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Light-stable Rhodopsin

I. A RHODOPSIN ANALOG RECONSTITUTED WITH A NONISOMERIZABLE 11-*cis* RETINAL DERIVATIVE*

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With the aim of preparing a light-stable rhodopsin-like pigment, an analog, II, of 11-*cis* retinal was synthesized in which isomerization of the C_{11}-C_{12} *cis*-double bond is blocked by a cyclohexene ring built around the C_{10} to C_{13}-methyl. The analog II formed a rhodopsin-like pigment (rhodopsin-II) with opsin expressed in COS-1 cells and with opsin from rod outer segments. The rate of rhodopsin-II formation from II and opsin was ~10 times slower than that of rhodopsin from 11-*cis* retinal and opsin. After solubilization in dodecyl maltoside and immunoaffinity purification, rhodopsin-II displayed an absorbance ratio ($A_{280\ nm}/A_{512\ nm}$) of 1.6, virtually identical with that of rhodopsin. Acid denaturation of rhodopsin-II formed a chromophore with λ_{max}, 452 nm, characteristic of protonated retinyl Schiff base. The ground state properties of rhodopsin-II were similar to those of rhodopsin in extinction coefficient (41,200 M^{-1} cm^{-1}) and opsin-shift (2600 cm^{-1}). Rhodopsin-II was stable to hydroxylamine in the dark, while light-dependent bleaching by hydroxylamine was slowed by ~2 orders of magnitude relative to rhodopsin. Illumination of rhodopsin-II for 10 s caused ~3 nm blue-shift and 3% loss of visible absorbance. Prolonged illumination caused a maximal blue-shift up to ~20 nm and ~40% loss of visible absorbance. An apparent photochemical steady state was reached after 12 min of illumination. Subsequent acid denaturation indicated that the retinyl Schiff base linkage was intact. A red-shift (~12 nm) in λ_{max} and a 45% recovery of visible absorbance was observed after returning the 12-min illuminated pigment to darkness. Rhodopsin-II showed marginal light-dependent transducin activation and phosphorylation by rhodopsin kinase.

Rhodopsin is the photoreceptor of vertebrate rod cells. Bovine rhodopsin consists of a single polypeptide chain of 348 amino acids whose primary sequence has been elucidated (1-3). The protein contains seven putative transmembrane α-helical segments, and the chromophore, 11-*cis* retinal (**I**, Fig. 1) is covalently linked to Lys-296 via a protonated Schiff base (4, 5). Light causes 11-*cis* to all-*trans* isomerization resulting in the formation of a series of photointermediates (6). One of the intermediates, metarhodopsin II, activates the guanine nucleotide binding protein (G-protein)[1] transducin, culminating in transmission of an electrical signal to the optic nerve (7, 8).

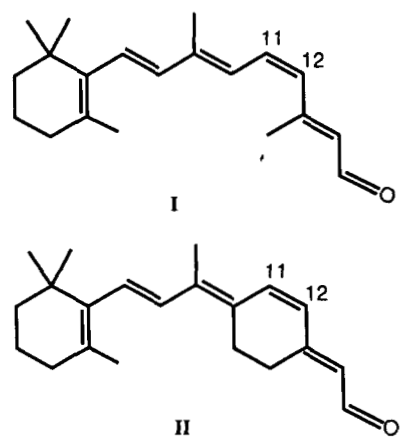

FIG. 1. **Structures of 11-*cis* retinal, I, and the cyclohexatrienylidene retinal analog, II.** Retinal analog **II** was synthesized as described under "Experimental Procedures."

Structural information on rhodopsin is extremely limited (8) and, therefore, x-ray diffraction analysis would be very desirable (9, 10). A light-stable rhodopsin analog would offer important advantages in these studies. Light stability could be achieved by using retinal analogs for reconstituting rhodopsin-like pigments that cannot undergo 11-*cis* to all-*trans* isomerization. Nakanishi and co-workers (11) have introduced a general method for restricting *cis* → *trans* isomerization in retinal analogs when they are bound to opsin. This is accomplished by constructing a carbocyclic ring of which the C_{11}-C_{12} *cis*-double bond forms a part. In two examples of such work, retinal analogs were prepared where the 11-*cis* configuration is maintained by formation of a cycloheptene or cyclopentene ring (11, 12). While these analogs were used successfully to reconstitute the corresponding rhodopsin analogs, the latter were unstable. Thus, hydroxylamine caused bleaching in the dark, and, furthermore, they were unstable in detergent solution (11, 13).

In attempts to prepare a stable rhodopsin analog, we synthesized a retinal analog, **II**, restricted in isomerization as

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[1] The abbreviations used are: G-protein, guanine nucleotide binding protein; ROS, rod outer segments; rho-**I**, opsin reconstituted with 11-*cis* retinal, **I**; rho-**II**, opsin reconstituted with retinal analog **II**; bR, bacteriorhodopsin; DM, *n*-dodecyl-β-D-maltoside; PSB, protonated Schiff base; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid.

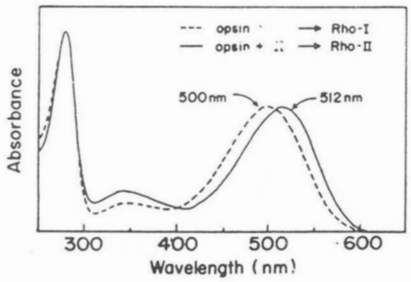

FIG. 3. **UV/visible absorption spectra of rhodopsin-II.** Opsin was reconstituted with **I** or **II**, solubilized in dodecyl maltoside, and purified by immunoaffinity adsorption as described under "Experimental Procedures." Spectra of rho-**I** (*broken line*) and rho-**II** (*continuous line*) in the dark are shown. The corresponding absorption maxima are indicated by *arrows*.

shown in Fig. 1. This analog efficiently reconstituted bovine opsin expressed in COS-1 cells as well as opsin from bovine rod outer segments (ROS).

In this paper, we report the characterization and properties of rhodopsin-**II** (rho-**II**), prepared by reconstitution of opsin with retinal analog **II**, and compare them with those of rhodopsin-**I** (rho-**I**), prepared by reconstitution of opsin with 11-*cis* retinal, **I**. While rho-**II** was much more resistant to light than rho-**I**, it was not completely light-stable.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Rhodopsin-II

Formation and Purification of Rhodopsin-II—A primary consideration toward the preparation of rho-**II** was the source of opsin to be reconstituted. Since quantitative removal of retinal from ROS rhodopsin is often difficult, the use of opsin derived from expression of the synthetic bovine opsin gene in COS-1 cells was preferred. This preparation of opsin, which had not been exposed to retinal, was used in chromophore generation with retinal **I** and the analog **II**.

