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Bioactivity of Visual Pigments with Sterically Modified Retinal Analogs

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Studies of several retinal analogs and the rhodopsin pigments incorporating them, lead to further clarification of the structural requirement of the retinal chromophore in the photoactivation process of rhodopsin. The data with a pigment incorporating an acyclic retinal show that the cyclohexene moiety is not required for formation of a stable pigment, which, however, has a reduced photoactivity. Our data also show that extra methyl groups at 13- and 14-positions of 11-*cis* retinal reduce the rate of retinal binding, photoactivity, and stability of the pigments. These data, taken together, give rise to a clear picture about the binding environment of the retinal chromophore and how retinal interacts with and activates the protein following its photoisomerization. The opsin/11-*cis* retinal complex has evolved into an ideal system, which is capable of converting photoenergy into Meta-II formation with high efficiency, critical for visual transduction.

Key Words: synthetic retinals; rhodopsin; phosphodiesterase assay; photoactivity.

INTRODUCTION

Bovine rhodopsin (*1*), λ_{max} 500 nm, the best studied of the visual pigments because of its availability in large quantities, was the first to be sequenced (*2–4*). Its single polypeptide chain consists of 348 amino acid residues arranged into seven transmembrane α-helices. An 11-*cis* retinal chromophore is linked to the terminal amino group of Lys 296 via a protonated Schiff base. The light-induced primary photochemical event, retinal 11-*cis* to all-*trans* isomerization (*5–7*), triggers a series of protein conformational changes driven by retinal–protein interaction, resulting in the deprotonation of the Schiff base to yield metarhodopsin II (Meta-II), λ_{max} 380 nm (*8–12*). Meta-II, the active form of rhodopsin, binds and activates the G-protein transducin, thus initiating the enzymatic cascade of the visual transduction process. Transducin activates phosphodiesterase, which in turn catalyzes the hydrolysis of cGMP.

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Despite the progress in our understanding of biochemical events of visual transduction, the rhodopsin structure and molecular mechanism of rhodopsin activation triggered by 11-cis-retinal isomerization is still obscure. In the dark static state, the chromophoric ionone ring is close to Trp 265 in the middle of helix F (*13*), showing that the chromophore lies near the center of the lipid bilayer with its polyene sidechain roughly horizontal to the membrane plane. Recent CD results have also indicated that the absolute sense of twist around the 12-s-bond is negative (*14*) (see Figs. 3 and 5). Examination of photochemical properties and enzymatic activities of 11-*cis* locked rhodopsin analogs (*15–19*) have demonstrated that activation by light requires complete 11-*cis* to all-*trans* isomerization with full involvement of retinal–receptor interactions.

The retinal chromophore with its trimethyl cyclohexene moiety, methylated polyene chain, nonplanar 6-s-*cis* and 12-s-*trans* bonds, unstable 11-*cis* geometry, and protonated Schiff base (PSB) linkage formed with Lys-296 is ideally suited for visual transduction and wavelength regulation (*20*). Examination of the biochemistry of pigments incorporating several retinal analogs has clarified further structural aspects of the chromophore with respect to the rhodopsin function. Our data below show that although the cyclohexene ring is not required for retinal binding to form a stable pigment, its interaction with the protein enhances the efficiency of the retinal photoisomerization and the protein activation. On the other hand, introduction of extra alkyl groups at 13- and 14-positions of 11-*cis* retinal reduces the rate of retinal incorporation into rhodopsin, the stability of the chromophore, and photoactivation of the pigments.

MATERIALS AND METHODS

General

Fresh bovine retinae were purchased from J. A. Lawson Co. (Lincoln, NE) and stored frozen at −70°C. ATP, ADP, dithiothreitol (DTT), Concanavalin A–Sepharose 4B, methyl α-D-mannopyranoside, protease inhibitors, 1-α-phosphatidylethanolamine (PE) from bovine brain, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (Hepes), 3-[(3-Cholamidopropyl)dimethylamino]-2-hydroxy-1-propanesulfonate (Chapso), phenylmethanesulfonyl fluoride (PMSF), and digitonin were purchased from Sigma. Other chemicals were purchased from Aldrich.

