[16] Synthetic Retinals: Convenient Probes of Rhodopsin and Visual Transduction Process

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Introduction

All the visual pigment proteins are integral membrane proteins, crossing the membrane seven times with the majority of the protein being composed of hydrophobic amino acids within the membrane. This has made these proteins difficult to study by many standard biophysical techniques. However, each of these opsins has a chromophore, generally 11-*cis*-retinal or one of a few closely related structures, that is attached to the protein via a protonated Schiff base linkage. The site of attachment is at a lysine in the center of the seventh helix. *In vitro,* exposure of these proteins to light in the presence of hydroxylamine results in the complete detachment of the retinal, and washing the membranes with bovine serum albumin (1%) removes any residual retinal. This generates the opsin apoprotein, which can reform rhodopsin on the addition of 11-*cis*-retinal in the dark. This provides a most convenient tool for the examination of rhodopsin structure and function as the protein has also been found to accommodate various retinal isomers and analogs, forming pigments or complexes which can then be tested for functionality and physical properties.

A large number of retinals have been synthesized and tested with opsins from various species (see a recent review[1]). Most of these experiments have been carried out *in vitro* although a few studies have shown that retinal analogs can be successfully incorporated into some species including vitamin A-deficient rats.[2] Very little work to date has been conducted on the cone opsins due to problems in obtaining reasonable quantities of the proteins. However, with the development of both improved expression systems and purification techniques, these studies are now being initiated on the cone opsins as well. Interestingly, the physiologic recordings conducted to date on isolated rod and cone photoreceptors utilizing retinal analogs indicate that there are some significant differences in the interactions of these respective opsins with their chromophores.[3] Recently studies have been initiated

[1] K. Nakanishi and R. K. Crouch, *Isr. J. Chem.* **35,** 253 (1996).
[2] R. Crouch, B. R. Nodes, J. I. Perlman, D. R. Pepperberg, H. Akita, and K. Nakanishi, *Invest. Ophthalmol. Vis. Sci.* **25,** 419 (1984).
[3] V. J. Kefalov, M. C. Cornwall, and R. K. Crouch, *J. Physiol.* (in press) (1999).

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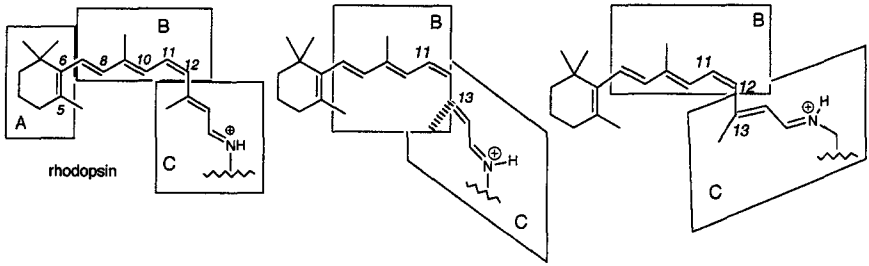


FIG. 1. The chromophore in rhodopsin is not planar, that is, planes A, B, and C are not coplanar.

on combining the use of retinal derivatives with opsins containing specific site mutations, which is indeed a powerful method of exploring the structural boundaries of the chromophore binding site.[4]

The constraints of the binding site of rod rhodopsin have been extensively examined using retinal analogs and from these studies the following generalizations can be made: (1) All isomers (including di- and tri-*cis* isomers), except for 13-*cis* and all-*trans*, can be accommodated; (2) the methyl at C-9 on the polyene chain is critical to the absorption properties and activity of the protein; and (3) the binding cavity has little tolerance for additional bulk in the region of the cyclohexyl ring; however, the ring itself is not essential for pigment formation if one methyl group is present, corresponding to the C-1 or C-6 methyl. The exact location of the chromophore within the protein has been probed by photoaffinity cross-linking studies and these results are discussed elsewhere in this volume. Due to the lack of significant quantities of the cone opsins, little is understood about the cone opsin-binding site.

We describe here some recent findings on rod rhodopsin using analogs of retinal that seek (1) to explain the unique circular dichroism (CD) spectrum of rhodopsin, (2) to define the absolute conformation around the 12-*s*-*trans* bond of the chromophore in bovine rhodopsin, and (3) to address the control of the activity of the protein by the retinal–protein interactions.

Circular Dichroism Spectrum of Rhodopsin

11-*cis*-Retinal is twisted around the C-6/C-7 and C-12/C-13 single bonds due to steric interactions between 5-CH_3/8-H and 13-CH_3/10-H, respectively. Namely, planes A/B and B/C (Fig. 1) are not coplanar. Then what is the absolute sense of twist between these planes? In rhodopsin, light

[4] M. Han, M. Groesbeek, S. O. Smith, and T. P. Sakmar, *Biochemistry* **37,** 538 (1998).

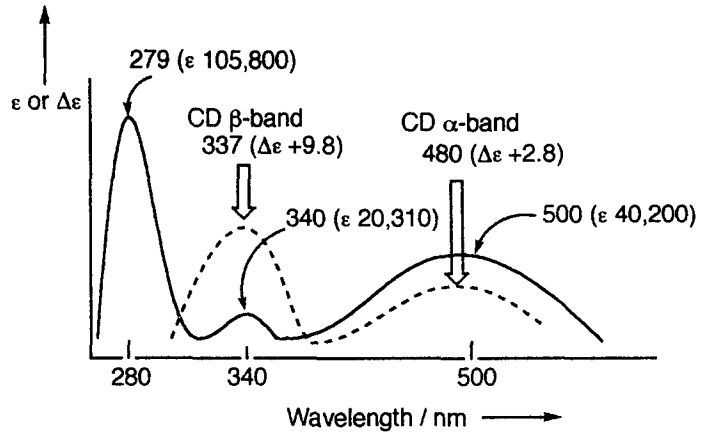


FIG. 2. UV–vis (solid line, ε) and CD (dashed line, $\Delta\varepsilon$) of bovine rhodopsin in 23 m*M* octylglucoside solution (pH 7.0).

induces isomerization of the 11-*cis*-retinylidene chromophore to its all-*trans* form. To accomplish this transformation, the C-11/C-12 double bond is required to rotate 180°. Taking the steric interaction between 13-CH_3 and 10-H into account, isomerization should involve a rotation with the 13-CH_3 moving away from the adjacent 10-H.[5]

Elucidating the absolute extent of twist around the 6-*s*-*cis* and 12-*s*-*trans* bonds of the retinal chromophore in rhodopsin is central for clarifying the changes in the chromophore–receptor interaction along the visual transduction pathway. In addition, the twists together with other factors, for example, the protonated Schiff base, the counteranion distance, and the electrostatic charge distribution within the binding site, dictate the regulations of absorption maxima of various visual pigments.[6,7]

