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 photoprocess 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), λmax 500 nm, is a single polypeptide consisting of 348 amino acids folded into seven membrane-spanning α-helices; its chromophore, the 11-cis isomer of all-trans-retinal 1, is bound to Lys-296 via a protonated Schiff base (SBH+) 3 (see Table 1). 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 photoprocess of Rh excitation is now considered to be photorhodopsin, λmax 500 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, λmax 543 nm, 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, λmax 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 result in hydrolysis of 102 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 λmax, i.e., the OS for Rh 3 (Table 1) λmax 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 pKₐ of 11-cis and all-trans-retinal SBH+ in the ground and excited states (Schafer et al., 1975) do demonstrate an increased polarity in the excited state. However, the extent to which

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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 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; 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 ret5 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 Ito et al., 1982; Fukada et al., 1984; De Grip et al., 1990; Akita et al., 1980). Except for literature data, all absorption spectra were recorded on a Perkin-Elmer Lambda 4B UV–vis spectrophotometer.

Table 1: Retinal Analog Structures and Spectroscopic Data

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<tr>
<th>X</th>
<th>1 All - trans retinal</th>
<th>11-cis</th>
<th>9, 11 - di-cis</th>
<th>7a, 13 - tri-cis</th>
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<tr>
<td>2</td>
<td>X = O</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>X = NH-Opsin</td>
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<tr>
<td>4</td>
<td>X = NH-n-Butyl</td>
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<th>5b</th>
<th>5c</th>
<th>6a</th>
<th>6b</th>
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<th>7d</th>
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<td>402</td>
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<td>494</td>
<td>496</td>
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<tr>
<td>OS (cm⁻¹)</td>
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<td>1930</td>
<td>2210</td>
<td>2550</td>
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<td>0.78</td>
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- Absorption maxima of a- and b-bands measured in ethanol (5), hexane (6, 8, 9), or methanol (7) and the corresponding A value ratios.
- λmax of the n-butylamine Schiff base measured in methanol and that of the protonated form with a methanolic solution of HCl. λmax of rhodopsin analogs measured in digitonin (5) and nonylglucoside (6, 7). 4 Opsin shift, defined as the difference (expressed in cm⁻¹) between the λmax of the SBH⁺ of the chromophore and that of the corresponding pigment. 5 Remesured value. 6a–d, 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.

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.

**EXPERIMENTAL PROCEDURES**

**Materials.** Digitonin and 4-(2-hydroxyethyl)-1-piperazinethanesulfonic 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, i-α-phosphatidylethanolamine (PE) from bovine brain, i-α-phosphatidycholine (PC) from frozen egg yolk, n-nonyl β-D-glucopyranoside (nonylglycologlucose), and 3-{(3-cholamidopropyl)dimethylamino]-2-hydroxy-1-propanesulfonate (CHAPS) 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 (Papamaster, 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 MgCl₂, 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 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 45 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 after 2 h at 0 °C. The mixture was centrifuged, and the supernatant was pooled 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 Na₂HPO₄/NaH₂PO₄, pH 7.0) containing 100 mM hydroxyamine (final concentration of 0.5 OD ROS/mL) and bleaching under room light for about 2 h, at ice-bath temperature. Excess hydroxyamine 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 A₂₇₈/A₅₀₂ ratio less than 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 Grijp, 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 CaCl₂, 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 A₂₇₈/A₅₀₂ ratio of Rh8 thus obtained was ca. 4.3. Alternatively, 20 mM nonylglycologlucose 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 A₂₇₈/A₅₀₂ 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 K₂HPO₄/KH₂PO₄, 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 5-720 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, Amicon) 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 & Evanczuski, 1982) on MeRh and MeRh8 were carried out using the same Me-opsin (bleached until chromatophore 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 hydrolyzes 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 MgCl₂, 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 (Bhattacharyya 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 mM MgCl₂/10 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 supernatant 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% 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, λ 26 360 in hexane) and a minor band at 250 nm (β-band or “cis-band”) with an A₁/A₂ 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 A₂/A₁ 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 λmax 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; P425 grew gradually and reached its maximum after 14 h. Incubation of 1 OD 11,13-dicis 8b with 1 mL of opsin for 17 h resulted only in the formation of P425. 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.

Incubation of 11,13-dicis-ret8 with opsin in 10 mM 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 maximum 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.
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 502 nm (MeRh8 or P502) 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).

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 un bleached 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 MeRh8. However, addition of 11-cis-retinal to MeRh8 preparation resulted in less than 5% rhodopsin regeneration. From these yields and from the 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\(_2\)OH.** 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...
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 Rh 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 NH₂OH (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–vis 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 32PO₄ 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 dihydroretinoids (Arnaboldi et al., 1979; Koutalos et al., 1989; Takahashi et al., 1990). The general trends 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. Phosphatidyethanolamine 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...
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 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 transducin/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 A478/A500 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 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 1); however, its stability to hydroxylamine (data not shown) and its bleaching and regrowth in the presence of excess NH₂OH 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 at 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; Vander 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 fulfill 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 moeity 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 substaled 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 C6-C9 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 494 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...
11-cis-Locked Eight-Membered Ring Rhodopsin

**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 activity. The present binding and assay results of rhodopsins derived from native and Rh8, respectively, and 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 polyene system.

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**REFERENCES**