After reconstitution of opsin with **I** and **II**, the pigments were solubilized in dodecyl maltoside (DM) and purified by immunoaffinity adsorption (16). As shown in Fig. 3, opsin reconstituted with **I** (rho-**I**) showed the characteristic rhodopsin chromophore (λ_{max}, 500 nm). A 12 nm red-shifted chromophore, relative to rho-**I**, was formed upon reaction of opsin with **II** (rho-**II**). The presence of a more prominent β-band (λ_{max}, 340 nm) is also evident in rho-**II**. This is presumably due to enhanced interactions between **II** and aromatic amino acid residues in the retinal binding pocket. The rate of formation of rho-**II** from **II** was ~10 times slower than the rate of formation of rho-**I** from **I**. The kinetics of the decrease in absorbance at ~390 nm were identical with the increase in absorbance at ~512 nm. A single isosbestic point at 430 nm was observed, suggesting that only two components viz. free retinal analog **II** and covalently linked chromophore, were present in the reconstitution mixture.

The purity of rho-**I** and rho-**II** was estimated by comparing the absorbance of the peaks in the UV and visible regions. The ratios of absorbance at 280 nm to 500 or 512 nm were 1.6, a value characteristic of purified bovine rhodopsin (30). Addition of **I** to preformed rho-**II** showed no increase in the visible absorbance (data not shown) and examination by SDS-PAGE and silver staining did not show any traces of contaminating proteins (Fig. 4). The amount of phospholipid remaining after immunoaffinity purification was found to be <1 mol/mol of rhodopsin. We conclude that analog **II** quantitatively forms a rhodopsin-like chromophore with opsin that is stable to solubilization in DM, delipidation, and immunoaffinity purification.

Characterization of the Schiff Base Linkage in Rhodopsin-II—The Schiff base linkage in rho-**II** was characterized by denaturation with acid or SDS. A species with a λ_{max} at 440 nm was obtained upon acid denaturation of rho-**I** (Fig. 5, *upper panel*), which is characteristic of a protonated retinyl Schiff base linkage in denatured rhodopsin (32). A 12 nm red-shifted species (λ_{max}, 452 nm), relative to rho-**I**, was obtained upon acid denaturation of rho-**II** (Fig. 5, *lower panel*). Identical results were obtained upon denaturation with SDS. The molar extinction coefficient and opsin shift for rho-**II** are 41,200 M^{-1} cm^{-1} and 2600 cm^{-1}, respectively, which is similar to values observed with rho-**I** (ϵ, 40,600 M^{-1} cm^{-1}, 2650 cm^{-1} opsin-shift) (33, 34).

The stability of the Schiff base in rho-**II** toward hydroxylamine was also studied. Like rho-**I**, rho-**II** was completely stable toward neutral hydroxylamine in the dark. Under

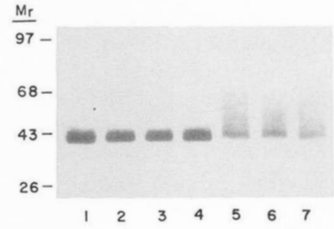

FIG. 4. **Characterization of purified rhodopsin-II.** Immunoaffinity-purified pigments (1 μg) were subjected to SDS-PAGE and visualized by silver staining as described under "Experimental Procedures." *Lane 1*, ROS opsin; *lane 2*, ROS rhodopsin; *lane 3*, rho-**I** reconstituted from ROS opsin and **I**; *lane 4*, rho-**II** reconstituted from ROS opsin and **II**; *lane 5*, COS-1 cell opsin; *lane 6*, rho-**I** reconstituted from COS-1 cell opsin and **I**; *lane 7*, rho-**II** reconstituted from COS-1 cell opsin and **II**. The glycosylation pattern of COS-1 cell opsin contributes to the apparent molecular weight heterogeneity observed on SDS-PAGE (31). Molecular weight markers are shown on the *left*.

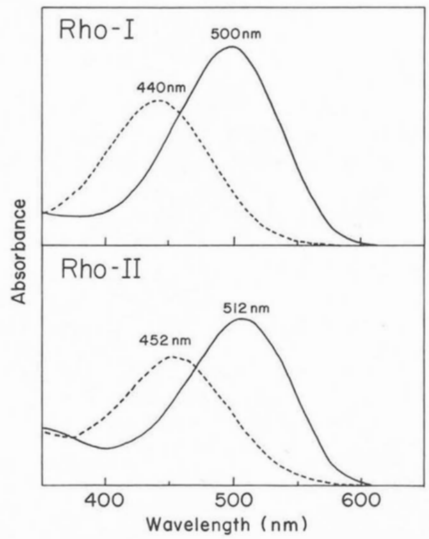

FIG. 5. **Acid denaturation of rhodopsin-II.** Rho-**I** (*upper panel*) and rho-**II** (*lower panel*) were denatured in the dark by addition of 2 N H_2SO_4 to a final pH of 1.9. The resulting absorption spectra are shown by *broken lines*.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Scheme 1, and Fig. 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

steady illumination (>495 nm), rho-I was quantitatively bleached by hydroxylamine within 10 s to form a 360 nm species. In contrast, complete bleaching of rho-II by hydroxylamine required >12.5 min of illumination (Fig. 6*A*). The reaction proceeded through a single isosbestic point (410 nm), implying the existence of only two species during the course of the reaction. Kinetic analysis of the light-dependent bleaching of rho-II by hydroxylamine showed that the loss of the 512 nm chromophore and formation of the ~360 nm species had virtually identical rate constants (~0.006 s^{-1}) (Fig. 6*B*).

When a partially regenerated preparation of rho-II was incubated with a slight excess of I, an increase in visible absorbance was observed. Upon illumination (>495 nm) for 10 s, only the chromophore corresponding to the protonated Schiff base (PSB) of I was quantitatively bleached, while the chromophore due to II was stable under the above conditions (data not shown). These findings indicate that the PSB with II is not readily displaced by I. Taken together, these results demonstrate that II adopts a structure similar to that of I when bound to opsin, although the Schiff base linkage in rho-II is considerably more stable.

Photochemical Behavior of Rhodopsin-II

In rho-I, the 500 nm chromophore was quantitatively converted to a 380 nm species upon illumination (>495 nm) for 10 s at 20 °C (Fig. 7, *upper panel*). Under identical illumination conditions, rho-II showed only a ~3 nm blue-shift in λ_{max} and ~3% decrease in visible absorbance (Fig. 7, *lower panel*). Further illumination caused a gradual blue-shift in λ_{max} (up to ~20 nm) with a concomitant decrease in visible absorbance up to ~40%. The appearance of a very broad absorption band between ~310 and ~475 nm was apparent. No isosbestic point was observed, suggesting the formation of multiple species,

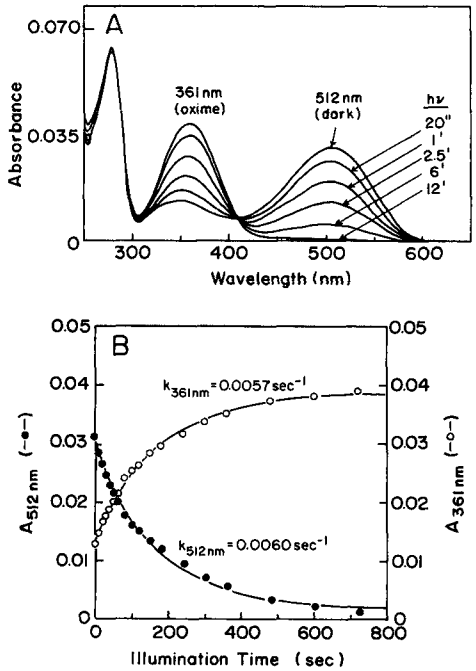

FIG. 6. **Stability of rhodopsin-II toward hydroxylamine.** *A*, time course of the light-dependent reaction of rho-II with hydroxylamine. After addition of hydroxylamine (100 mM, pH 7.0), the pigments were illuminated at >495 nm for the indicated time periods at 20 °C. *B*, kinetics of chromophore bleaching of rho-II upon incubation with hydroxylamine under steady illumination. Loss of the ~512 nm chromophore (●) and formation of the ~361 nm species (○) have the same rate constant (~0.006 s^{-1}).