The nonplanar conformation of the retinal chromophore also accounts for the unique CD spectrum of rhodopsin. Native rhodopsin exhibits two positive Cotton effects in its CD spectrum at 480 nm ($\Delta\varepsilon$ = +2.8, α-band) and 337 nm ($\Delta\varepsilon$ = +9.8, β-band), respectively (Fig. 2). A rhodopsin incorporating the retinal analog ret5 with a five-membered ring bridging C-10 and C-13, in which planes B/C are coplanar, exhibits CD with negligible α-band.[8] In contrast, the pigment formed from a retinal analog in which planes A/B are kept coplanar by a five-membered ring bridging C-8 and

[5] G. G. Kochendoerfer and R. A. Mathies, *Isr. J. Chem.* **35,** 211 (1995).
[6] K. Nakanishi, *Am. Zool.* **31,** 479 (1991).
[7] K. Nakanishi, *Pure Appl. Chem.* **63,** 161 (1991).
[8] Y. Fukada, Y. Shichida, T. Yoshizawa, M. Ito, A. Kodama, and K. Tsukida, *Biochemistry* **23,** 5826 (1984).

C-5, showed a weak β-band.[9,10] Based on these observations, Ito and coworkers have assigned the origin of α- and β-band to distortions around the 12-*s-trans* and 6-*s-cis* bonds, respectively.[11] Although it is generally accepted that the two positive Cotton effects (CE) reflect the interaction between the twisted chromophore and its protein environment, the absolute senses of twist around the 6-*s-cis* and 12-*s-trans* bonds, or the absolute conformation of the retinylidene chromophore in rhodopsin, remains to be established.

The absolute twist around the 12-*s*-bond has been determined by two independent approaches: (1) chiroptical spectroscopy based on exciton-coupled CD and (2) bioorganic studies based on incorporation of an 11,12-cyclopropyl retinal analog. The twist around the 6-*s* bond is under investigation.

Chiroptical Spectroscopy

The approach was to modify the retinal structure so that the absolute sense of twist around the 12-*s* bond could be detected spectroscopically. Exciton-coupled CD has proven to be an extremely versatile and sensitive method for stereochemical studies.[12] However, since the method depends on the through-space chiral coupling between two or more isolated chromophores, it cannot be applied to the native retinal system, which consists of a single twisted polyene system. Saturation of the 11-ene breaks the chromophore into two exciton conjugated systems, a triene and a diene, which might interact or couple through space within the protein binding site and thus give rise to an exciton couplet reflecting the C-12/C-13 twist. As shown in Fig. 3, a positive helicity between B/C planes would yield a positive couplet, and vice versa.

11,12-Dihydrorhodopsins

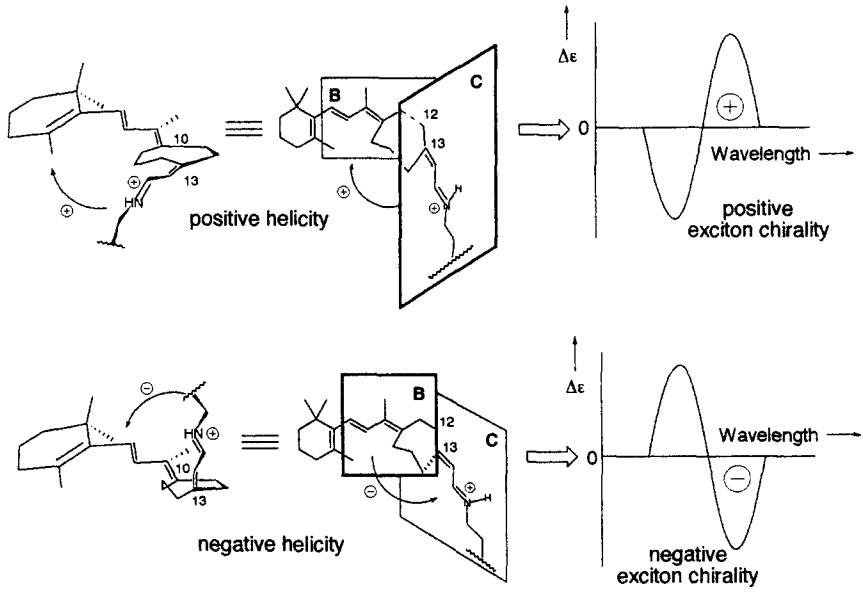
Figure 4 shows the UV of 11,12-dihydroretinal (2H-ret) (**1**) and its protonated Schiff base (PSB) with *n*-butylamine (2H-PSB) (**2**) in methanol, and the UV (A) and CD (B) of 11,12-dihydrorhodopsin (2H-Rh) (**3**) in octylglucoside solution. The pigment incorporating 11,12-dihydroretinal,

[9] M. Ito, T. Hiroshima, K. Tsukida, Y. Shichida, and T. Yoshizawa, *J. Chem. Soc. Chem. Commun.* 1443 (1985).

[10] Y. Katsuta, M. Sakai, and M. Ito, *J. Chem. Soc. Perkin Trans. I,* 2185 (1993).

[11] M. Ito, Y. Katsuta, Y. Imamoto, Y. Shichida, and T. Yoshizawa, *Photochem. Photobiol.* **56,** 915 (1992).

[12] K. Nakanishi, N. Berova, and R. W. Woody, "Circular Dichroism: Principles and Applications." VCH, New York, 1994.



Positive helicity in B / C planes gives positive couplet.
Negative helicity in B / C planes gives negative couplet.

FIG. 3. Expected signs of exciton couplet resulting from the two directions of twist between planes B and C.

2H-Rh (**3**) absorbs at 279 nm (ε 27,000) and exhibits a *negative bisignate* CD, 295 nm (Δε − 1.2) and 275 nm (Δε + 2.5), *A* value −3.7 (Fig. 4). This bisignate nature of the CD is in contrast to native rhodopsin, λ_{max} 500 nm, which exhibits two positive CD Cotton effects at 480 nm (Δε + 2.8, α-band) and 337 nm (Δε + 9.8, β-band, Fig. 2). This bisignate CD is thus interpreted as being due to the coupling between the triene and diene (protonated Schiff base, PSB) moieties and reflects the absolute sense of twist between planes B and C.

11-*Cis*-Locked Seven-Membered Ring 11,12-Dihydrorhodopsins (2H-Rh7)

The similarity in the CD and UV–vis spectra of the pigment incorporating ret7 (**4**), that is, Rh7, with native rhodopsin indicated that the conformations of the seven-membered chromophore and the native chromophore were alike.[13] Moreover, because saturation of the double bond at the center

[13] K. Nakanishi, A. H. Chen, F. Derguini, P. Franklin, S. Hu, and J. Wang, *Pure Appl. Chem.* **66**, 981 (1994).