Preparation and Purification of 11-cis-retinal Analogs

Acyclic retinal **2** was prepared via two- and five-carbon extension reactions from the acyclic β-ionone analog obtained from aldol condensation of 2-isopropyl-3-methyl-2-crotonaldehyde with acetone (*21*). 14-Methyl retinal **3** and 13-ethyl retinal **4** were synthesized as described (*22*, *23*). After HPLC purification, the all-*trans* retinals were dissolved in acetonitrile (1 mg/ml) and irradiated for 2–3 h at 0°C by a 500 W Sylvania tungsten lamp with a 430-nm cut-off filter. The 11-*cis* isomers were isolated by semiprep YMC Silicagel HPLC (250 × 5 mm, SIL) using 5% ethyl acetate in hexane, isocratic elution, and their structures were confirmed by NMR.

Preparation of Visual Pigments and Enzymes

Bovine Rod Outer Segment (ROS) were isolated from 200 frozen retinae by the flotation method (*15*,*24*) in sucrose solution prepared from isotonic buffer A

(10 mM Tris, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 0.1 mM PMSF, 1 mM DTT, pH 8.0). The enzymes were extracted twice by hypotonic buffer B: 10 mM Tris, pH 8.0, 2 mM EDTA, 2 mM dithiothreitol, 2 mg each of soybean trypsin inhibitors, aprotinin, benzamidine, leupeptin, and pepstatin A in 100 ml buffer. The stripped ROS and the enzyme extract thus obtained were further concentrated for bioassay. The stripped ROS can be stored at −70°C for future use. Bleaching of ROS was performed in hypotonic Hepes buffer (pH 7.0) containing 100 mM hydroxylamine on ice-bath under day light for 4 h. Excess hydroxylamine was removed through five washings with 40 ml each of buffer B. For qualitative preparation of rhodopsin analogs for phosphodiesterase assay and CD measurements, freshly prepared opsin was suspended (ca. 3 OD/ml) in 10 mM Hepes buffer C (10 mM Hepes, 50 mM DTPA, 0.1 mM PMSF, 1 mM DTT, pH 7.5), and this was treated with 2 OD equivalents of retinal dissolved in a small amount of ethanol (<2% v/v). In case of acyclic retinal, the mixture was incubated in the dark at room temperature and stirred overnight. For 14-methyl retinal (**3**) and 13-ethyl retinal (**4**), a 10-h incubation at 37°C was required. As a control, native rhodopsin was regenerated under the same conditions. To obtain pigments for spectroscopic measurement and stability studies, the regenerated pigments in Hepes buffer were spun down at 45,000 rpm, 30 min, and solubilized in 2% digitonin, followed by purification on a Concanavalin A–Sepharose gel column at 4°C under dim red light as described (*25*). The binding rate was estimated from the increment of the pigment absorption around 500 nm (*26*).

UV/VIS and CD Measurement

UV/Vis spectra of the pigments were recorded on a Perkin–Elmer Lambda 6B UV/Vis spectrophotometer. CD measurements were performed on a JASCO J-720 spectropolarimeter from 250 to 600 nm, 1 cm cell; an average of four scans were performed rapidly per measurement to decrease the possible bleaching of rhodopsin samples by the measuring light. The concentration of the rhodopsin analog samples was adjusted to 0.4 OD/ml. The pigment spectra were obtained by subtracting the bleached ROS spectra as a baseline.

Quantum Efficiency Measurements

Quantum efficiency measurements (*27*) were performed by monitoring the bleaching of purified pigments under irradiation, each for 1–3 min, total time of bleaching 30 min. The samples in cuvettes, 0.2 OD/ml and containing 10 mM NH_2OH, were irradiated by a 160 W Sylvania tungsten halogen lamp stabilized by a 250 W SOLA constant voltage transformer. The dynamics of bleaching was recorded using a Perkin–Elmer IF 320 UV/Vis spectrophotometer. The irradiation light was filtered by a 530 ± 10-nm interference filter and controlled by a shutter and built-in mirror, which was mounted at 45° with respect to both measuring light and irradiation light. The slopes of the plot of $-\log (10^{At} - 1)$ against total irradiation time were directly proportional to the photosensitivity of the pigments. Quantum efficiency of the pigments was calculated from the known quantum

efficiency of rhodopsin (0.67) (*28*) and the molar extinction coefficient of the corresponding pigments.