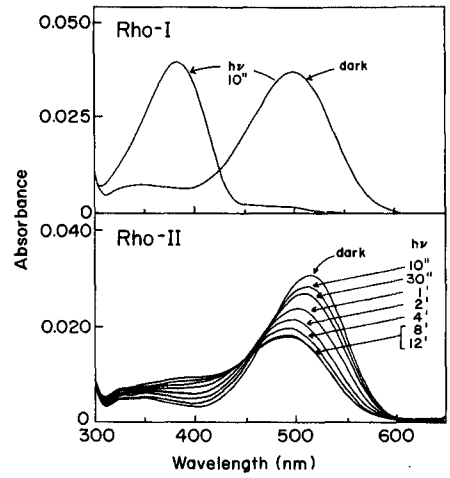

FIG. 7. **UV/visible absorption spectra of rhodopsin-II in the dark and on continuous illumination.** Rho-I (*upper panel*) and rho-II (*lower panel*) were illuminated (>495 nm) for the indicated time periods at 20 °C until no further spectral changes were observed.

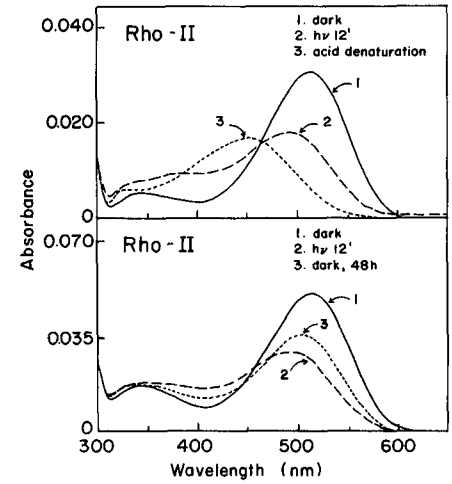

FIG. 8. **Characterization of "light-saturated" rhodopsin-II.** Spectra of rho-II before (*continuous line*) and after (*broken line*) 12 min of continuous illumination (>495 nm). *Upper panel*, the illuminated pigment was denatured by addition of 2 N H_2SO_4 to a final pH of 1.9 (*dotted line*). *Lower panel*, the illuminated pigment was returned to darkness for 48 h at 20 °C (*dotted line*).

possibly due to isomerization at double bond(s) other than the C_{11}-C_{12} ene. Photochemical changes were apparent on illumination up to a total time of 12 min. We have therefore defined an illumination time of 12 min as "saturating illumination" and used this period for comparing the light sensitivities of other rhodopsin analogs. Similar light sensitivity was observed upon illumination of ROS opsin reconstituted with II. The presence of a Schiff base in this "light-saturated" pigment was examined by acid denaturation. The formation of a species with a λ_{max} ~450 nm was observed (Fig. 8, *upper panel*). Further, if the light-saturated pigment was returned to dark for ~48 h ("dark adaptation"), a 12-nm red-shift in λ_{max} and a partial recovery (~45%) of visible absorbance was evident (Fig. 8, *lower panel*). Thus, prolonged illumination of rho-II does not result in hydrolysis of the Schiff base linkage between opsin and II.

Rhodopsin-II Activates Transducin and Undergoes Phosphorylation

Transducin Activation—Activation of transducin by rho-II was examined by the light-dependent hydrolysis of GTP.

Determination of the specific activities revealed that rho-**II** prepared from either COS-1 cell opsin or ROS opsin exhibited GTPase activity ~5–10% relative to ROS rhodopsin (Table I). Opsin from either source did not show any detectable activity.

Phosphorylation by Rhodopsin Kinase—Kinetic analysis of the light-dependent phosphorylation of rho-**II** by rhodopsin kinase showed that rho-**II** was maximally phosphorylated at a level of 0.8 mol of phosphate/mol of pigment in 1 h at 20 °C (Fig. 9*A*). The level of phosphorylation in the ROS rhodopsin and rho-**I** samples under the same conditions were 3.6 and 3.0 mol of phosphate/mol of pigment, respectively. In agreement with the transducin activation results, the level of light-dependent phosphorylation observed with rho-**II** was ~10% relative to that of ROS rhodopsin. An autoradiograph of the phosphorylated pigments is presented in Fig. 9*B*. These results demonstrate that rho-**II** shows limited activity.

DISCUSSION

In the preceding paper (35), we described the preparation of a light-insensitive bacteriorhodopsin (bR)-like pigment by reconstituting bacterio-opsin with a retinal analog in which the C_{13}-C_{14} *trans*-double bond is blocked from isomerization. In the present work, we have used a similar approach to prepare a light-stable rhodopsin-like pigment. Opsin prepared by expression of a synthetic gene in COS-1 cells has been reconstituted with a retinal analog where the 11-*cis* configuration is maintained by inclusion of the C_{11}-C_{12} *cis*-double bond in a cyclohexene ring (Fig. 1).

The main concern in the present work was the selection of an analog of retinal that would reconstitute opsin efficiently to form a chromophore that would be stable both in the dark and in light. Nakanishi and co-workers (11) described a number of nonisomerizable analogs of 11-*cis* retinal in which the C_{11}-C_{12} *cis*-double bond formed a part of a carbocyclic ring. One such analog contained a cycloheptene (ret-7) ring while another a cyclopentene (ret-5) ring (11, 12). The pigments, Rh7 and Rh5, formed from these analogs, respectively, were deficient in several ground state properties of rhodopsin. Thus, they bleached in the presence of hydroxylamine in the dark and were unstable in detergent solution (11, 13). Furthermore, both Rh7 and Rh5 exhibited markedly reduced opsin shifts (1950 and 1350 cm^{-1}, respectively) relative to rho-**I** (2650 cm^{-1}). Thus, both Rh7 and Rh5 showed considerable perturbation in their structures.

TABLE I

Transducin activation as measured by GTP hydrolysis

GTP hydrolysis was measured as described under "Experimental Procedures."