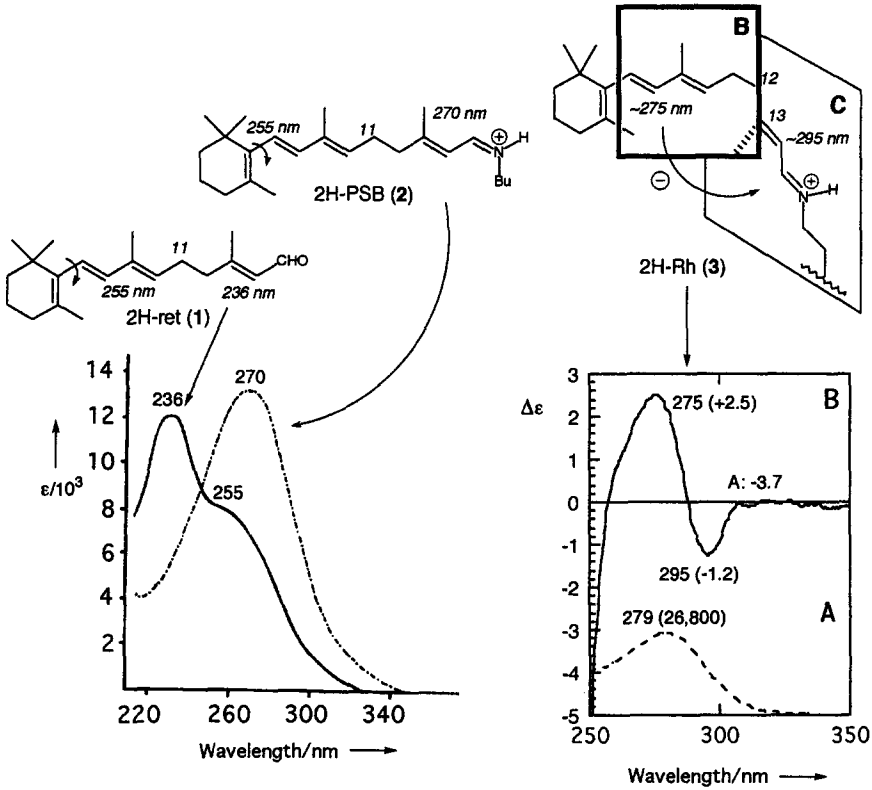


FIG. 4. 11,12-2H-ret (**1**). UV of 11,12-2H-ret and its protonated Schiff base in methanol and UV/CD of 11,12-2H-Rh in octylglucoside solution.

of the polyene chain increases the chromophoric flexibility within the binding site, a chain was bridged across C-10 and C-13 to fix the C-11/C-12 as *cis* in the seven-membered ring analogs **5** and **6** (2H-ret7). Figure 5 shows 13-*trans*-2H-ret7 (**5**), its PSB (**7**) with *n*-butylamine, and their respective UV spectra in methanol. The enal and nonplanar triene moieties in 2H-ret7 (**5**) absorb at 244 and 270 nm (shoulder) in methanol, respectively (Fig. 5). In the protonated Schiff base with *n*-butylamine, the absorption maximum of the enal is shifted to 272 nm, whereas the triene absorption stays at 270 nm.

Figure 6 shows the UV, CD, and projected structures of 13-*trans*-2H-Rh7 (**8**) and 13-*cis*-2H-Rh7 (**9**), that is, the rhodopsin analogs incorporating **5** or **6**, respectively. They absorb maximally at 283 nm (ε 27,000) and 284 nm (ε 29,000), respectively.

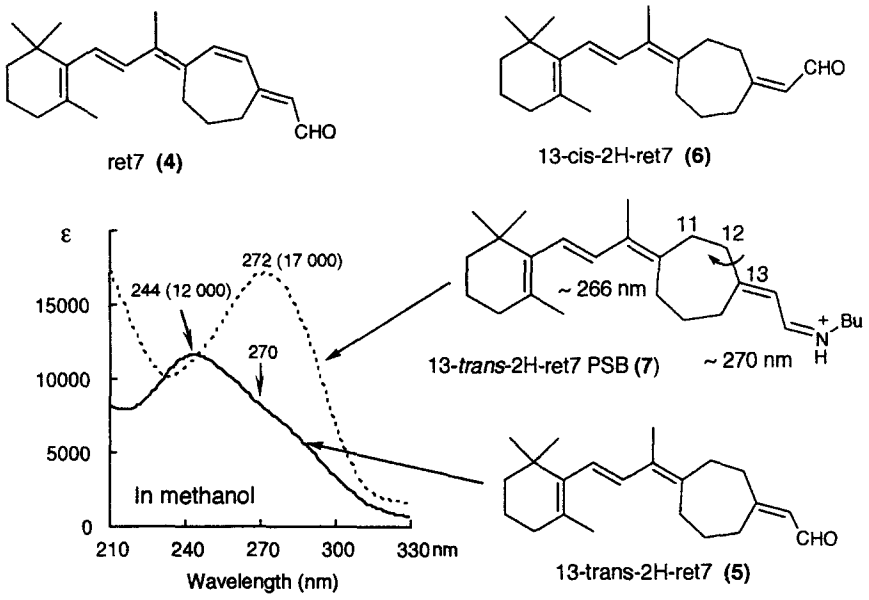


FIG. 5. Structures of ret7 (**4**), 13-*trans*-2H-ret7 (**5**), and 13-*cis*-2H-ret7 (**6**). UV of 13-*trans*-2H-ret7 (**5**) and 13-*trans*-2H-ret7 PSB (**7**).

13-*trans*-2H-Rh7 (**8**) exhibits a *negative split Cotton effect* CD at 297 nm ($\Delta\varepsilon$ − 6.5) and 273 nm ($\Delta\varepsilon$ + 5.2), that is, *A* value (amplitude) −11.7. Namely, the triene absorption originally at 270 nm is red-shifted to ca. 275 nm arising from its more planar shape in the protein, whereas the 272 nm maximum of the PSB group is also red-shifted to 295 nm due to its interaction with the global electrostatic charge within the binding site. This is further supported by the larger *A* value of −14.2 for the pigment 13-*cis*-2H-Rh7 (**9**) as compared to the *A* value of −11.7 for the pigment 13-*trans*-2H-Rh7 (**8**) (Fig. 5). The projection angle of the two interacting chromophores (through the C-13/C-10 axis), is ca. 60° and ca. 20°, respectively, for 13-*cis* and 13-*trans* isomers. In agreement with the known fact that the *A* value of bisignate CD curves is maximal around 70°,[12] it follows that the *A* value of the pigment derived from the 13-*cis* isomer is larger.