Phosphodiesterase Assay

Phosphodiesterase assay was performed according to the protocol developed by Liebman and Ivanczsuk (*29*) under dim red light with some modifications described below. The pigment (80 μM/ml in 10 mM Hepes buffer) was mixed with isotonic buffer D (10 mM Hepes, 60 mM KCl, 30 mM NaCl, 2 mM $MgCl_2$, 0.1 mM PMSF, 1 mM DTT, pH 8.0) to give a final concentration of 6 mM/ml. The suspension was then treated with 1.2 equivalent of enzyme extract, usually 10–30 μl, depending on the final volume of the concentrated enzyme extract and the total amount of ROS used for extraction. After incubation for 10 min at room temperature, GTP was added and pH of the mixture was adjusted to 8.00 $\pm$ 0.05 by 1 M KOH solution. After addition of cGMP the mixture was monitored for 0.5 min to check the level of dark activity using a Sensorex S900c fast pH electrode coupled through an Orion 811 pH meter to a Servo Chessell 321 chart recorder. After exposure of the mixture to a suitably attenuated flash of light, Xenon flash-Metz-Mecablitz 45 CL, the light-induced phosphodiesterase activity or light activity was monitored for 1–2 min. For these measurements, 10 mM GDP, 10 mM GTP, and 50 mM cGMP stock solutions in isotonic Hepes buffer prepared in 1- to 2-ml aliquots were used. The flash bleached about 20% of rhodopsin under bioassay conditions. The intensity of light was attenuated to 25, 2.5, 0.625, and 0.25% by combination of neutral density filters, Kodak 0.6, 1, and 2 ND. The conversion factor between pH change and the proton released was obtained by HCl titration of a mixture of 900 μl of the isotonic buffer and 100 μl hypotonic buffer, pH 8.0, which corresponded to buffer components of the bioassay mixture. The conversion factor ΔpH/ΔmM HCl was 0.162 mM^{-1}. The curves of pH versus time were converted to cGMP hydrolyzed versus time.

RESULTS

Binding of Retinal Analogs with Apoprotein

Acyclic retinal **2**, 14-methylretinal **3**, and 13-ethylretinal **4** bind to opsin to form acyclic Rh, 13-Et-Rh, and 14-Me-Rh, respectively, the UV/Vis spectra of which are shown in Fig. 2. The binding of retinals is much faster in 10 mM Hepes than in isotonic buffer. In 10 mM Hepes buffer the binding of acyclic retinal **2** was over within 10 min, while in isotonic buffer at 10°C, the binding of **2** and 11-*cis* retinal **1** required, respectively, 10 and 30 h. The rapid binding of acyclic **2** should be noted because there appears to be no report of a synthetic retinal analog having a higher binding rate than 11-*cis* retinal.

14-Methylretinal **3** and 13-ethylretinal **4** bind to opsin much slower than 11-*cis* retinal. At 37°C in 10 mM Hepes, the regeneration was complete in 3 and 1 h, respectively, for **3** and **4**, while at 25°C, both required a 24-h regeneration period. Thus the extra alkyl groups in 14-methylretinal **3** and 13-ethylretinal **4** hinder the 11-*cis* retinal from entering the binding cavity.

CD Spectra of the Pigments

The retinal chromophore in rhodopsin, λ_{max} 498 nm, shows strong positive CD Cotton effects at 490 (α-band) and 340 nm (β-band) (Fig. 3a, curve 2). The observed

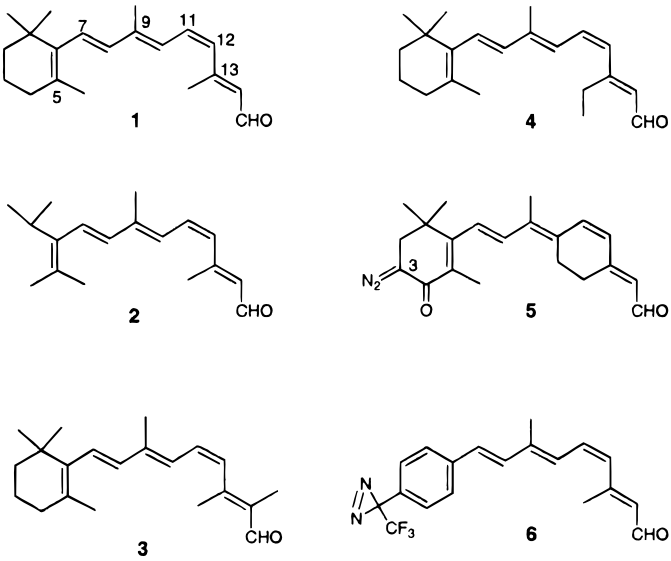

FIG. 1. Structures of 11-*cis* retinal and its analogs.