Sample[a]	Specific activity[b]		Activation[c]
	Light	Dark	
	mol ^{32}Pi released/mol rho/min		%
ROS rhodopsin	24.0 ± 1.3	1.0 ± 0.5	100
ROS rho-I	20.0 ± 1.6	1.0 ± 0.5	82
ROS rho-II	2.2 ± 0.3	1.0 ± 0.5	5
ROS opsin	0.3 ± 0.1	1.1 ± 0.7	—
COS-1 cell rho-I	22 ± 0.8	0.9 ± 0.4	93
COS-1 cell rho-II	3.3 ± 0.3	0.9 ± 0.5	10
COS-1 cell opsin	0.6 ± 0.2	1.0 ± 0.7	—

[a] The amount of pigment assayed was based on the molar extinction coefficient. The extinction coefficient of opsin was assumed to be 65,000 M^{-1} cm^{-1}.

[b] Each value is the average of four independent determinations ± standard deviation. The reaction rate for the hydrolysis of GTP in the light and the dark was plotted as a function of the amount of pigment used in the assay. The specific activities were calculated from the slope of the lines.

[c] The values were normalized to the activity of purified ROS rhodopsin.

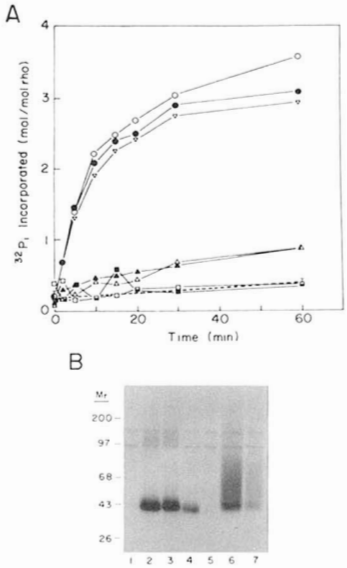

FIG. 9. **Rhodopsin kinase-catalyzed phosphorylation of rhodopsin-II.** *A*, time course of rhodopsin kinase-catalyzed phosphorylation. Light-dependent phosphorylation by rhodopsin kinase was performed as described under "Experimental Procedures." Aliquots from the reactions were removed at the indicated time periods, and the stoichiometry of ^{32}P incorporation was determined. ○, ROS rhodopsin; ●, rho-**I** reconstituted from ROS opsin and **I**; ▽, rho-**I** reconstituted from COS-1 cell opsin and **I**; △, rho-**II** reconstituted from ROS opsin and **II**; ▲, rho-**II** reconstituted from COS-1 cell opsin and **II**; □, ROS opsin; ■, COS-1 cell opsin; – –, dark controls. *B*, autoradiograph of the phosphorylated pigments. Aliquots from the phosphorylation reactions were removed after 60 min and analyzed by 10% reducing SDS-PAGE and autoradiography. *Lane 1*, ROS opsin; *lane 2*, ROS rhodopsin; *lane 3*, rho-**I** reconstituted from ROS opsin and **I**; *lane 4*, rho-**II** reconstituted from ROS opsin and **II**; *lane 5*, COS-1 cell opsin; *lane 6*, rho-**I** reconstituted from COS-1 cell opsin and **I**; *lane 7*, rho-**II** reconstituted from COS-1 cell opsin and **II**. The glycosylation pattern of COS-1 cell opsin contributes to the apparent molecular weight heterogeneity observed on SDS-PAGE (31). The higher molecular weight phosphorylated species present in all the lanes correspond to proteins which co-purify with rhodopsin kinase. Molecular weight markers are shown on the *left*.

In order to prepare a stable rhodopsin-like pigment, we have synthesized an analog in which the C_{11}-C_{12} *cis*-double bond is a part of a cyclohexene ring (**II**, Fig. 1 and Scheme 1). Although the rate of formation of the rho-**II** chromophore from opsin and **II** was ~10 times slower than the formation of rho-**I** with **I**, reconstitution of rho-**II** was quantitative (Fig. 3). This was so both when the opsin from the synthetic gene or from ROS were used. The spectral characteristics of the pigment from the two sources were identical. The extinction coefficient (41,200 M^{-1} cm^{-1}) and opsin-shift (2600 cm^{-1}) of rho-**II** are very similar to those of rho-**I** (ϵ, 40,600 M^{-1} cm^{-1}; opsin shift 2650 cm^{-1}). In the dark, both rho-**I** and rho-**II** were completely stable toward hydroxylamine. In the presence of light, the rate of hydroxylamine bleaching of rho-**II** was >75 times slower than that of rho-**I** (Fig. 6). The similarities in these ground state properties between rho-**I** and rho-**II** suggest that **II** adopts a structure that cannot be very different from **I** when bound to opsin.

As was expected, rho-**II** was far more light-resistant than rho-**I**. Nevertheless, rho-**II** did not show complete stability to light. Upon illumination, rho-**II** showed photochemical changes up to a period of about 12 min (Fig. 7), there being no change upon further illumination. Dark adaptation of

"light-saturated" rho-**II** resulted in a partial recovery of the "lost" visible absorbance while acid denaturation yielded a species with a λ_{max} ~450 nm (Fig. 8). These findings suggest that the Schiff base linkage between opsin and **II** was preserved upon continuous illumination and that the observed blue shift in λ_{max} and loss of visible absorbance was not due to cleavage of **II** from the protein. It appears that the cyclohexene ring which maintains the C_{11}-C_{12} double bond in a *cis* configuration is still capable of interacting with light to cause deformation or bending around the unblocked polyene double bonds and/or rotation around single bonds. Liu *et al.* (36) have proposed a model for the isomerization process based on a concerted rotation of the C_{10}-C_{11} single bond and isomerization of the C_{11}-C_{12} *cis*-double bond.

Rho-**II** was able to activate transducin and undergo rhodopsin kinase catalyzed phosphorylation to ~10% of the extent of ROS rhodopsin (Table I, Fig. 9). Presumably, rho-**II** is still able to undergo a light-induced conformational change although to a much reduced extent. Thus, a "meta-rhodopsin II like" conformation may be generated upon interaction of rho-**II** with light, which will bind and activate transducin. Similarly, this structure will allow phosphorylation of rho-**II**.

Recently, DeGrip and co-workers (29) independently reported the reconstitution of rho-**II** and its thermal and photochemical properties. They indicated a similar opsin shift (2660 cm^{-1}) for their preparation, but a significantly lower extinction coefficient (34,200 M^{-1} cm^{-1}). Their preparation of rho-**II** also showed limited signal transduction capacity.

The present results show that the use of analog **II** does not yield a completely light-stable rhodopsin. It is possible that a light-stable rhodopsin may be obtained by alternative manipulation of 11-*cis* retinal. However, as reported in the following paper (37), the objective has been achieved by the use of retinal analog **II** and a suitable amino acid substitution mutant of the opsin. The light-stable rhodopsin analogs now prepared should be useful for a variety of structural studies, as well as for crystallization of rhodopsin.