The amplitude of 2H-Rh is smaller than those of the 2H-ret7-derived pigments, probably due to the more flexible nature of the side chain. Nevertheless, the fact that the CD spectra of all these dihydro pigments are negative bisignate curves shows that the triene and PSB moieties constitute a negative couplet. Thus it is concluded that the C-12/C-13 bond of the

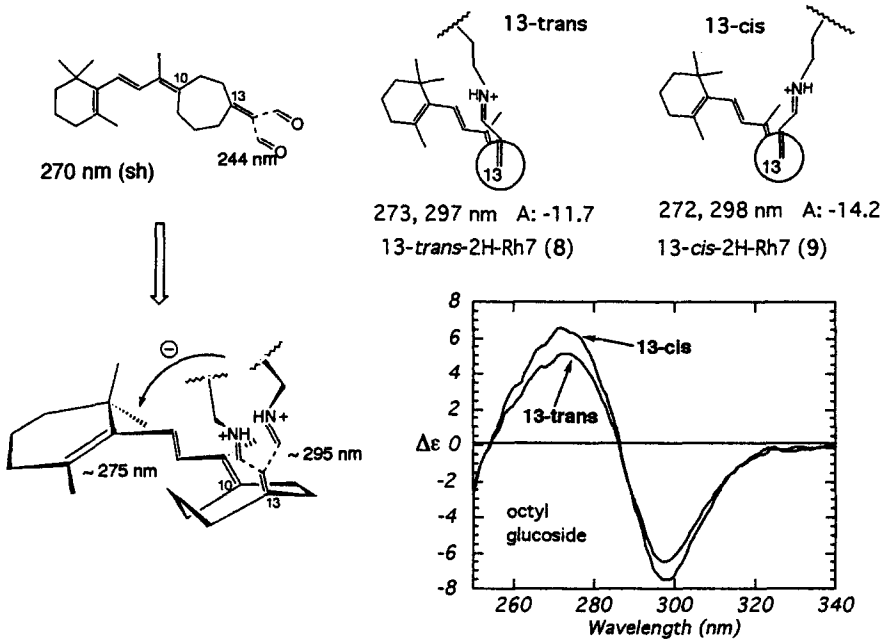


FIG. 6. Projected structures of 13-*trans*-2H-Rh7 (**8**) and 13-*cis*-2H-Rh7 (**9**) and their CD.

chromophore is twisted in a negative sense as depicted in Fig. 4 (for 2H-Rh) and Fig. 11 (given later).[14]

Absolute Conformation of 12-*s*-*trans* Bond of Retinal in Rhodopsin; 11,12-Cyclopropylretinal and Derived Pigment

The exciton-coupled circular dichroic studies of 11,12-dihydrorhodopsin pigments just described led to the absolute conformation around the 12-*s*-*trans* bond of the retinal chromophore in bovine rhodopsin.[14] Namely, the negative CD couplets of 11,12-dihydrorhodopsin pigments indicate that planes B and C are oriented as shown in Fig. 7.

This negative helicity agrees with the theoretical calculation by Kakitani and co-workers[15] and the results from the solid-state nuclear magnetic resonance (NMR) studies by Smith and co-workers.[16,17] However, Buss *et*

[14] Q. Tan, J. Lou, B. Borhan, E. Karnauknova, N. Berova, and K. Nakanishi, *Angew. Chem. Int. Ed. Engl.* **36,** 2089 (1997).
[15] H. Kakitani, T. Kakitani, and S. Yomosa, *J. Phys. Soc. Japan* **42,** 996 (1977).
[16] M. Han and S. O. Smith, *Biochemistry* **34,** 1425 (1995).
[17] M. Han and S. O. Smith, *Biochemistry* **36,** 7280 (1997).

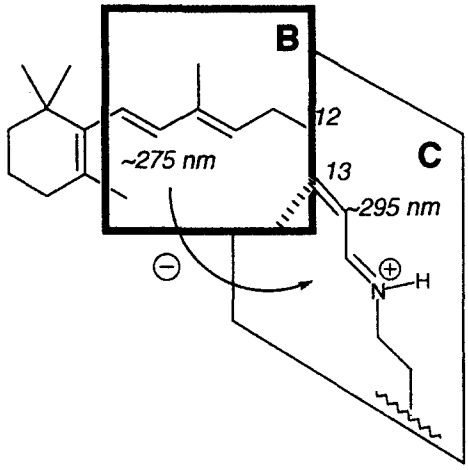


FIG. 7. Absolute sense of twist around C-12/C-13 single bond of the retinal chromophore in rhodopsin based on negative CD couplets of 11,12-dihydrorhodopsin pigments.

al. proposed a positive twist around the 12-*s-trans* bond in retinal on the basis of semiempirical and nonempirical calculations of the 11-*cis*-retinylidene chromophore.[18] To resolve the discrepancy between these theoretical calculations and to verify the assignments obtained from exciton-coupled CD and solid-state NMR data,[14,16,17] further investigation of the retinal conformation in rhodopsin was carried out. The following work represents the first case of enantioselective binding of a chiral retinal analog to bovine opsin, that is, one of the enantiomers forms a pigment with opsin while the other does not bind to opsin.[19] This bioorganic study unambiguously establishes the chirality of the retinal binding site in rhodopsin.

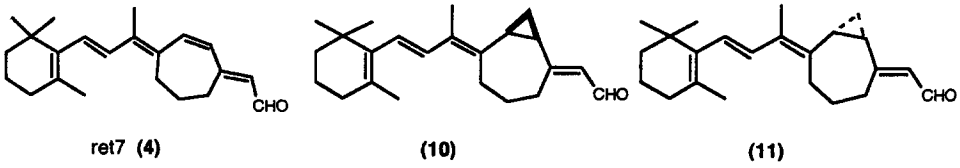
11-*cis*-Seven-membered ring-locked retinal analogs with a cyclopropyl ring incorporated to the C-11/C-12 bond (**10** and **11**) have been designed for opsin binding studies. The rationale in choosing a seven-membered-ring locking the retinal into 11-*cis* conformation lies in the earlier observation that the UV–vis and CD spectra of rhodopsin pigment containing 11-*cis*-cycloheptatrienylidene retinal or ret7 (**4**) closely resemble those of the native rhodopsin.[20] Incorporation of a cyclopropyl ring into the C-11/C-12 bond introduces a chiral element to the retinal chromophore and induces

[18] V. Buss, K. Kolster, F. Terstegen, and R. Vahrenhorst, *Angew. Chem. Int. Ed. Engl.* **37,** 1893 (1998).

[19] J. Lou, M. Hashimoto, N. Barrow, and K. Nakanishi, *Org. Lett.* **1,** 51 (1999).

