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Analysis of the Retinoid Isomerase Activities in the Retinal Pigment Epithelium and Retina

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Abstract

Light sensitivity in the vertebrate retina is mediated by the opsin visual pigments inside rod and cone photoreceptor cells. These pigments consist of a G protein-coupled receptor and the photo-sensitive ligand, 11-*cis*-retinaldehyde (11-*cis*-RAL). Absorption of a photon by an opsin pigment induces isomerization of the 11-*cis*-RAL chromophore to all-*trans*-retinaldehyde (all-*trans*-RAL), rendering the pigment insensitive to light. The bleached opsin regains light sensitivity by recombining with another 11-*cis*-RAL. The vertebrate eye contains a biochemical mechanism for regenerating 11-*cis*-RAL chromophore from all-*trans*-RAL, called the visual cycle. The visual cycle takes place within cells of the retinal pigment epithelium (RPE). A second visual cycle also appears to be present in Müller glial cells of the retina. A critical step in the regeneration of 11-*cis*-RAL chromophore is thermal re-isomerization to the 11-*cis* configuration of an all-*trans*-retinyl ester (all-*trans*-RE) or an all-*trans*-retinol (all-*trans*-ROL). In RPE cells, this step is carried out by an enzyme called Rpe65 isomerase. This chapter provides methods for assaying Rpe65 isomerase. Although Rpe65 utilizes an all-*trans*-RE such as all-*trans*-retinyl palmitate (all-*trans*-RP) as substrate, it can be assayed in RPE homogenates by providing all-*trans*-ROL substrate and allowing the endogenous lecithin:retinol acyl transferase (LRAT) to synthesize all-*trans*-REs using fatty acids from phosphatidylcholine in the membranes. Alternatively, all-*trans*-RP can be provided directly as substrate, although this requires the isomerase reaction to be carried out in the presence of detergent, since fatty-acyl esters of all-*trans*-ROL are insoluble. Methods are provided in this chapter for assaying Rpe65 in RPE homogenates with both all-*trans*-ROL and all-*trans*-RP substrates. A second visual cycle appears to be present in the retinas of cone-dominant species such as chicken. This retinal pathway may augment the RPE to provide 11-*cis*-RAL to cone photoreceptors under conditions of bright light where the rate of opsin photoisomerization is high. The isomerase in this pathway (isomerase-2) utilizes all-*trans*-ROL and palmitoyl coenzyme A (palm CoA) as substrates to synthesize 11-*cis*-retinyl palmitate (11-*cis*-RP). Isomerase-2 appears to be present in Müller cells but has not yet been identified. Methods are provided in this chapter for assaying isomerase-2 in chicken retina homogenates.

Keywords

Cellular retinaldehyde-binding protein; chicken; chromophore; cone; enzyme assay; isomerase; lecithin retinol acyl transferase; Müller cell; photoreceptor; retina; retinal pigment epithelium; retinol; retinyl ester; rhodopsin; rod; Rpe65; visual cycle

⁴Isomerase-2 assay conditions also detect Rpe65 activity, while the Rpe65 assay conditions with all-*trans*-RP substrate do not detect isomerase-2 activity.