chirality can be attributed to the intrinsic asymmetry of the chromophore induced by the protein chiral environment. As shown in Fig. 3b, the 11-*cis* chromophore is twisted around the 6-s and 12-s bonds due to the steric interaction between 5-Me and 8-H, and negatively twisted between 13-Me and 10-H (*14,17,30,31*). Ito and coworkers

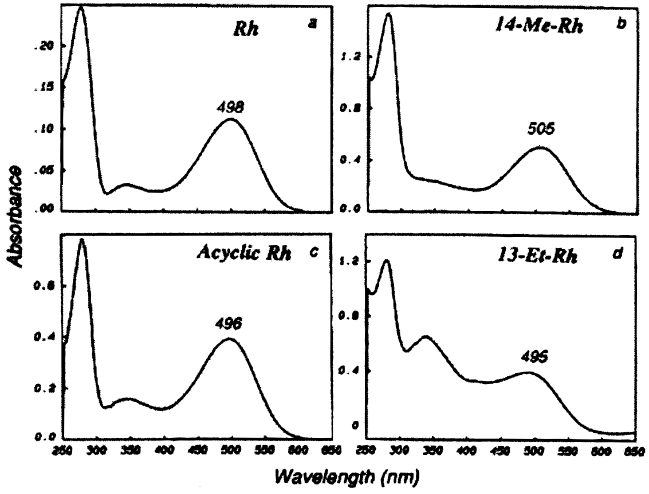

FIG. 2. UV/Vis spectra of the pigments: (a) rhodopsin (Rh), (b) 14-methylrhodopsin (14-MeRh), (c) acyclic rhodopsin (acyclic Rh), (d) 13-ethylrhodopsin (13-Et-Rh, which contained an impurity absorbing around 340 nm and hard to be washed off the Concanavalin A–Sepharose column during purification).

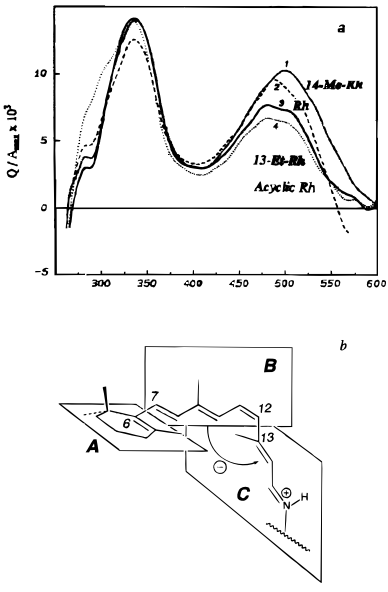

FIG. 3. (a) Circular dichroic spectra (CD) of rhodopsin and the pigments: (1) 14-methylrhodopsin; (2) rhodopsin; (3) 13-ethylrhodopsin; (4) Acyclic rhodopsin. (b) Retinal adopts a 6-*s*-*cis* and 12-*s*-*trans* conformation in rhodopsin. The distortions around 6–7 and 12–13 single induce the CD β-band (around 340 nm) and CD α-band (around 480 nm), respectively.

observed that a rhodopsin incorporating a retinal analog in which B/C planes adopt a planar structure via a five-membered bridge between C-10 and 13-Me exhibit a negligible 480 nm CD band; similarly, the pigment formed from a retinal with planar A/B conformation via a five-membered bridge between 5-Me and C-8 showed a weak 337 nm CD band. Based on these observations, the α- and β-bands have been ascribed to the distortions around 12-s and 6-s bonds, respectively (*32*,*33*).

The overall simlarity of the UV/Vis (Fig. 2) and CD (Fig. 3a) spectra of all four rhodopsin pigments indicates that the conformations and electrostatic surroundings of the retinal chromophore within the protein chiral environment are similar. It is noteworthy that the 340 nm CD β-band of the pigment incorporating acyclic retinal **2** (Fig. 3a, curve 4) is similar to that of rhodopsin (Fig. 3a, curve 2), showing that the incomplete retinal cyclohexene ring still preserves the key elements to interact with the protein leading to a similar twist around the 6-s bond.

Certain differences in CD spectra are also noted. 13-Et-Rh has a smaller CD α-band around 480 nm (Fig. 3a, curve 3), suggesting a less twisted 12-s bond as compared to rhodopsin. This is notable since a larger 12-s twist was expected as the steric hindrance between 10 and 13 position is larger in 11-*cis* 13-Et retinal than in native 11-*cis* retinal. Clearly factors other than the intrinsic properties of retinal, namely, the protein–retinal interactions, play important roles in determining the retinal chromophore conformation.