[20] H. Akita, S. P. Tanis, M. Adams, V. Balogh-Nair, and K. Nakanishi, *J. Am. Chem. Soc.* **102,** 6370 (1980).

a predetermined rigid twist around the C-12/C-13 bond. The two enantiomeric cyclopropyl retinal analogs adopt opposite twists around the C-12/C-13 bond, and hence it is expected that the enantiomer with the right geometry will bind preferably to opsin, while binding of the other enantiomer will be less favored or will not proceed at all.



ret7 (4) (10) (11)

Binding Studies with Opsin

Both cyclopropyl retinal analogs **10** and **11** absorb maximally at 266 nm in methanol, their *n*-butylamine Schiff bases absorb at 260 nm, while the protonated Schiff bases absorb at 284 nm in methanol. Retinals **10** and **11** were incubated with bovine opsin solubilized in CHAPSO/HEPES buffer in a UV sample cuvette and the progress of binding was monitored by UV–vis spectra. The concentrations of opsin and retinal analog in the two binding experiments were about the same. Generally, the binding of a retinal analog to opsin results in a red-shifted absorption band in UV that originates from formation of the protonated Schiff base within the retinal binding pocket of rhodopsin, and this is what was observed in the binding of **10** to opsin. A new band absorbing at 312 nm was formed during a 1-hr incubation at room temperature, indicating formation of cyclopropylrhodopsin pigment (Fig. 8), while for **11**, no change in UV was observed, clearly indicating that **11** does not bind to opsin (Fig. 8). The optical purity

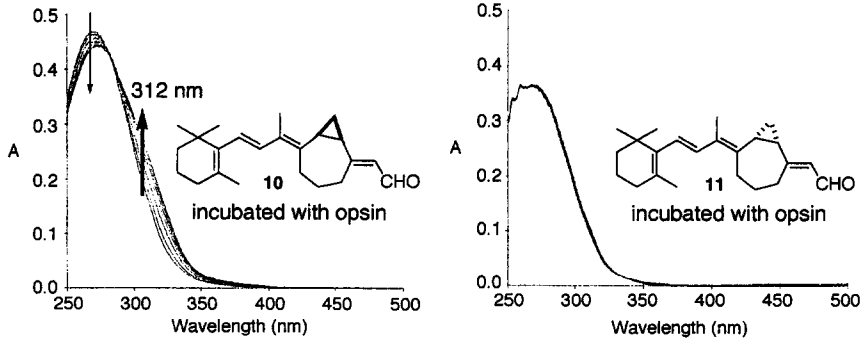


FIG. 8. Binding of **10** to opsin was monitored by UV; no binding was observed for **11**.

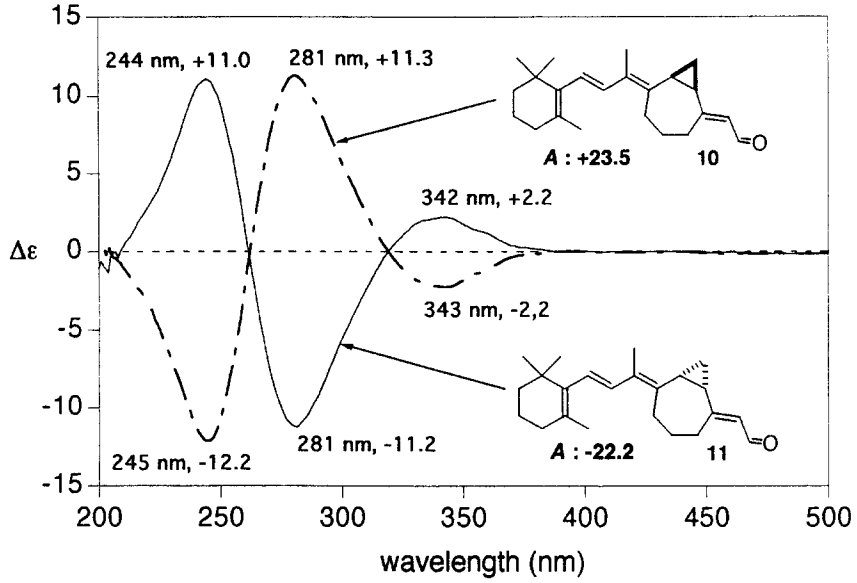


FIG. 9. CD of cyclopropylretinal analogs **10** and **11** in methanol.

of both enantiomers is about the same as seen by similar intensities of the Cotton effects in the CD (Fig. 9).

Conformational Analysis of the Cyclopropylretinal Analogs

The binding experiments of **10** and **11** with opsin clearly demonstrated the chiral preference of the retinal binding site. Analog **10** binds to opsin and forms a pigment absorbing at 312 nm, whereas **11** does not bind to opsin at all. Thus the conformation of **10** should represent the shape of the retinal binding pocket in rhodopsin. Molecular modeling by MacroModel using MM3 force field yielded the lowest energy conformations of **10** and **11** as shown in Fig. 10. The twists around C-12/C-13 single bond in **10** and **11** are opposite to each other, and for **10**, the analog that binds to opsin, there is a negative twist around the C-12/C-13 bond. This negative twist is in agreement with the above-mentioned conclusion based on exciton-coupled CD of 11,12-dihydrorhodopsin.

The pigment formed from **10** absorbs maximally at 312 nm, which is red-shifted by more than 25 nm as compared to the λ_{max} of the pigment formed from the seven-membered ring-locked 11,12-dihydroretinal analog **5** (which absorbs maximally at 285 nm).[14] Such a red shift in absorption maximum is due to the partial conjugation effect resulting from the cyclo-

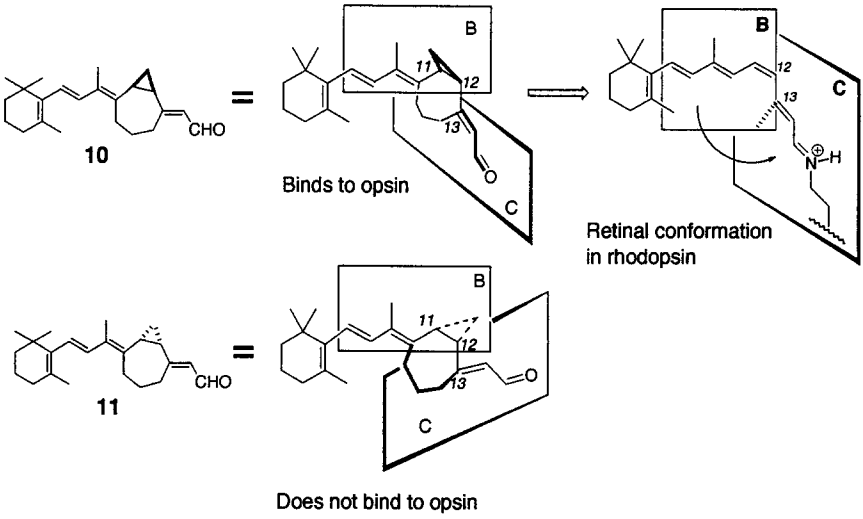


FIG. 10. Conformation of cyclopropylretinal analogs, MacroModel using MM3 force field.

propyl ring. Namely, the CD of the cyclopropyl rhodopsin pigment (not shown) does not originate from exciton coupling, which requires zero or negligible molecular orbital overlaps. Theoretical calculation of the chiroptical data of the cyclopropylretinal analogs is currently under way.