1. Introduction

Rods and cones are two types of photoreceptor cells present in the vertebrate retina. These cells contain a light-sensitive membranous process called the outer segment (OS). OS membranes are packed with rhodopsin or cone-opsin visual pigments. Opsins are members of the G protein-coupled receptor superfamily. The light-absorbing ligand or chromophore in most vertebrate opsins is 11-*cis*-retinaldehyde (11-*cis*-RAL), which is covalently coupled to a Lys residue in the protein. Absorption of a photon by an opsin pigment induces isomerization of the 11-*cis*-RAL to all-*trans*-retinaldehyde (all-*trans*-RAL). After a brief period of activation, the pigment decays to yield free all-*trans*-RAL and apo-opsin. Apo-opsin regains light sensitivity by recombining with a new molecule of 11-*cis*-RAL to form another rhodopsin or cone-opsin pigment. The released all-*trans*-RAL is converted back to 11-*cis*-RAL by a multi-step enzyme pathway called the visual cycle (Fig. 19.1). The first catalytic step of the visual cycle, reduction of all-*trans*-RAL to all-*trans*-retinol (all-*trans*-ROL), takes place within the photoreceptor OS. The remaining steps take place within cells of the retinal pigment epithelium (RPE). The all-*trans*-ROL is released by photoreceptors into the extracellular space where it binds to inter-photoreceptor retinoid-binding protein (IRBP). RPE cells take up all-*trans*-ROL where it is esterified to a fatty acid derived from phosphatidylcholine through the action of lecithin:retinol acyl-transferase (LRAT) to yield an all-*trans*-retinyl ester (all-*trans*-RE) such as all-*trans*-retinyl palmitate (all-*trans*-RP). These all-*trans*-REs are substrates for Rpe65, which catalyzes the critical all-*trans* to 11-*cis* isomerization step. The resulting 11-*cis*-retinol (11-*cis*-ROL) product of Rpe65 binds to cellular retinaldehyde-binding protein (CRALBP) before being oxidized by one of several 11-*cis*-retinol dehydrogenases (11-*cis*-RDHs) in RPE cells to yield 11-*cis*-RAL chromophore. 11-*cis*-RAL also binds to CRALBP. Finally, the 11-*cis*-RAL is released by RPE cells into the extracellular space, where it too binds IRBP, is taken up by the photoreceptors, and re-combines with apo-opsin to form a new light-sensitive pigment.

Rpe65 is strongly associated with internal membranes although it contains no membrane spanning segments. Similarly, the substrates for Rpe65, all-*trans*-REs, are insoluble in water and present within the lipid bilayer. The remaining activities of the visual cycle in RPE cells, LRAT, and 11-*cis*-RDHs are integral membrane proteins of the endoplasmic reticulum (ER). Rpe65 activity can be assayed in total homogenates or microsomal fractions of RPE cells. Microsomal fractions contain higher isomerase-specific activity but lower total activity. The microsomal fraction also contains significantly lower endogenous retinoids. With mice it is convenient to assay isomerase activity in homogenates of eyecups (the posterior half of the eyeball containing RPE, choroid, and sclera after removing the cornea, lens, vitreous, and retina). The isomerase activity of Rpe65 in RPE homogenates can be assayed *in vitro* by two approaches. The first involves the use of all-*trans*-ROL substrate, which is much more water-soluble than all-*trans*-RP. This approach depends on LRAT to synthesize all-*trans*-REs and insert them into Rpe65-containing membranes. Rpe65 utilizes these all-*trans*-REs as substrate to synthesize 11-*cis*-ROL. The second approach is to provide directly all-*trans*-RP as substrate. Here, the reaction must be carried out in the presence of detergent to solubilize the substrate.

Müller glial cells of the retina express several proteins involved in the processing of visual retinoids. These include (i) CRALBP (1,2), (ii) RPE-retinal G protein-coupled receptor (RGR-opsin), a non-photoreceptor opsin that effects light-dependent regulation of the visual cycle in RPE cells (3), (iii) CRBP1 (4), and (iv) retinol dehydrogenases types 10 and 11. However, Müller cells do not express Rpe65 (5) nor LRAT (6), which are both present in RPE cells. Therefore, Müller cells do not simply duplicate the function of RPE cells in the regeneration of visual chromophore. Several lines of evidence suggest that an alternate source of chromophore precursor is available to cones but not to rods. When frog retinas

were separated from the RPE, cone opsins, but not rhodopsin, regenerated spontaneously after a photobleach (7–10). After photobleaching, isolated salamander cones recovered sensitivity with addition of either 11-*cis*-ROL or 11-*cis*-RAL, while isolated rods only recovered sensitivity with addition of 11-*cis*-RAL (11). Müller cells in primary culture were shown to take up all-*trans*-ROL and synthesize 11-*cis*-ROL, which they secreted into the medium (12). Salamander cones were shown to dark adapt and regenerate visual chromophore in isolated retinas separate from the RPE (13). The intrinsic capacity of cones to recover sensitivity and regenerate visual chromophore in salamander retinas was lost after exposure to a selective Müller cell toxin (α -aminoadipic acid), suggesting that Müller cells play a role in these processes (13).