Stability against Heat and NH_2OH

The stability of visual pigments against heat and NH_2OH, which reflect the overall stability of the chromophore and accessability of the Schiff base region to hydroxylamine (*25*), were measured by monitoring the 500 nm peak of purified pigment in 2% digitonin at 37°C, and in 100 mM NH_2OH at 0°C, respectively.

The stability of 14-Me-Rh is drastically reduced, both thermally and against NH_2OH (Figs. 4a and 4b); this can be accounted for, respectively, by increased steric congestion (*21*) and exposure of the protonated Schiff base linkage. In contrast, in the case of 13-Et-Rh, the increased steric congestion could be balanced by a tighter binding arising from increased hydrophobic interaction, thus leading to unchanged stability relative to Rh (Fig. 4a). However, as in the case of 14-Me-Rh, the protonated Schiff base linkage could be more exposed, leading to increased susceptibility to NH_2OH.

As shown in Fig. 4, acyclic Rh was slightly more stable than Rh against temperature and NH_2OH, which in addition to the faster binding of acyclic retinal **2**, suggests a more energy-favorable chromophore–opsin interaction.

Photoactivity

The photoactivities of the pigments were evaluated by the PDE activity representing the entire photoactivation process, and the quantum efficiency of the primary photoactivation event, i.e., the retinal 11-ene isomerization. The results of these measurements are summarized in Table 1.

The very high quantum efficiency (0.67) of rhodopsin is dependent on the retinal–receptor interaction as well as the intrinsic properties of 11-*cis* retinal. It has been postulated that the steric interaction between the substituents at positions 10 and 13 in 11-*cis* retinal facilitates the rapid photoisomerization of the visual chromophore (*34*). Indeed, removal of this steric effect leads to a remarkable loss in quantum efficiency, reducing it to 0.30 for 13-desmethyl (13 dm) rhodopsin (*35*). Introduction of a methyl group at C-10 in 13 dm rhodopsin increases the quantum efficiency back

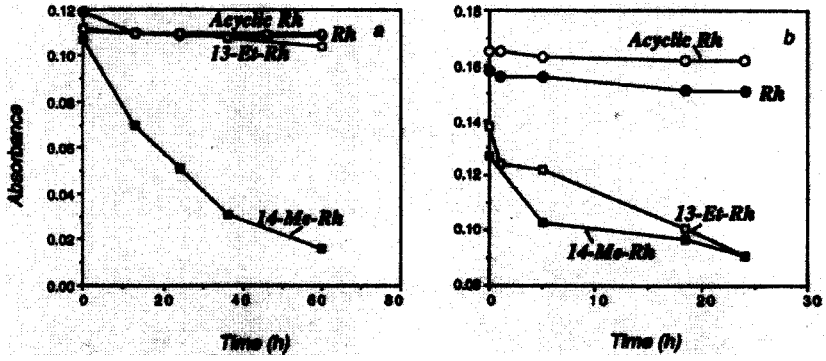

FIG. 4. The stability tests against (a) heat, 37°C, and (b) NH_2OH. Both rhodopsin and the analogs were purified on Concanavalin A–Sepharose column in 2% digitonin with 100 mM methyl α-mannoside and the absorbance at the λ_{max} was monitored. For NH_2OH-stability test, rhodopsin, and each pigment samples contained 100 mM NH_2OH.

TABLE 1

UV/Vis Data, Quantum Efficiency, and Light-Dependent PDE Activity of Visual Pigments

	Rhodopsin	Acyclic Rhodopsin	14-Methyl Rhodopsin	13-Ethyl Rhodopsin
ε_{max}	40,600	45,200 ± 600[a]	38,000 ± 5000[b]	36,000 ± 200[a]
γ_{max}(nm)	498	496	505	495
Relative PDE Activity	100%	80%	80%	100%
Quantum Efficiency	0.67[c]	0.48	0.55[c]	0.52

[a] The ε values at the absorption peaks of acyclic rhodopsin and 13-ethylrhodopsin were obtained by comparing the absorbance of regenerated pigments from 11-*cis*-retinal (**1**), 11-*cis* acyclic retinal (**2**), and 11-*cis* 13-ethylretinal (**3**), with the same opsin preparation in 10 mM Chapso/10 mM Hepes (pH 7.0).

[b] Ref. (*49*); the quantum efficiency was recalculated based on the ε and relative photosensitivity of rhodopsin and 14-methylrhodopsin at 560 nm.