Two independent studies, chiroptical and bioorganic, show that the absolute sense of twist between planes B and C is negative as depicted in Fig. 11. Figure 11 also incorporates the conclusion of earlier photoaffinity labeling studies with 3-diazo-4-keto-11-*cis*-retinal, which showed that C-3 of the ionone ring is close to Trp-265 located around the middle of helix F.[21]

Role of 13-Methyl Group in Control of Rhodopsin Activity

The removal of the 13-methyl group from the polyene side chain has a remarkable effect on the properties of rhodopsin. As noted earlier, in the 11-*cis* isomer of retinal, steric hindrance occurs between the C-10 hydrogen and the C-13 methyl (Fig. 11) and polyene side chain twists to accommodate this interaction. Removal of the 13-methyl relieves this strain. Early reports on the pigment formed with 11-*cis*-13-demethylretinal showed that phosphodiesterase was activated in the absence of light.[22] In collaboration

[21] H. Zhang, K. Lerro, T. Yamamoto, T. Lien, L. Sastry, M. Gawinowicz, and K. Nakanishi, *J. Am. Chem. Soc.* **116,** 10165 (1994).

[22] T. G. Ebrey, M. Tsuda, G. Sassenrath, J. L. West, and W. H. Waddell, *FEBS Lett.* **116,** 217 (1980).

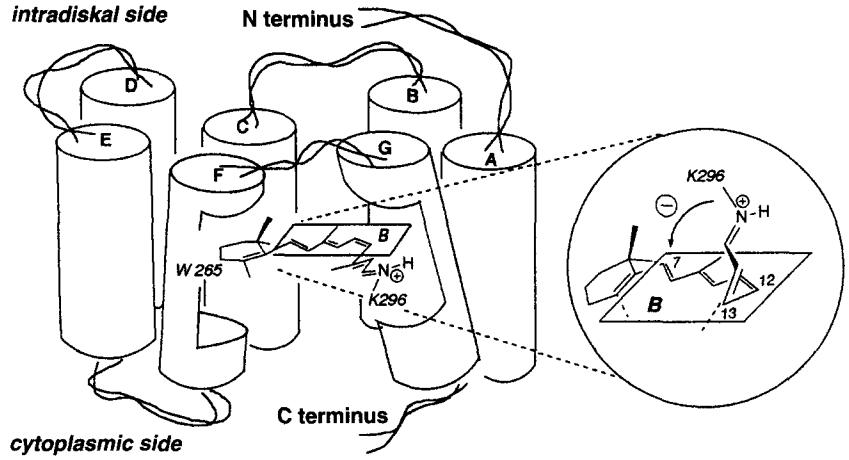


FIG. 11. Location of chromophore and the negative twist around C-12/C-13 bond in rhodopsin ground state.

with Palczewski, Saari, and colleagues, biochemical studies yielded the surprising result that on the addition of 11-*cis*-13-demethylretinal to the apoprotein opsin, rhodopsin kinase is activated (Fig. 12). Over time, this activity decreases. The native chromophore, 11-*cis*-retinal, shows no activity in the same time range. The all-*trans* isomer of the 13-demethylretinal, like the all-*trans* isomer of retinal itself, combines with opsin to form a fully active state, with phosphorylation occurring at the same sites as in rhodopsin itself. The pigment formation between 11-*cis*-13-demethylretinal and opsin is quite slow (one-ninth the rate of rhodopsin). The complex formed between the 11-*cis*-13-desmethyl retinal and the opsin places the protein into a conformation that evidently is recognized by the rhodopsin kinase as a metarhodopsin-II conformation. This results in activation of rhodopsin kinase and phosphorylation of the pigment. If the pigment is allowed to fully regenerate (24 hr) before exposure to rhodopsin kinase, no activation is obtained until the pigment is photolyzed and has similar activity to photolyzed rhodopsin. Therefore, either the 13-methyl group has a critical interaction with the protein, which facilitates the Schiff base formation, or the change in the conformation of the polyene chain as a result of the relief of the strain due to the interaction between the 10-hydrogen and the 13-methyl places the retinal in a conformation that is no longer optimum for interaction with the critical lysine.

The use of this retinal derivative has been critical to the understanding that retinal has a most precise role in the control of the activity of this G-protein receptor. A photon of light of a specific wavelength isomerizes

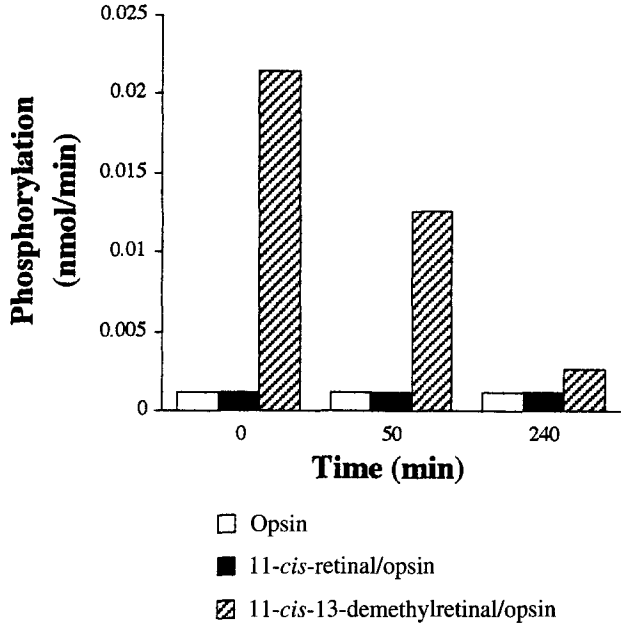


FIG. 12. Levels of phosphorylation of opsin–retinal complexes.

the critical 11-*cis* double bond to the all-*trans* conformation, bringing the receptor from its inactive state to the active state. 11-*cis*-Retinal therefore acts as an inverse agonist, locking the receptor in an inactive conformation, which of course is critical in keeping the receptor "quiet" when not receiving light. The light-induced isomerization of the chromophore would appear to be designed to break the glutamate-113/lysine-296 Schiff base salt bridge and change the conformation of the receptor to allow maximal interaction with the G protein. In the all-*trans* conformation, the retinal is acting as an agonist, activating the receptor. At least two forms of the rod rhodopsin containing the all-*trans* conformation are proposed to have activity: (1) the metarhodopsin-II (or R*) form, which is very effective in activating the transduction process (see recent review) and (2) a second form of metarhodopsin-II, proposed to be phosphorylated and bound to arrestin, having an activity of 10^{-5} that of R*.[23] The apoprotein opsin with no ligand in the binding site also has some basal activity, estimated at 10^{-7} that of R*.[24] While this is a decidedly low level,[25] it is quite meaningful on the physiologic scale, as has been demonstrated using both isolated rods and cones. A

[23] C. S. Leibrock, T. Reuter, and T. D. Lamb, *Eye* **12,** 511 (1998).
[24] M. C. Cornwall, and G. L. Fain, *J. Physiol.* **480,** 261 (1994).
[25] T. J. Melia, Jr., C. W. Cowan, J. K. Angleson, and T. G. Wensel, *Biophys. J.* **73,** 3182 (1997).

number of laboratories have also determined that combination of the apoprotein opsin with all-*trans*-retinal forms a complex that has activity resembling that of the photolyzed rhodopsin as measured by phosphodiesterase,[26] phosphorylation by rhodopsin kinase, and arrestin binding.[27] The activity of this complex does not depend on the formation of a Schiff base.[28] The role of such a complex in the physiologic process is as yet unclear.