A retinoid isomerase activity, distinct from the activity catalyzed by Rpe65, is detectable in homogenates of cone-dominant ground squirrel and chicken retinas separated from the RPE. This isomerase-2 activity catalyzes the conversion of all-*trans*-ROL into 11-*cis*-ROL and 11-*cis*-retinyl esters (11-*cis*-REs) such as 11-*cis*-retinyl palmitate (11-*cis*-RP) (6,14). The protein responsible for isomerase-2 activity has not yet been identified. Formation of 11-*cis*-ROL is favored when the reaction is carried out in the presence of CRALBP. Addition of palmitoyl coenzyme A (palm CoA) to the reaction mixture favors formation of 11-*cis*-RP. These observations suggest that isomerase-2 catalyzes the direct interconversion of all-*trans*-ROL and 11-*cis*-ROL. At equilibrium, all-*trans*-ROL is favored over 11-*cis*-ROL by ~1000:1 ($\Delta G^\circ + 4.1$ kcal/mol). Thus, removal of 11-*cis*-ROL from the equilibrium mixture is required to drive all-*trans* to 11-*cis* isomerization of the retinoid. This appears to be accomplished by subsequent esterification of 11-*cis*-ROL by a palm CoA-dependent retinyl ester synthase and acyl-CoA:retinol acyltransferase (ARAT). High-affinity binding of 11-*cis*-ROL by CRALBP also drives all-*trans* to 11-*cis* isomerization. The proteins responsible for the isomerase-2 and ARAT activities have not yet been identified.

2. Materials

2.1. Buffers and Solutions for Rpe65 Isomerase Assays

1. HEPES buffer: 20 mM HEPES (pH 7.4), 150 mM NaCl. One tablet of Roche Complete Protease Inhibitor (EDTA free) is dissolved in 50 ml of HEPES buffer.
2. 15% (w/w) bovine serum albumin (BSA) in HEPES buffer: 1.5 g of BSA dissolved in 10 ml of HEPES buffer.
3. 0.5 M sodium cholate in HEPES buffer: 215 mg of sodium cholate is dissolved in 1.0 ml of HEPES buffer.
4. 400 μ M all-*trans*-ROL in ethanol.
5. 400 μ M all-*trans*-RP in hexane (HPLC grade).
6. 1.0% SDS solution: 1.0 g of sodium dodecyl sulfate is dissolved in 100 ml of double distilled water.

2.2. Buffers and Materials for Isomerase-2 Assay

1. 20% BSA.
2. 0.1 M NaOAc, pH 6.0.
3. 5.0 mM palm CoA in 0.1 M NaOAc, pH 6.0.
4. 1.0 mM all-*trans*-ROL in dimethylsulfoxide (DMSO).
5. Homogenization buffer: 10 mM HEPES (pH 7.5), 100 mM NaCl, 1.0 mM MgCl₂, 1.0 mM CaCl₂, protease inhibitor cocktail.

6. Assay buffer: 10 mM Tris-HCl (pH 8.0), 2.0 mM MgCl₂, 2.0 mM CaCl₂, 1.0 mM dithiothreitol (DTT).
7. Glass-to-glass homogenizer.
8. Pierce Micro BCA Protein Concentration Kit.

2.3. HPLC Analysis of Retinoids

1. Liquid chromatogram equipped with photodiode array detector, such as Agilent 1100.
2. Silica column for normal-phase HPLC such as Supelcosil LS-SI 5 µm, 4.6 mm ×250 mm ID.