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REFERENCES

1. Ovchinnikov, Y. A., Abdulaev, N. G., Feigina, M. Y., Artamonov, I. D., Zolotarev, A. S., Kostina, M. B., Bogachuck, A. S., Moroshinikov, A. I., Martinov, V. I., and Kudelin, A. B. (1982) *Bioorg. Khim.* **8,** 1011–1014
2. Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S.-L., Rao, J. K. M., and Argos, P. (1983) *Biophys. Struct. Mechn.* **9,** 235–244
3. Nathans, J., and Hogness, D. S. (1983) *Cell* **34,** 807–814
4. Dratz, E. A., and Hargrave, P. A. (1983) *Trends Biochem. Sci.* **8,** 128–131
5. Wald, G. (1968) *Nature* **219,** 800–807
6. Hubbard, R., and Kropf, A. (1958) *Proc. Natl. Acad. Sci. U. S. A.* **44,** 130–134
7. Stryer, L. (1986) *Annu. Rev. Neurosci.* **9,** 87–119
8. Dohlman, H. G., Caron, M. G., and Lefkowitz, R. J. (1987) *Biochemistry* **26,** 2657–2664
9. Michel, H. (1982) *EMBO J.* **1,** 1267–1271
10. Demin, V. V., Yurkova, W. V., Kuzin, A. P., Barnakov, A. N., and Abdulaev, N. G. (1987) *Retinal Proteins*, pp. 519–524, VNU Science Press, Utrecht, The Netherlands
11. Akita, H., Tanis, S. P., Adams, M., Balogh-Nair, V., and Nakanishi, K. (1980) *J. Am. Chem. Soc.* **102,** 6370–6372
12. Ito, M., Kodama, A., Tsukida, K., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1982) *Chem. Pharm. Bull.* **30,** 1913–1916
13. Fukada, Y., Shichida, Y., Yoshizawa, T., Ito, M., Kodama, A., and Tsukida, K. (1984) *Biochemistry* **23,** 5826–5832
14. IUPAC-IUB Joint Commission on Biochemical Nomenclature (1982) *Eur. J. Biochem.* **129,** 1
15. Ferretti, L., Karnik, S. S., Khorana, H. G., Nassal, M., and Oprian, D. D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 599–603
16. Oprian, D. D., Molday, R. S., Kaufman, R. J., and Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 8874–8878
17. Wilden, U., and Kuhn, H. (1982) *Biochemistry* **21,** 3014–3022
18. Shichi, H., and Somers, R. L. (1978) *J. Biol. Chem.* **253,** 7040–7046
19. Katre, N. V., Wolber, P. K., Stoeckenius, W., and Stroud, R. M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4068–4072
20. Nakayama, T. A., and Khorana, H. G. (1990) *J. Biol. Chem.* **265,** 15762–15769
21. Laemmli, U. K. (1970) *Nature* **227,** 680–685
22. Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) *Anal. Biochem.* **118,** 197–203
23. Bartlett, G. (1959) *J. Biol. Chem.* **234,** 466–468
24. Fung, B. K.-K., Hurley, J. B., and Stryer, L. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 152–156
25. Wessling-Resnick, M., and Johnson, G. L. (1987) *J. Biol. Chem.* **262,** 3697–3705
26. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356
27. Sitaramayya, A. (1980) *Biochemistry* **25,** 5460–5468
28. Van der Steen, R., Groesbeek, M., Van Amsterdam, L. P. J., Lugtenberg, J., Van Costrum, J., and De Grip, W. J. (1989) *Recl. Trav. Chim. Pays Bas.* **108,** 20–27
29. De Grip, W. J., Van Oostrum, J., Bovee-Geurts, P. H. M., Van der Steen, R., Van Amsterdam, L. P. J., Groesbeek, M., and Lugtenberg, J. (1990) *Eur. J. Biochem.* **191,** 211–220
30. Hargrave, P. A. (1982) *Prog. Ret. Res.* **1,** 1–51
31. Karnik, S. S., Sakmar, T. P., Chen, H.-B., and Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8459–8463
32. Kito, Y., Suzuki, T., Azuma, M., and Sekoguti, Y. (1968) *Nature* **218,** 955–957
33. Wald, G., and Brown, P. K. (1953) *J. Gen. Physiol.* **37,** 189–200
34. Arnaboldi, M., Motto, M. G., Tsujimoto, K., Balogh-Nair, V., and Nakanishi, K. (1979) *J. Am. Chem. Soc.* **101,** 7082–7084
35. Bhattacharya, S., Marti, T., Otto, H., Heyn, M. P., and Khorana, H. G. (1992) *J. Biol. Chem.* **267,** 6757–6762
36. Liu, R. S. H., Asato, A. E., Denny, M., and Mead, D. (1984) *J. Am. Chem. Soc.* **106,** 8298–8301
37. Ridge, K. D., Bhattacharya, S., Nakayama, T. A., and Khorana, H. G. (1992) *J. Biol. Chem.* **267,** 6770–6775

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Supplementary Material to:

Light-Stable Rhodopsin I. A Rhodopsin Analog Reconstituted with a Non-isomerizable 11-*cis* Retinal Derivative

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EXPERIMENTAL PROCEDURES

Materials - Protease inhibitors, bovine serum albumin (BSA), dithiothreitol (DTT), and CNBr-activated Sepharose 4B were purchased from Sigma Chemical Co. Dodecyl maltoside (DM) was purchased from Anatrace and its purity determined by thin layer chromatography (TLC) and ^{1}H-NMR. When necessary, DM was purified by flash chromatography over silica gel. 11-*cis* Retinal was a gift from Dr. P. Sorter (Hoffman-LaRoche). Adenosine 5'-[γ-^{32}P] triphosphate, guanosine 5'-[γ-^{32}P] triphosphate and guanosine 5'-[γ-(3^{5}S)thio] triphosphate (GTPγS) were obtained from Dupont-New England Nuclear. Hexylagarose was purchased from IGN Biomedicals, Inc. Centricon CX-30 immersible filters were from Millipore and bovine retinae were from J. A. Lawson Co. Acetic anhydride, β-ionone and 3-ethoxy-2-cyclohexen-1-one were purchased from Aldrich Chemical Co. and distilled before use. Diisopropylamine (Aldrich) was distilled from CaH_2. n-Butyl lithium (n-BuLi, 1.6 M in hexane), 1,5-diazabicyclo[4.3.0]-non-5-ene (DBN), dimethylamino pyridine (DMAP), diisobutyl aluminum hydride (DIBAL, 1.0 M in hexane), hydroxylamine hydrochloride, lithium aluminum hydride (LAH), sodium hydride (NaH), and diethyl cyanomethylphosphonate were purchased from Aldrich and used without further purification. Silica gel (Merck 60, 230-400 mesh) was employed during preparative flash chromatography. Analytical analyses were carried out using silica gel coated plates (Baker Flex). Freshly distilled anhydrous solvents were employed whenever necessary.