Retinal is central to the entire visual process because it is the ligand of the visual pigments and is critical for the control of the activity of these pigments. The molecule itself is quite small (molecular weight <300) but it very precisely controls the conformation of these proteins, which in turn determines the interaction of these pigments with the various proteins involved in the transduction cascade. 11-*cis*-Retinal can be thought of in the classical pharmacologic sense as being a reverse agonist, locking rhodopsin in the inactive conformation.

Opsin	→	rhodopsin	→	metarhodopsin-II
no chromophore		11-*cis*-retinal		all-*trans*-retinal
(*partially active*)		(*inactive*)		(*active*)

The apoprotein opsin, which is lacking the chromophore and therefore this "lock" in the inactive conformation, can assume conformations that are partially active. The role of light in the process is to isomerize the 11-*cis* bond of retinal to the all-*trans* isomer, placing rhodopsin into the active meta-II conformation. In this form, the chromophore is acting as an agonist. The results reported here, as well as work in other laboratories, demonstrate that complexes formed with all-*trans* isomers of retinal and this desmethyl analog can also place the opsin protein into a meta-II conformation and thus mimic the action of light on native rhodopsin. In addition, there are retinals analogs which act as antagonists as they block the binding site but do not activate the pigment. The large body of literature on studies of pigments formed with retinal analogs has been fundamental to establishing this critical role of retinal in orchestrating the activity of this G-protein receptor.

General Procedures

General Information

Chemicals including solvents are purchased from Aldrich (Milwaukee, WI), Fisher (Suwanee, GA), and Sigma (St. Louis, MO). Water for bio-

[26] Y. Fukada and T. Yoshizawa, *Biochim. Biophys. Acta* **675,** 195 (1981).
[27] K. P. Hofmann, A. Pulvermuller, J. Buczylko, P. Van Hooser, and K. Palczewski, *J. Biol. Chem.* **267,** 15701 (1992).
[28] S. Jager, K. Palczewski, and K. P. Hofmann, *Biochemistry* **35,** 2901 (1996).

chemical procedures is double distilled and deionized. Dark-adapted retinas are obtained from W. Lawson Co. (Lincoln, NE). Centrifugation is performed on a Du Pont Sorvall RC-58 refrigerated superspeed centrifuge or a Beckman L8-M ultracentrifuge using appropriate rotors. Anhydrous solvents are either purchased or prepared by distillation over appropriate dehydrating reagents. Glassware for anhydrous purposes is flame-dried. The progress of reactions is checked by thin-layer chromatography using 250-μm precoated silica gel TLC plates from EM Separation Technology, which are visualized by phosphomolybdic staining or UV light. Flash column chromatography is carried out using 32-63 mesh silica gel from ICN. All isomers of retinal analogs are isolated by HPLC on a YMC-Pack SIL column with a size of 250 × 10 mm ID. All UV–vis spectra are recorded on a Perkin-Elmer (Norwalk, CT) Lambda 6 UV–vis spectrophotometer, and CD spectra on a JASCO J-720 spectropolarimeter, 1-cm light path cell.

Synthesis of Retinal Analogs

1. Retinal derivatives are synthesized by several laboratories. A general overall scheme employing the Horner reaction is shown in Fig. 13. Several different agents are employed both for the reduction steps

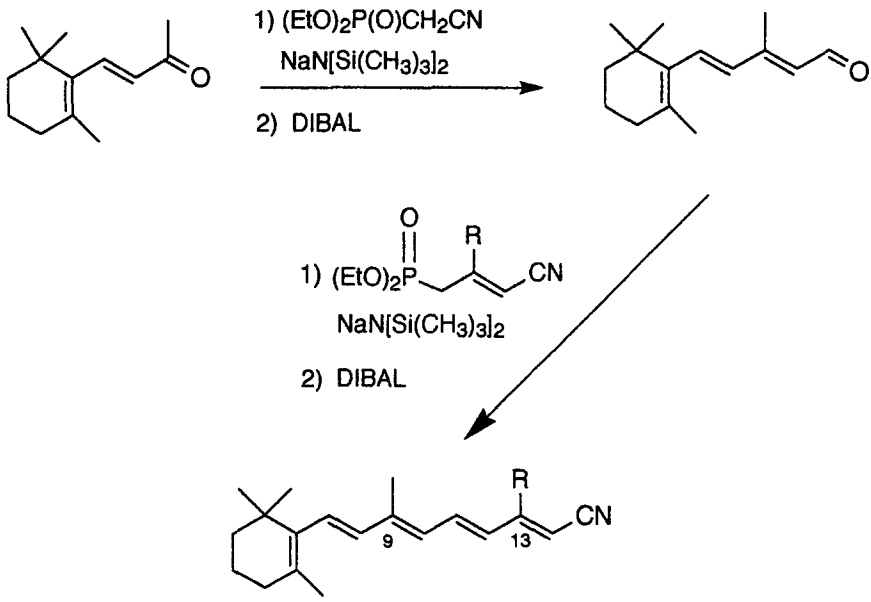


FIG. 13. General scheme for synthesis of retinal analogs.