3. Methods

3.1. Assays for Rpe65-Isomerase in RPE

3.1.1. Preparation of Bovine RPE Homogenate

1. Fifty freshly slaughtered bovine eyeballs are dark adapted on ice for 1 h.
2. On ice, section the anterior one-third of the eyeball and discard the lens, vitreous, and retina.
3. Invert the eyecup and gently brush the RPE into HEPES buffer at 4°C.
4. Collect the pooled RPE cells in HEPES buffer by centrifugation at 500×g for 10 min.
5. Discard the supernatant and resuspend the pellet in 5.0 ml of HEPES buffer.
6. Disrupt the cells in a nitrogen cavitation bomb at 500 psi.
7. Remove cell nuclei and intact cells by centrifugation at 1,000×g for 10 min.
8. Flash-freeze 50 µl aliquots of the supernatant (homogenate) in liquid nitrogen and store at −80°C for use in isomerase assays.
9. At time of use, determine the total protein concentration with a Micro BCA Assay Kit.

3.1.2. Rpe65-Isomerase Assay Using All-Trans-ROL as Substrate

1. Expose bovine RPE homogenate to handheld 365-nm UV light source for 5 min on ice to bleach endogenous retinoids (*see* Note 2).
2. Add 160 µl of 15% BSA in HEPES buffer to 50 µl of RPE homogenate (50–100 µg total protein) on ice.
3. Add 190 µl of HEPES buffer. Transfer samples to darkness (*see* Note 1).
4. Add 10 µl of 400 µM all-*trans*-ROL in ethanol and mix.
5. Incubate samples at 37°C in darkness for 1 h.

²Bleaching of endogenous retinoids in the homogenate with UV light can be eliminated to yield slightly higher isomerase-specific activity. Eliminating this step will result in a higher 11-*cis*-ROL background.

¹All manipulations of retinoids should be done in the dark-room under dim red light.

3.1.3. Extraction and Analysis of Retinoids by Liquid Chromatography

1. Add 100 μ l 1% SDS, vortex briefly, incubate at room temperature 10 min to denature proteins.
2. Add 1 ml ice-cold methanol, vortex briefly.
3. To each sample add 2 ml hexane and vortex. Centrifuge at 3000 $\times$ g for 5 min to separate phases. Transfer the hexane layer (top) to a clean 12-mm $\times$ 75-mm test tube. Repeat extraction of aqueous phase and combine hexane extracts (4 ml total). These extracts may be stored at -20°C in the dark or analyzed immediately.
4. Filter extracts through a polypropylene 200- μ m disposable filter column (e.g., Fisher Scientific #11-387-50).
5. Dry the extract in the dark under a stream of filtered nitrogen or argon gas. Dissolve the dried retinoids in 100 μ l of hexane.
6. Separate retinoids by chromatography on a silica column using gradient elution (0.2–10% dioxane in hexane) at 2.0 ml/min in an Agilent 1100 liquid chromatograph equipped with a UV photodiode array detector. The wavelength of maximal absorbance (λ_{max}) for 11-*cis*-ROL is 318 nm; therefore this wavelength should be monitored during chromatography. The λ_{max} for all-*trans*-ROL and all-*trans*-RP is 325 nm. Confirm identification of UV-absorbing peaks by acquisition of UV spectra and co-elution with authentic retinoid standards.
7. Quantitate each retinoid by comparing the sample peak area to a calibration curve established with an authentic retinoid standard. Figure 19.2 shows a representative chromatogram of retinoids following an assay for isomerase activity in RPE.
8. Determine the isomerase-specific activity by subtracting endogenous 11-*cis*-ROL (from the assay mixture at t_0) from 11-*cis*-ROL in the post-incubation assay mixture ($t_{15\text{-min}}$) and dividing by the incubation time and total protein in the assay mixture.