Methods

Synthesis of 7-Methyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-3,6-ethano-2E, 4Z,6E,8E-nonatetraenal, II (Fig. 1)

This synthetic procedure does not employ or produce 11-*cis* retinal, I, in any steps of the synthesis. All operations of the synthesized polyenes were performed in dim light. The steps in the synthesis of II are shown in Scheme 1. ^{1}H NMR spectra were recorded on a Varian XL-300 spectrometer. Chemical shifts (δ) are reported in parts per million downfield from $(CH_3)_4Si$. Spectral assignments for V and VI were based on the retinoid numbering system (14). Mass spectroscopic measurements were carried out by FAB mass spectral analysis.

4-[(2E)-1-Hydroxy-1-methyl-3-(2,6,6-trimethyl-1-cyclohexenyl)-2-propenyl]-2-cyclohexene-1-one, V, (Scheme 1)

n-BuLi (4.15 ml of a 1.6 M solution in hexane) (6.6 mmol) was added gradually to a stirred solution of 668 mg (6.6 mmol) of diisopropylamine in 10 ml of THF at -78^0 C under argon. After 20 min at -78^0 C (when formation of LDA is complete *in situ*), 841 mg (6 mmol) of 3-ethoxy-2-cyclohexen-1-one, III, in 10 ml of THF was added dropwise from a pressure equalizing dropping funnel. The reaction mixture was then stirred for an additional 30 min at -78^0 C. At this point, 1.154 g (6 mmol) of β-ionone in 10 ml of THF was added dropwise. The mixture was stirred for 30 min and then transferred to a flask containing 501 mg (13.2 mmol) of LAH in 25 ml of anhydrous Et_2O (0^0 C) and stirred for 1 h at 0^0 C under argon. The mixture was warmed to ambient temperature and stirred for an additional 15 min when the reduction was complete. The reaction mixture containing the dilithium salt of IV was cooled to 0^0 C, and ice-cold H_2O/H_2SO_4 (10:1) was slowly added (~ 40 min). The organic layer was separated and washed with H_2O (2 x 25 ml), 10% aq. NaCl (2 x 10 ml), and dried over anhydrous $MgSO_4$. Evaporation of the solvents from the organic layer yielded a syrupy liquid of pale yellow color, V, which was purified over silica gel using a mixed solvent system of Et_2O/hexane (1:3) and isolated as a diastereomeric mixture [896 mg (3.1 mmol, 51%)]. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.0 (s, 6H, 2 x 1-CH_3), 1.38, 1.45 (2 x s, 2 x 3H, 2 x 9-CH_3), 1.41 - 1.72 (m, 5H, 2-CH_2, 3-CH_2, and OH), 1.68 (s, 3H, 5 CH_3), 1.9 - 2.1 (m, 4H, 4-CH_2, 14-CH_2), 2.4 - 2.9 (m, 3H, 15-CH_2, and 10-CH), 5.49, 5.52 (2 x d, *J* ~ 16 Hz, 1H each, 2 x H-8), 6.02 (m, 1H, H-12), 6.15, 6.17 (2 x d, *J* ~ 16 Hz, 1H each, 2 x H-7), 7.19 (m, 1H, H-11).

(4-*E* and 4-*Z*) 4-[1-methyl-3-(2,6,6-trimethyl-1-cyclohexenyl)-2-propenylidene]-2-cyclohexen-1-one, VI, (Scheme 1)

To a solution of 403 mg (1.4 mmol) of V in 25 ml of anhydrous toluene were added 286 mg (2.8 mmol) of Ac_2O and 342 mg (2.8 mmol) of DMAP. The reaction mixture was stirred for ~ 72 h at ambient temperature. Acetylation was complete as judged by TLC [Et_2O/hexane (1:1)]. DBN (348 mg, 2.8 mmol) was added directly and the resulting mixture was heated under reflux for ~ 2 h. The brownish red solution was cooled to room temperature. H_2O (25 ml) was added, and the layers allowed to separate. The aq. layer was extracted with Et_2O (2 x 25 ml), the organic extracts combined, washed with H_2O (2 x 25 ml), saturated aq. NaCl (2 x 25 ml), and dried over anhydrous $MgSO_4$. The material obtained by evaporation of the solvents from the organic fraction was purified over silica gel using Et_2O/hexane (1:4) as eluent. The product [250 mg (0.92 mmol, 65%)] was isolated as a purified diastereomeric mixture of isomers, VI. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.04, 1.05 (pair of s, 6H, 2 x 1-CH_3), 1.48 (m, 2H, 2-CH_2), 1.64 (m, 2H, 3-CH_2), 1.74 and 1.75 (pair of s, 3H, 5-CH_3), 2.04 (t, *J* ~ 6 Hz, 2H, 4-CH_2), 2.05 and 2.08 (pair of s, 3H, 9-CH_3), 2.52 and 2.54 (pair of t, *J* ~ 7 Hz, 2H, 14-CH_2), 2.85 and 2.88 (pair of t, *J* ~ 7 Hz, 2H, 15-CH_2), 5.89 and 5.93 (d, *J* ~ 10 Hz, 1H, H-12), 6.36 and 6.49 (pair of d, *J* ~ 16 Hz, 1H, H-7), 6.62 and 6.68 (pair of d, *J* ~ 16 Hz, 1H, H-8), 7.61 and 7.70 (pair of d, *J* ~ 10 Hz, 1H, H-11).

7-Methyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-3,6-ethano-2E,4Z,6E,8E-nonatetraenenitrile, VII, (Scheme 1)

NaH [45 mg (1.125 mmol)], in the form of a 60% mineral oil suspension, was added to a septum capped flame-dried flask containing ~ 10 ml dry THF and cooled in an ice-water bath to ~ 0^{0}C. Diethyl cyanomethylphosphonate [212 mg (1.17 mmol)] in ~ 10 ml of dry THF was added and the contents were stirred for ~ 15 min at ~ 0^0 C and then warmed to ambient temperature. Stirring was continued for an additional 30 min before cooling to ~ 0^0 C. The isomeric mixtures of enones, VI, [250 mg (0.92 mmol)], in 10 ml of dry THF was added dropwise to the phosphonate carbanion generated *in situ* with constant stirring. After complete addition of enones, the reaction mixture was warmed to ambient temperature and stirring was continued for ~ 2 h, when TLC [Et_2O/hexane (1:9)] indicated completion of coupling. Then 5 ml of ice-cold H_2O was added to the reaction mixture and the layers were allowed to separate. The aq. layer was extracted 3 times with Et_2O and the organic layers combined. The combined organic extract (~ 20 ml) was washed with H_2O (2 x 20 ml), saturated aq. NaCl (2 x 20 ml), and dried over anhydrous $MgSO_4$. Solvents were evaporated from the organic layer and the resulting liquid was purified over silica gel using Et_2O/hexane (2:98) as eluent. The purified product [285 mg (1.09 mmol, 97%)] was isolated as a mixture of four nitrile isomers, VII. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.02, 1.03 [s, 6H, 2 x 9-[6-CH_3's)], 1.46 [m, 2H, 9-(5-CH_2)], 1.63 [m, 2H, 9-(4-CH_2)], 1.72, 1.73 [s, 3H, 9-(2-CH_3)], 1.99-2.03 (mixture of s + t, 5H, 7-CH_3 + 9-(3-CH_2)], 2.65 (mixture of t, 2H, 6-CH_2), 2.73 (mixture of t, 2H, 3-CH_2), 5.08 (s, 1H, H-2), 6.12, 6.18 (pair of d, *J* ~ 10 Hz, 1H, H-4), 6.29, 6.38 (pair of d, *J* ~ 16 Hz, 1H, H-9), 6.53-6.68 (mixture of d, *J* ~ 16 Hz, 1H, H-8), 6.93, 7.03 (pair of d, *J* ~ 10 Hz, 1H, H-5).