[c] Ref. (*28*).

to 0.59 (*36*). In our experiments, however, increasing the steric hindrance by replacing the 13-Me with a bulkier 13-Et reduces the quantum efficiency of the rhodopsin pigment to 0.52, suggesting that other factors promoting the retinal photoisomerization, likely the correct protein–receptor interactions, have been disturbed by the extra bulk at 13-position.

Despite a lower quantum efficiency for the primary photoactivation, 13-EtRh exhibits a light-dependent PDE activity similar to native rhodopsin. It is possible that a 13-Et is more efficient than a 13-Me for inducing the protein conformational changes following retinal isomerization, therefore offsetting the loss of quantum efficiency of the retinal isomerization to reach a similar overall photoactivity.

With acyclic Rh and 14-MeRh, the PDE activities and the quantum efficiencies are lower than with rhodopsin. It is not clear whether the low activities of PDE originate from low quantum efficiencies or a less active protein conformation at the Meta-II stage, or both. Nevertheless, an incomplete cyclohexene ring and an extra bulk at 14-position must disrupt the normal receptor–retinal interaction and, therefore, the efficiency of the photoactivation process.

DISCUSSION

The retinal binding pocket has been shown to be flexible to accommodate a variety of retinal analogs. It was reported in 1984 (*37*) that retinal analogs without the cyclohexene ring could still bind to opsin, while Liu and coworkers (*21*,*38*) made semiquantitative comparisons of the binding rate of synthetic retinal analogs, among which acyclic retinal **2** showed efficient binding; this has been confirmed in our studies. 14-Methylretinal analog **3** (*23*) also binds with opsin to form stable rhodopsin pigments. Opsin can also incorporate retinal analogs containing 5- to 9-membered rings bridging C-10 and C-13 (*15*), thus showing that the binding domain in the vicinity of C-13 is relatively loose. The facile formation of 13-Et-Rh is thus not surprising.

It has been shown that although the protein binding cavity is flexible to accommodate structurally modified retinals, it frequently leads to less efficient photoactivation and/or less stable pigments in the ground state (*21*,*39*). This is also supported by the

present finding that the stability and quantum efficiencies of 14-Me-Rh and 13-Et-Rh are lower than those of rhodopsin; the light-dependent PDE activity of 14-Me-Rh is also smaller.

The fast incorporation and high stability of acyclic retinal **2** suggests that deletion of part of the cyclohexene ring yields a flexible molecule where the chromophore–opsin interaction that could impede the binding process is absent. The photoactivity of the formed pigment, however, is lower, demonstrating that the ionone moiety with its hydrophobic methyl groups is essential for an efficient retinal → protein transmittal of structural changes induced by the chromophoric isomerization.

The changes in retinal chromophore and opsin 3D interactions trigerred by the 11-*cis* to all-*trans* isomerization is central for activation of rhodopsin. Our photoaffinity labeling experiment using the ring-locked 11-*cis* retinal analog **5**, which cross-linked cleanly to Trp-265 (in the middle of helix F, Fig. 5) has shown that in the ground state, the cyclohexene ring of the retinal chromophore is in close contact with helix F (*13*). In contrast, photoaffinity labeling of rhodopsin by Nakayama and Khorana using a photoisomerizable trifluoromethyldiazirene analog **6** led to two and six cross-linking sites on helices F and C, respectively(*40*), whereas in their site-directed spin labeling studies, Hubbell and Khorana and coworkers observed specific rigid-body motions of helices C and F upon photoactivation (*41–44*). Recent photocrosslinking studies performed sequentially at −196°C to 0°C with the Batho-, Lumi-, Meta-I, and Meta-II Rh intermediates, using 11-*cis* 3-diazo-4-oxoretinal (diazoketo **5** without the bridging ethylene chain) have shown that the cross-linked amino acids in Batho-Rh is Trp-265 (i.e., same as in resting state rhodopsin); after Lumi-Rh and subsequent intermediates, the diazoketo photolabile group binds exclusively to Ala-169 (in helix D). Namely, the transition of Batho-Rh to Lumi-Rh is accompanied by a flip-over of the cyclohexene moiety of the retinal chromophore so that the bridging changes from Trp-265 to Ala-169. This shows that helices C and D move, respectively, outward