3.1.4. Rpe65-Isomerase Assay Using all-*trans*-RP as Substrate

1. Expose RPE homogenate to UV light (*see* Note 2).
2. Add 160 μ l 15% BSA in HEPES buffer to 50 μ l RPE homogenate (50–100 μ g total protein) on ice.
3. In the dark, dry 10 μ l of 400 μ M all-*trans*-RP in hexane under a stream of filtered nitrogen gas in a separate tube (*see* Note 1).
4. Add 5 μ l of 500 mM sodium cholate solution plus 185 μ l of HEPES buffer to the dried all-*trans*-RP substrate. Vortex for 2 min.
5. Add substrate solution to RPE homogenate (final assay volume 400 μ l). Incubate in the dark for 1 h at 37°C .
6. Add 100 μ l of 1.0% SDS to each sample to quench the reaction. Incubate at 25°C for 10 min.
7. Add 1.0 ml methanol to precipitate proteins.
8. Pellet the precipitated proteins by centrifugation at 1000 $\times$ g in a benchtop centrifuge.
9. Extract the supernatant twice with 2.0 ml hexane. Pool extracts and filter through a polypropylene 200- μ m disposable filter column.

10. Dry the extract in the dark under a stream of filtered nitrogen gas.
11. Dissolve the dried retinoids in 100 μ l of hexane. Analyze by HPLC as described in **Section 2.4.6**.

3.2. Assay for Retinol Isomerase-2 in Chicken Retinas

3.2.1. Preparation of Chicken Retina Homogenates

1. Obtain heads from freshly slaughtered chickens (*see* Notes 3^{–5}).
2. Remove the eyes from the chicken head using a scalpel, surgical scissors, large scissors, and two forceps, one with and without grasping points on the end.
3. On ice, remove the anterior ~1/3 of the eye with a scalpel. Place the opened eyeballs into small Petri dishes containing phosphate-buffered saline (PBS) on ice and place in the dark for 1 h to dark adapt the retina. This facilitates removal of the retina from the RPE.
4. Transfer to a clean Petri filled with fresh PBS in light. Using a Pasteur pipet, gently squirt PBS underneath the yellowish retina to separate it from the black RPE and choroid. After separating, free the retina from the optic nerve head by snipping with microsurgery scissors. Remove any adhering black RPE from the retina with forceps.
5. Transfer the retina to a fresh, tared 1.5 ml tube, determine the tissue wet weight, store on ice for immediate use.
6. Homogenize approximately two retinas (~25 mg) in 1 ml of ice-cold homogenization buffer using the glass-to-glass homogenizer.
7. Assay the total protein content with the Micro BCA kit. Dilute the concentration to 2 mg/ml using Assay buffer. Store on ice.

3.2.2. Isomerase-2 Assay

1. Set up a labeled 12-mm $\times$ 75-mm glass test tube for each assay which will have a total volume of 500 μ l.
2. Combine 250 μ l of protein homogenate or microsomes, 200 μ l of assay buffer, and 25 μ l of 20% BSA to each tube, mix, and preincubate at 37°C for 5 min.
3. Add 15 μ l of palm CoA, mix, and preincubate for 2 min.
4. Add 10 μ l of all-*trans*-ROL substrate to begin the reaction, mix, and incubate at 37°C for 10–20 min (*see* Note 1).
5. After incubating, transfer the assay mixtures to 12-mm $\times$ 75-mm extraction tubes. Follow the retinoid extraction protocol described in **Section 2.4**.
6. Analyze retinoids by HPLC as described in **Section 2.4**.

³Commercially slaughtered chickens are commonly scalded after killing to facilitate removal of feathers. This treatment will inactivate enzymes in the eye. Ensure that the chicken heads have not been exposed to hot water or steam.

⁵Rpe65 and isomerase-2 activities can be distinguished by assaying homogenates of chicken retina and RPE under conditions described in **Sections 2.5** and **3.3**. Chicken retina homogenates contain virtually no all-*trans*-RP-dependent isomerase activity, while this activity is abundantly present in RPE homogenates (Fig. 19.3a). In contrast, chicken retina homogenates show much greater synthesis of 11-*cis*-ROL than RPE during incubation with all-*trans*-ROL and palm CoA (Fig. 19.3b).

