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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

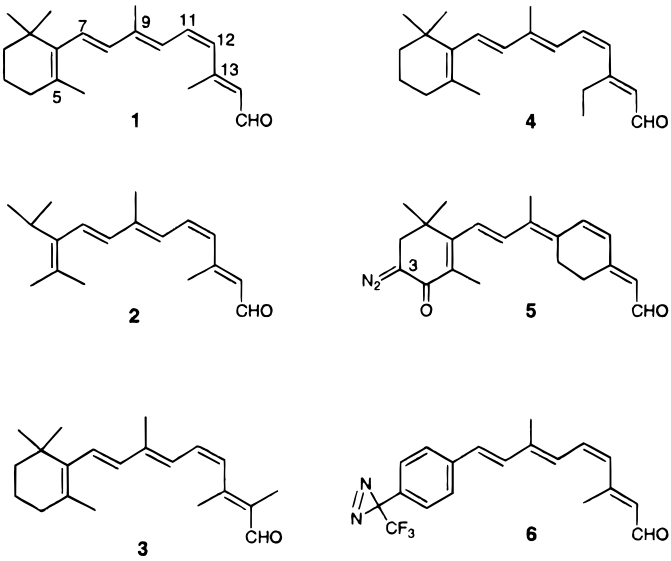

FIG. 1. Structures of 11-*cis* retinal and its analogs.

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

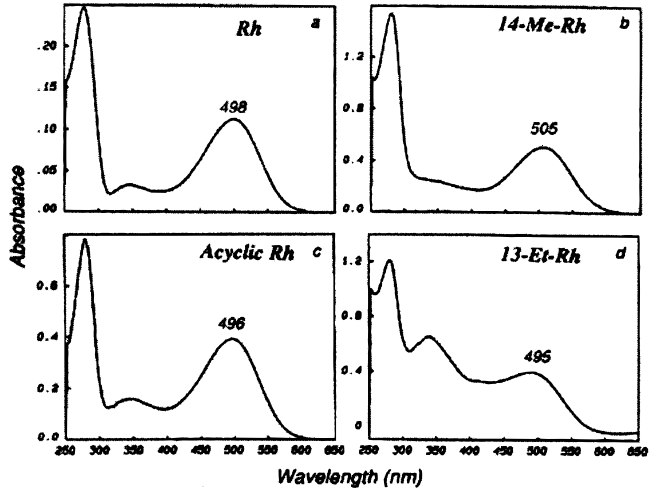

FIG. 2. UV/Vis spectra of the pigments: (a) rhodopsin (Rh), (b) 14-methylrhodopsin (14-MeRh), (c) acyclic rhodopsin (acyclic Rh), (d) 13-ethylrhodopsin (13-Et-Rh, which contained an impurity absorbing around 340 nm and hard to be washed off the Concanavalin A–Sepharose column during purification).