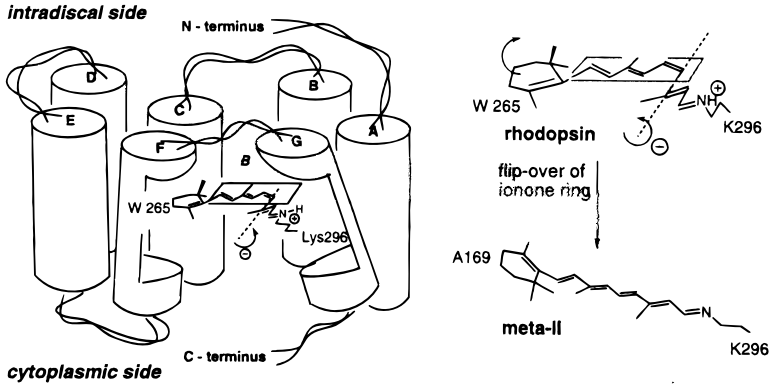

FIG. 5. The ionone ring (C-3) of the chromophore in the rhodpsin binding site is close to Trp-265 located around the middle of helix F; the negative absolute twist around the 12-*s*-bond is also depicted. In lumi-Rh and subsequent intermediates, the ionone ring flips over and C-3 becomes close to Ala-169 in helix D. Protein conformational changes accompanying these changes activated the G protein.

and inward and it is these movements of helices and extramembrane loops that activate the G protein (M. Souto and B. Borhan, manuscript in preparation).

With flexible analog **2** the incomplete cyclohexene ring is in less tight contact with helices, and therefore the retinal/opsin interaction is inefficient in moving the helices for activation. This is in agreement with our recent studies (*45*) regarding the mechanism of transient dark activity of opsin induced by 11-*cis* 13 desmethyl retinal (13 dm). Namely, it was shown that interaction of the retinal ring moiety with the opsin β-ionone binding domain (*46–47*) is required for the formation of the Meta II-like active species of the protein in the dark. In summary, the opsin/11-*cis* retinal complex has evolved into an ideal system, which is capable of converting photoenergy into Meta-II formation with high efficiency, critical for visual transduction.

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REFERENCES

1. Ottolenghi, M., and Sheves, M., (Eds.) (1995) *Isr. J. Chem.* **35** (Special issue of Photophysics and Photochemistry of Retinal Proteins).
2. Ovchinnikov, Y. A. (1982) *FEBS Lett.* **148,** 179–191.
3. Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S. L., Rao, J. K., and Argos, P. (1983) *Biophys. Struct. Mech.* **9,** 235–244.
4. Tang, L., Ebrey, T. G., and Subramaniam, S. (1995) *Isr. J. Chem.* **35,** 193–209.
5. Wald, G. (1968) *Nature* **219,** 800–807.
6. Stryer, L. (1991) *J. Biol. Chem.* **266,** 10711–10714.
7. Chabre, M. (1985) *Annu. Rev. Biophys. Biophys. Chem.* **14,** 331–360.
8. Hofmann, K. P. (1986) *Photobiochem. Photobiophys.* **13,** 309–327.
9. Yoshizawa, T., and Shichida, Y. (1982) *Methods Enzymol.* **81,** 333–354.
10. Yoshizawa, T., and Shichida, Y. (1982) *Methods Enzymol.* **81,** 634–641.
11. Kliger, D., and Lewis, J. (1995) *Isr. J. Chem.* **35,** 289–307.
12. Kochendoerfer, G., and Mathies, R. (1995) *Isr. J. Chem.* **35,** 211–226.
13. Zhang, H., Lerro, K. A., Yamamoto, T., Lien, T. H., Sastry, L., Gawinowicz, M. A., and Nakanishi, K. (1994) *J. Am. Chem. Soc.* **116,** 10165–10173.
14. Tan, Q., Lou, J., Borhan, B., Karnaukhova, E., Berova, N., and Nakanishi, K. (1997) *Angew. Chem. Int. Eng. Ed.* **36,** 2089–2093.
15. Hu, S., Franklin, P. J., Wang, J., Ruiz Silva, B. E., Derguini, F., Nakanishi, K., and Chen, A. H. (1994) *Biochemistry* **33,** 408–416.
16. Mizukami, T., Kandori, H., Shichida, Y., Chen, A. H., Derguini, F., Caldwell, C. G., Biffe, C. F., Nakanishi, K., and Yoshizawa, T. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4072–4076.
17. Fukada, Y., Shichida, Y., Yoshizawa, T., Ito, M., Kodama, A., and Tsukida, K. (1984) *Biochemistry* **23,** 5826–5832.
18. De Grip, W. J., Van Oostrum, J., Bovee-Geurts, P. H. M., Van der Steen, R., Van Amsterdam, L. J. P., Groesbeek, M., and Lugtenburg, J. (1990) *Eur. J. Biochem.* **191,** 211–220.
19. Bhattacharya, S., Ridge, K. D., Knox, B. E., and Khorana, H. G. (1992) *J. Biol. Chem.* **267,** 6763–6769.
20. Nakanishi, K. (1991) *Pure Appl. Chem.* **63,** 161–170.
21. Liu, R. S. H., and Asato, A. E. (1990) *in* Chemistry and Biology of Synyhetic Retinoids (Dawson, M. I., and Okamura, W. H., Eds.), pp. 51–75, CRC Press, Cleveland, OH.
22. Nakanishi, K. (1985) *Pure Appl. Chem.* **57,** 769–776.
23. Chan, W. K., Nakanishi, K., Ebrey, T. G., and Honig, B. (1974) *J. Am. Chem. Soc.* **96,** 3642–3644.
24. Papermaster, D. S. (1982) *Methods Enzymol.* **81,** 49–52.
25. De Grip, W. J. (1982) *Methods Enzymol.* **81,** 256–265.
26. Cusanovich, M. A. (1982) *Methods Enzymol.* **81,** 443–447.
27. Dixon, S. F., and Cooper, A. (1987) *Photochem Photobiol* **46,** 115–119.