3.2.3. Saponification

1. After drying under a stream of nitrogen gas, dissolve the samples in 980 μ l of ethanol (*see* Note 6).
2. Add 20 μ l of 6 M KOH and vortex briefly (120 mM ethanolic KOH final).
3. Incubate at 55°C for 10 min.
4. Place the tubes on ice and add 1 ml of cold dH₂O.
5. Extract retinoids into hexane as described in **Section 2.4**.
6. Analyze samples by normal-phase HPLC as described in **Section 2.4**.

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⁶The products of the isomerase-2 reaction are a mixture of 11-*cis*-ROL, 11-*cis*-REs, and all-*trans*-REs. Different fatty-acyl forms of retinol isomers co-elute during normal-phase HPLC, which makes quantitation difficult. More accurate quantitation of isomerase-2 activity can be obtained by saponification (hydrolyzing in base) to yield the free retinols, which are readily separated by HPLC.

Fig. 19.1.

Visual cycle in RPE cells. 11-*cis*-RAL in rhodopsin is isomerized by absorption of a photon ($h\nu$). The resulting all-*trans*-RAL is reduced to all-*trans*-ROL by all-*trans*-RDH in the rod outer segment. The all-*trans*-ROL is taken up by an RPE cell where it is esterified to a fatty acid by LRAT. The resulting all-*trans*-RE is isomerized and hydrolyzed by Rpe65 to 11-*cis*-ROL. The 11-*cis*-ROL is oxidized to 11-*cis*-RAL by 11-*cis*-RDH. Both 11-*cis*-ROL and 11-*cis*-RAL are bound to CRALBP within the RPE cell. During transit through the extracellular space, all-*trans*-ROL and 11-*cis*-RAL are bound to IRBP (not shown).

Fig. 19.2.

Representative retinoid chromatograms. **(a)** Chromatogram of retinoid standards showing UV absorption at 318 nm. Identified 11-*cis*-RP, all-*trans*-RP, 11-*cis*-RAL, all-*trans*-RAL, 11-*cis*-ROL, and all-*trans*-ROL peaks are labeled. **(b)** Chromatogram of chicken retina homogenate in the isomerase-2 assay mixture before incubation (t_0) showing 20–25 min elution times. This chromatogram shows endogenous 11-*cis*-ROL and 13-*cis*-ROL, plus all-*trans*-ROL substrate (off scale) added to the assay mixture. **(c)** Chromatogram of chicken retina homogenate in isomerase-2 assay mixture following 15 min incubation at 37°C ($t_{15\text{-min}}$). Note the higher 11-*cis*-ROL peak. The difference in 11-*cis*-ROL before and after incubation reflects the isomerase-2 activity. 13-*cis*-ROL increases during the incubation due to thermal isomerization of 11-*cis*-ROL product and all-*trans*-ROL substrate. **(d)** UV spectrum of 11-*cis*-ROL. The wavelength of maximum absorption (λ_{max}) for 11-*cis*-ROL is 318 nm. **(e)** UV spectrum of 13-*cis*-ROL (λ_{max} , 328 nm). **(f)** UV spectrum of all-*trans*-ROL (λ_{max} , 325 nm).

Fig. 19.3.

Rpe65 and isomerase-2 activities in chicken retina and RPE. **(a)** Isomerase-specific activities (pmol 11-*cis*-ROL per min per mg protein) in chicken retina and chicken RPE homogenates using the Rpe65 assay with all-*trans*-RP substrate. Rpe65 activity is almost undetectable in retina homogenates. **(b)** Isomerase-specific activities in chicken retina and chicken RPE homogenates using the isomerase-2 assay with all-*trans*-ROL and palm CoA substrates. Retinoids were saponified after assay incubation. Note the much higher activity in chicken retina versus RPE homogenates. Also note the difference in scale of specific activities between figure panels **(a)** and **(b)**. During dissection, all visible traces of pigmented RPE material were removed from the retina samples. However, contaminating retina could not be removed from the RPE samples since retina is non-pigmented. Thus, RPE samples in this experiment probably contained contaminating retina material, while the retina samples were relatively free of RPE contamination.