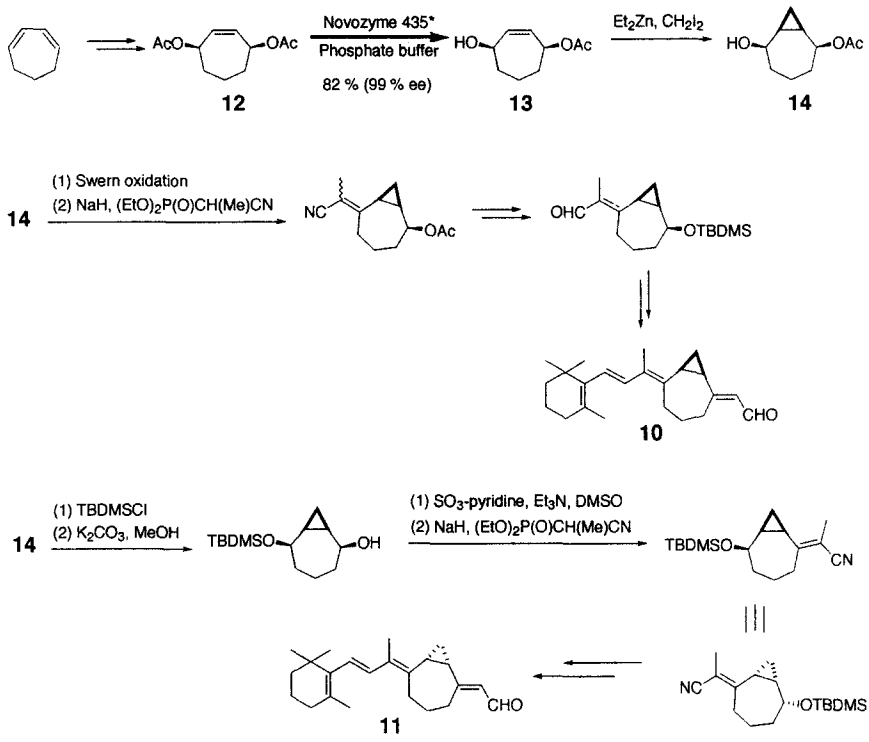


FIG. 14. Synthesis of cyclopropylretinal analogs.

as well as oxidizing agents. Other types of Wittig-based schemes are also employed (see Ref. 1 for citations to the individual preparations).

2. The cyclopropyl retinal analogs are synthesized as enantiomerically pure isomers following the route summarized in Fig. 14. The key step involves enantioselective hydrolysis of diacetate **12** by lipase Novozyme 435 to give monoacetate **13** in 82% yield (99% ee).[29] The Simmons-Smith reaction of **13** gives cyclopropyl alcohol **14**, which can be converted to both **10** and **11** through separate reaction sequences as shown in Fig. 14.

Preparation of Schiff Bases

n-Butylamine (300 μl) is added to a mixture of 0.3 μmol of retinal analog and 5 mg anhydrous K_2CO_3. After stirring for 24 hr at room temperature, *n*-

[29] C. R. Johnson and S. J. Bis, *Tetrahedron Lett.* **33**, 7287 (1992).

butylamine is removed *in vacuo,* the residue is stirred with 1 ml hexane and the K_2CO_3 filtered off. The hexane is removed *in vacuo.* The remaining Schiff base is dissolved in 1 ml absolute methanol, treated with 0.1 ml of methanolic HCl, and the UV measured.

ROS Preparation

Bovine rod outer segments (ROS) are isolated according to a standard procedure with modifications. All operations are performed at 4° under dim red light (>680 nm). Sucrose solutions are prepared with isotonic buffer (10 m*M* Tris, pH 8.0, 60 m*M* KCl, 30 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* dithiothreitol). The frozen retinas (~100) are defrosted overnight and gently shaken in 80 ml 35% sucrose solution for 1 min and centrifuged at 5000 rpm in a Sorvall SS-34 rotor for 10 min. The pellet is resuspended in 80 ml 35% (w/v) sucrose solution and centrifuged similarly. The two supernatants are combined, diluted with isotonic buffer to 26% (w/v) sucrose concentration, and centrifuged at 15,000 rpm in a Sorvall SS-34 rotor for 30 min. The pellet is resuspended in 26% (w/v) sucrose solution, and added gently to the top of a 26–35% (w/v) discontinuous sucrose gradient by a Pasteur pipette. After centrifugation at 23,000 rpm for 45 min in a Beckman SW28 swinging bucket rotor, the ROS are found at the interface of the gradient. The ROS are collected with a syringe fitted with a flat-tipped needle and washed three times with isotonic buffer. For the experiments with rhodopsin kinase, the membranes are also washed with 500 m*M* NaCl (35 min; 15,000 rpm) and then with 10 m*M* 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP), pH 7.5, containing 50 m*M* NaCl (35 min; 15,000 rpm). This procedure removes the soluble and membrane-associated proteins. The membranes are stored frozen in 10 m*M* BTP, pH 7.5, with 50 m*M* NaCl at −20°.

ROS Bleaching and Opsin Preparation

The ROS are suspended in 67 m*M* phosphate buffer (pH 7.0) containing 0.1 *M* hydroxylamine to a final concentration of 0.5–2 OD/ml. At 0° the suspension is illuminated by a projector equipped with a 470-nm cutoff filter for 30 min, or under room light for 2 hr. The bleaching is complete when the red color changes into pale yellow, and the absorption at 500 nm vanishes. Excess hydroxylamine and oxime are removed by washing with 5% (w/v) bovine serum albumin (BSA) in 0.02 *M* Tris buffer (pH 7.0) three times followed by three washes with 67 m*M* phosphate buffer (pH 7.0).

Preparation and Characterization of Artificial Pigments

All procedures are carried out at 25° in the dark. Opsin (1 OD) suspended in 426 μl 67 m*M* phosphate buffer (pH 7.0) is added to two screw-capped vials with magnetic stir bars. To one vial, 1.5 molar equivalence of retinal analog in 5 μl ethanol is added. To the other vial, 5 μl ethanol is added as a control reference. Both mixtures are stirred for 5 hr and then centrifuged at 25,000 rpm for 15 min. In some cases the pellets are washed with 1% BSA to remove excess chromophore. The pellets are either suspended in phosphate buffer or dissolved in 1 ml 23 m*M* octylglucoside solution (67 m*M* phosphate buffer, pH 7.0) and centrifuged at 25,000 rpm for 10 min. The supernants are analyzed by UV and CD measurements. The amount of opsin in each supernatant is determined from the A_{500} value after reconstitution with 11-*cis*-retinal.

Activation of Rhodopsin

Several assays for the measurement of the activation of rhodopsin or rhodopsin analog pigments have been reported in the literature. Activation of rhodopsin kinase provides one convenient monitor for level of activation of rhodopsin. Further, this method provides the potential of determining if the activated conformation of the analog pigment is identical to that of the native rhodopsin as the sites of phosphorylation can be determined.[30] The assay has been described in detail elsewhere.[31] Rhodopsin kinase is expressed in insect *Spodoptera frugiperda* cells and purified by chromatography on DEAE-cellulose. The activity is measured by the P_i transferred per minute per milligram rhodopsin or analog pigment using [γ-^{32}P]ATP. The reaction is terminated by the addition of potassium phosphate buffer (250 m*M*, pH 7.2) containing 200 m*M* EDTA, 100 m*M* KF, 5 m*M* adenosine, and 200 m*M* KCl.

Acknowledgments

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[30] D. I. Papac, J. E. Oatis, Jr., R. K. Crouch, and D. R. Knapp, *Biochemistry* **32,** 5930 (1993).

[31] K. Palczewski, *Methods Neurosci.* **15,** 217 (1993).

[32] J. Buczylko, J. C. Saari, R. K. Crouch, and K. Palczewski, *J. Biol. Chem.* **271,** 20621 (1996).