28. Dartnall, H. J. A. (1972) *in* Sensory Physiology (Dartnall, H. J. A., Ed.), Vol. VII/1, pp. 122–145.
29. Liebman, P. A., and Ivanczsuk, A. Y. (1982) *Methods Enzymol.* **81,** 532–542.
30. Kropf, A. K., Whittenberger, B. P., Goff, S. P., and Waggoner, A. S. (1973) *Exp. Eye Res.* **17,** 591–606.
31. van der Steen, R., Groesbeek, M., van Amsterdam, L. J. P., Lugtenburg, J., van Oostrum, J., and De Grip, W. J. (1989) *Recl. Trav. Chim. Pays-Bas.* **108,** 20–27.
32. Ito, M., Kodama, A., Tsukida, K., Fudaka, Y., Shichida, Y., and Yoshizawa, T. (1982) *Chem. Pharm. Bull.* **30,** 1913–1916.
33. Ito, M., Katsuta, Y., Imamoto, Y., Shichida, Y., and Yoshizawa, T. (1992) *Photochem. Photobiol.* **56,** 915–919.
34. Peteanu, L. A., Schoenlein, R. W., Wang, Q., Mathies, R. A., and Shank, C. V. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11762–11766.
35. Ternieden, S., and Gartner, W. (1996) *J. Photochem. Photobiol. B.* **33,** 83–86.
36. Koch, D., and Gartner, W. (1997) *Photochem. Photobiol.* **65,** 181–186.
37. Crouch, R., Nodes, B. R., Perlman, J. I., Pepperberg, D. R., Akita, H., and Nakanishi, K. (1984) *Invest. Ophthalmol. Vis. Sci.* **25,** 419–428.
38. Liu, R. S. H., Asato, A. E., Denny, M., and Mead, D. (1984) *J. Am. Chem. Soc.* **106,** 8298–8300.
39. Kochendoerfer, G. G., Verdegem, P. J., van der Hoef, I., Lugtenburg, J., and Mathies, R. A. (1996) *Biochemistry* **35,** 16230–16240.
40. Nakayama, T. A., and Khorana, H. G. (1990) *J. Biol. Chem.* **265,** 15762–15769.
41. Altenbach, C., Yang, K., Farrens, D. L., Farahbakhsh, Z. T., Khorana, H. G., and Hubbell, W. L. (1996) *Biochemistry* **35,** 12470–12478.
42. Farahbakhsh, Z. T., Ridge, K. D., Khorana, H. G., and Hubbell, W. L. (1995) *Biochemistry* **34,** 8812–8819.
43. Yang, K., Farrens, D. L., Altenbach, C., Farahbakhsh, Z. T., Hubbell, W. L., and Khorana, H. G. (1996) *Biochemistry* **35,** 14040–14046.
44. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) *Science* **274,** 768–770.
45. Tan, Q., Nakanishi, K., and Crouch, R. K., (1998) *J. Am. Chem. Soc.* **120,** 12357–12358.
46. Matsumoto, H., and Yoshizawa, T. (1975) *Nature* **258,** 523–526.
47. Jäger, S., Palczewski, K., and Hofmann, K. P. (1996) *Biochemistry* **35,** 2901–2929.