7-Methyl-9-(2,6,6-trimethyl-1-cyclohexenyl)-3,6-ethano-2E,4Z,6E,8E-nonatetraenal, II (Scheme 1)

Hexane (10 ml) was added to a septum capped flame-dried flask containing 50 mg (0.17 mmol) of VII and cooled to -78^0 C. DIBAL-hexane [0.21 mmol (2.1 ml of 0.1 M)] was slowly added and the contents stirred for 2 h at -78^0 C. After warming to ~ 0^0 C, H_2O/SiO_2 (1:5, w/w) in Et_2O/hexane (1:1) was added, and the suspension stirred for 1 h. A mixture of anhydrous $MgSO_4$ and K_2CO_3 (0.1 g of each) was added, the mixture stirred, filtered and the residue washed with Et_2O/hexane (1:1). Filtrate and leachings were combined and concentrated to remove organic solvents. The resulting oily red residue was flash chromatographed over silica gel (230-400 mesh) in Et_2O/hexane (1:9).

Individual isomers of II were isolated by HPLC of the pre-purified (flash chromatography) synthetic mixture on a system equipped with a spectrophotometer and a Zorbax-SIl column (9.4 x 250 mm; Dupont) with hexane/ethyl acetate/isopropanol (98.6:1.3:0.1) as eluent. The flow rate was 5 ml/min and the absorbance was monitored at 368 nm. All analog isomers were stored at -70^0 C under argon and repurified by HPLC immediately before chromophore generation. The purified 11-*cis* isomer of II was characterized by mass spectral analysis and ^{1}H NMR and UV-visible absorption spectroscopy. MS m/z 297 (M^+); ^{1}H NMR (300 MHz, $CDCl_3$ δ 1.05 [s, 6H, 2 x 9-(6-CH_3's)], 1.40 (m, 2H, 9-(5-CH_2)], 1.58 [m, 2H, 9-(4-CH_2)], 1.72 [s, 3H, 9-(2-CH_3)], 2.01-2.04 [s + t, 5H, 7-CH_3 + 9-(3-CH_2)], 2.67 (t, *J* ~ 7 Hz, 2H, 6-CH_2), 2.94 (t, *J* ~ 7 Hz, 2H, 3-CH_2), 5.86 (d, *J* ~ 8 Hz, 1 H, H-2), 6.22 (d, *J* ~ 10 Hz, 1 H, H-4), 6.37 (d, *J* ~ 16 Hz, 1 H, H-9), 6.56 (d, *J* ~ 16 Hz, 1H, H-8), 7.0 (d, *J* ~ 10 Hz, 1H, H-5), 10.03 (d, *J* ~ 8 Hz, 1 H, H-1); UV-vis λ_{max}, 390 nm (EtOH).

Expression of the Synthetic Opsin Gene in COS-1 Cells and Preparation of Opsin Membranes - The wild-type synthetic bovine opsin gene (15) was transiently expressed in COS-1 cells as described (16). After three days of growth, the cells were harvested and subjected to hypotonic lysis by resuspension in 15 mM Tris-HCl, pH 7.5, containing 2 mM $MgCl_2$, 1 mM DTT, and 10 μg/ml of aprotinin, benzamidine-HCl, leupeptin, and pepstatin. After 45 min at 4^0 C, the suspension was centrifuged and the resulting pellet was homogenized and layered on a 20%/50% sucrose gradient. The crude membranes, which band at the 20%/50% interface, were resuspended in 20 mM NaH_2PO_4, pH 6.5, containing 150 mM NaCl and 10 μg/ml of above mentioned inhibitors. After centrifugation, the membranes were homogenized in the same buffer and used immediately for chromophore generation.

Preparation of ROS Rhodopsin and Opsin - ROS membranes were prepared under dim red light (> 650 nm) from frozen bovine retinae according to Wilden and Kuhn (17). Urea-washed ROS membranes (U-ROS) were prepared by the method of Shichi and Somers (18) and were bleached by combining the methods of Katre, *et al.* (19) and of Nakayama and Khorana (20). Briefly, U-ROS were resuspended in 10 mM NaH_2PO_4, pH 6.5, containing 50 mM NH_2OH and 0.1 mM PMSF to a final concentration of 0.1 mg/ml. The suspension was illuminated with a 300 W light-source through a > 495 nm long-pass filter for 10 min. Under these conditions, all of the 500 nm species was converted to ~ 360 nm species, which is characteristic of retinaloxime. Retinaloxime was removed by washing the membranes five times with 10 mM NaH_2PO_4, pH 6.5, containing 2% BSA and 0.1 mM PMSF. Residual BSA was removed by washing three times with 10 mM NaH_2PO_4, pH 6.5, containing 0.1 mM PMSF and two times with 20 mM NaH_2PO_4, pH 6.5, containing 150 mM NaCl and 0.1 mM PMSF. ROS opsin membranes were stored at -70^0 C under argon.

Chromophore Formation and Immunoaffinity Purification of Rhodopsin Pigments - Pigments were generated by incubating opsin membranes for 8 - 12 h with a ~ 5-fold molar excess of I or II at 20^0 C under dim red light. The retinals were added in the form of an ethanolic solution under an argon blanket. Excess retinal was removed by centrifugation in 20 mM NaH_2PO_4, pH 6.5, containing 150 mM NaCl and 0.1 mM PMSF. After solubilization in 10 mM Tris-HCl, pH 7.0, containing 1% (w/v) DM, 150 mM NaCl and 0.1 mM PMSF, the samples were purified by immunoaffinity chromatography on 1D4-Sepharose as previously described (15). Purified protein samples were analyzed by SDS-PAGE (21) with a 5% stacking and a 10% resolving gel and visualized by silver staining (22). The wet ashing method of Bartlett (23) was used to quantitate the amount of phospholipid remaining after immunoaffinity purification of the analog rhodopsin pigments.

Spectral Characterization of Rhodopsin Pigments - Spectrophotometric measurements were performed with a Perkin-Elmer λ 7 UV-visible spectrophotometer. All spectra were recorded in 10 mM Tris-HCl, pH 7.0, containing 0.1% (w/v) DM and 150 mM NaCl at 20^0 C unless otherwise noted. Purified pigments were illuminated at 20^{0}C for the indicated times with a 300 W light source through a > 495 nm long-pass filter. Samples were acid denatured by adjusting the pH of the sample to ~ 1.9 with 2 N H_2SO_4. Chromophore stability toward hydroxylamine was determined by adding an aliquot of a freshly prepared 2 M solution adjusted to pH 7.0 (final concentration, 100 mM). All visible absorption maxima are ± 3 nm.

