Rh (569 nm)[1] suggests more strain in the former.

Rh5, with the most rigid chromophore, does not form any photointermediate under laser flash photolysis due to the complete inhibition of rotation around 11–12 double bond; consequently, it only emits strong fluorescence (Kandori et al., 1989). Rh6, on which flash photolysis measurements are being carried out, is expected to behave more like Rh5 than Rh7 since it shares more similarities with Rh5 in the ground state in terms of similar CD spectra and light sensitivities. The more flexible chromophore in Rh7 undergoes 11-ene photoisomerization upon flash photolysis to form photo-Rh7 absorbing at 580 nm; however, the extent of distortion of the transoid-11-ene is likely to be higher in photo-Rh7 than in photo-Rh. The quantum efficiency of photo-Rh7 formation was only 25% that of Rh, reflecting the strong tendency of excited Rh7 to return to its ground state. In photo-Rh8, the 11-ene distortion is probably close to that of native photo-Rh in view of similar high quantum efficiencies (Mizukami et al., 1993); the increased ring flexibility allows photo-Rh8 to thermally relax one step further from photorhodopsin, to the batho species, which, however, does not relax further and converts to ground state Rh8.

In spite of efficient formation of the transient Rh8 species, photorhodopsin, MeRh8 was incapable of activating the enzymatic cascade. These results show that complete isomerization of the polyene is necessary to induce conformational changes in the opsin leading to activity. Furthermore, experiments with the dihydro series show that, upon replacement of the double bonds with single bonds, the rigidity of the overlapping π system of the polyene is lost; since the critical isomerization that initiates conformational changes occurs at the 11-ene, the closer the site of the reduced bond to the 11-ene, the greater the activity loss. Thus satisfactory conformational change in the protein requires participation of the entire rigid polyene system.

CONCLUSION

Among the four isomers of 11-*cis*-locked eight-membered ring retinal analogs, only 11-*cis*-ret8 **8a** with a spatial polyene arrangement similar to native 11-*cis*-retinal forms a pigment. The fact that the UV–vis and CD spectra of MeRh8 are very similar to that of native Rh shows that the chromophoric environments in the two pigments are quite similar. Furthermore, the maxima at 570 and 585 nm of the photorhodopsins derived from native and Rh8, respectively, and more importantly, their quantum yields, are both high and similar. The photoexcited state of Rh8 involves charge redistribution of the chromophore, yet the pigment exhibits no activity. The present binding and assay results of rhodopsins incorporating ret8 and dihydroretinals, coupled with previous flash photolytic studies on Rh5~Rh8, have demonstrated that although charge redistribution occurs in the excited state, the visual transduction process requires complete 11-*cis* to *all-trans* isomerization involving the entire rigid π system.

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