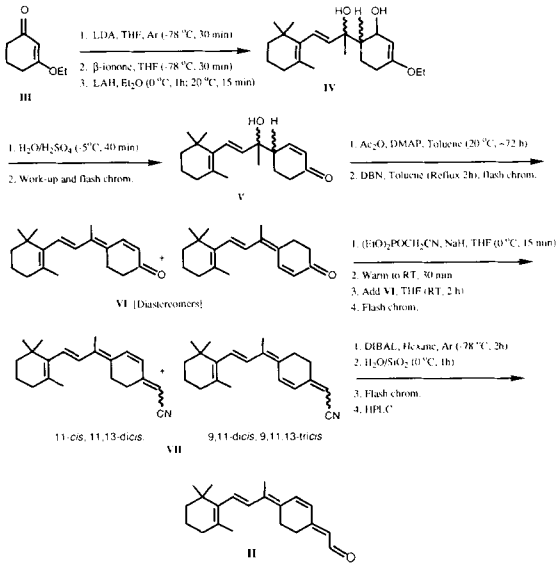
Transducin Activation Assay - Transducin was purified from bovine retinae as previously described (24) and stored in 50% glycerol at -20^0 C. The concentration of active transducin was determined from [^{35}S]GTPγS binding (25) and protein determination (26). The stoichiometry of binding was determined to be 0.98 mol of [^{35}S]GTPγS/mol of transducin. Purified pigments were assayed for their light-dependent ability to activate transducin by the GTPase assay (16). The reaction mixtures (100 μl) contained 125, 250 or 500 fmol of the purified pigments in 20 mM Tris-HCl, pH 7.2, containing 0.012% (w/v) DM, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 200 pmol of transducin. After illumination for 1 min with a 300 W light source (> 495 nm) at 20^{0}C, the reaction was initiated by the addition of 20 μM [γ-^{32}P]GTP. At 2 min intervals, an aliquot (20 μl) was removed and free [γ-^{32}P] phosphate was extracted and quantitated. Corresponding dark reactions were performed under dim red light.

Rhodopsin Kinase Catalyzed Phosphorylation - ROS crude extract enriched for rhodopsin kinase was prepared according to Sitaramayya (27) with some modifications. Briefly, ROS membranes were extracted with 70 mM NaH_2PO_4, pH 7.0, containing 200 mM KCl, 3 mM EGTA, 3 mM EDTA, 5 mM DTT, and 50 μg/ml of aprotinin, benzamidine-HCl, leupeptin and pepstatin under dim red light. The membranes were centrifuged, the supernatant removed, and centrifuged again to ensure removal of all rhodopsin. The supernatant, which served as the kinase preparation, was concentrated 15 - 20 fold using centricon CX30 immersible filters. To 250 pmol of purified pigments was added 200 - 250 units of kinase extract (1 unit = 1 mol of P_i incorporated/mol U-ROS) and a solution of 70 mM NaH_2PO_4, pH 7.0, containing 100 μM [γ-^{32}P]ATP, 3 mM $MgCl_2$, 150 μM DTT, and 10 μM GTP. The final DM concentration was 0.012 % (w/v) and the reaction volume was 2.0 ml. Phosphorylation was initiated by illuminating with a 300 W light source (> 495 nm) at 20^{0}C. At various times, up to 60 min, aliquots (200 μl) were removed and precipitated with 5 mM phosphoric acid containing 7.0 % (w/v) trichloroacetic acid (TCA) and 25 μg BSA. Samples phosphorylated under dim red light for 60 min served as dark controls. After 2 h at 4^0 C, the samples were centrifuged, and the precipitate washed five times with 1 ml of TCA. The pellets were dissolved in 0.5 ml of protosol and counted. Additionally, aliquots from the 60 min time point were analyzed by reducing SDS-PAGE (21) with a 5 % stacking and 10 % resolving gel and an autoradiograph obtained. The ^{32}P (mol) incorporated was calculated from the amount of acid-precipitable counts and the specific activity of the [γ-^{32}P]ATP.

RESULTS

Synthesis of Retinal Analog II

For the synthesis of retinal analog II, the reaction sequences outlined in Scheme 1 were utilized. The synthetic procedures are similar to those reported (28, 29), but have been significantly modified to render it more efficient and concise. An enol ether, 3-ethoxy-2-cyclohexen-1-one, III, was coupled with β-ionone in an aldol type condensation to introduce a cyclohexene ring that eventually formed the central part of II. As the anion of the aldol adduct is sensitive to acid or base during neutralization, LAH reduction was directly carried out in one-pot to generate the dilithium salt of IV. IV was then neutralized with dilute H_2SO_4 to produce the diastereomeric mixture of hydroxy enone, V. This modification allowed an enhancement in the overall yield of V from ~ 23 % (28) to ~ 51 %. V was then acetylated and subjected to base-catalyzed alimination of HOAC to generate two diastereomeric enones, VI (65 %). The diastereomeric enones were directly coupled with diethylcyano-methyl phosphonate to yield a mixture of four isomeric nitriles, VII. After purification by preparative flash chromatography, these nitriles were reduced with DIBAL to give the four geometric isomers of retinal analog II. The yields from the last two steps were greater than 95%. The four geometric isomers of II could be effectively separated to baseline resolution by normal phase HPLC (Fig. 2). Pure II, which corresponds to peak 3 in the chromatogram, has an absorption maximum of ~ 390 nm in EtOH. Spectral assignments (chemical shifts and coupling constants) for individual isomers were in complete agreement with the published data (28, 29).



Scheme 1. **Synthesis of the cyclohexatrienylidene retinal analog, II.** Reagents, reaction conditions, and the direction of each synthetic transformation are indicated with the arrow.

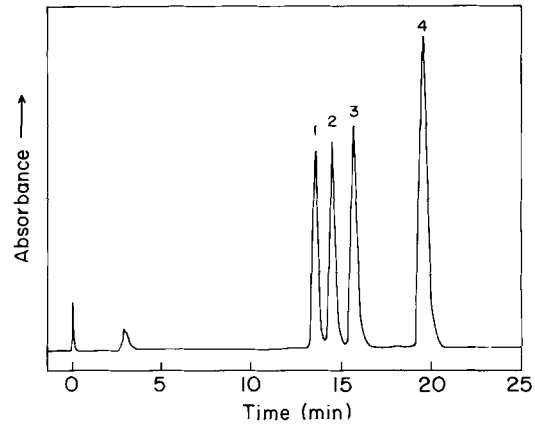


Fig. 2. **HPLC separation of the geometric isomers of retinal analog II.** Normal phase separation of the geometric isomers of II was performed on Zorbax silica with a mixed solvent system of hexane/ethyl acetate/isopropanol (98.6, 1.3, 0.1) at a flow-rate of 5 ml/min. Fractions were monitored at 368 nm and those corresponding to peak 3 were pooled, the solvents removed by evaporation, and the residue dissolved in EtOH.