Retinyl Esters Are the Substrate for Isomerohydrolase†

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ABSTRACT: Regeneration of 11-*cis* retinal from all-*trans* retinol in the retinal pigment epithelium (RPE) is a critical step in the visual cycle. The enzyme(s) involved in this isomerization process has not been identified and both all-*trans* retinol and all-*trans* retinyl esters have been proposed as the substrate. This study is to determine the substrate of the isomerase enzyme or enzymatic complex. Incubation of bovine RPE microsomes with all-*trans* [^{3}H]-retinol generated both retinyl esters and 11-*cis* retinol. Inhibition of lecithin retinol acyltransferase (LRAT) with 10-N-acetamidodecyl chloromethyl ketone (AcDCMK) or cellular retinol-binding protein I (CRBP) diminished the generation of both retinyl esters and 11-*cis* retinol from all-*trans* retinol. The 11-*cis* retinol production correlated with the retinyl ester levels, but not with the all-*trans* retinol levels in the reaction mixture. When retinyl esters were allowed to form prior to the addition of the LRAT inhibitors, a significant amount of isomerization product was generated. Incubation of all-*trans* [^{3}H]-retinyl palmitate with RPE microsomes generated 11-*cis* retinol without any detectable production of all-*trans* retinol. The RPE65 knockout (*Rpe65*$^{-/-}$) mouse eyecup lacks the isomerase activity, but LRAT activity remains the same as that in the wild-type (WT) mice. Retinyl esters in WT mice plateau at 8 weeks-of-age, but *Rpe65*$^{-/-}$ mice continue to accumulate retinyl esters with age (e.g., at 36 weeks, the levels are 20× that of WT). Our data indicate that the retinyl esters are the substrate of the isomerization reaction.

Photoreceptor pigments are formed by the opsin proteins and their ligand 11-*cis* retinal covalently attached by a Schiff base linkage, usually in the protonated form. Absorption of a photon results in isomerization of 11-*cis* retinal to all-*trans* retinal. This isomerization changes the ligand from an inverse agonist to the antagonist form, resulting in a conformational change in the opsin and triggering the phototransduction cascade (*1*). The generation/regeneration of 11-*cis* retinal, in a process termed the visual cycle (*1*), is essential to support normal vision. The ligand acts both as a chromophore (i.e., changing the absorption properties of the protein and thus making it possible for light of specific wavelengths to activate the various pigments) and as an inverse agonist (i.e., keeping these G-protein coupled receptors in their inactive states). Complete or partial absence of the ligand (e.g., vitamin A deficiency; or, in the case of the mutation of certain proteins involved in the metabolism, binding or transport of visual cycle retinyl compounds) has been shown to lead to various clinical retinal degeneration disorders, including Leber congenital amaurosis and retinitis pigmentosa (*2*−*5*).

The all-*trans* retinal formed as a result of photoactivation dissociates from opsin and is reduced to all-*trans* retinol by a dehydrogenase (*6*, *7*). The all-*trans* retinol then moves from the photoreceptors to the retinal pigment epithelium (RPE[1]). The all-*trans* retinol is isomerized into 11-*cis* retinol by an enzyme or enzymatic complex which is as yet not identified, or may be esterified by lecithin:retinol acyl transferase (LRAT) (*8*, *9*) and stored in this form. There is some controversy as to the substrate for the isomerization reaction. On one hand, it has been proposed that all-*trans* retinol is first acylated and the all-*trans* retinyl esters serve as the substrate for a putative enzyme termed isomerohydrolase (*10*). These workers suggest that the energy liberated in the process of ester hydrolysis provides the energy for the concerted isomerization reaction (*10*). The generated 11-*cis* retinol is oxidized to 11-*cis* retinal by 11-*cis* retinol dehydrogenase (*11*) and transported to the photoreceptors to

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[1] Abbreviations: AcDCMK, 10-N-acetamidodecyl chloromethyl ketone; BSA, bovine serum albumin; BTP, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; CRALBP, cellular retinaldehyde-binding protein; CRBP, cellular retinol-binding protein I; DCMK, dodecyl chloromethyl ketone; LRAT, lecithin retinol acyltransferase; RBP, retinol-binding protein; RDH, retinol dehydrogenase; REH, retinyl ester hydrolase; RPE, retinal pigment epithelium; *Rpe65*$^{-/-}$, RPE65 knockout; WT, wild-type.

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regenerate the visual pigments. On the other hand, an alternative mechanism involving a retinyl carbocation, which has a much lower energy barrier for the trans to cis isomerization, has been proposed (*12*). These workers propose that 11-*cis* retinol is formed directly from all-*trans* retinol and retinol esterification is used as a mechanism for storing the retinol.

The isomerization reaction has been difficult to study as the enzyme/enzymatic complex has only limited solubility and has not yet been characterized. The isomerase activity, residing in the microsomal fraction of the RPE, is very sensitive to all detergents used to date (*13*). We have shown previously that RPE65 is essential for the isomerase activity (*14*).

RPE65 is a membrane-associated protein expressed predominantly in the RPE (*15*, *16*), but also found in cone photoreceptors (*17*, *18*). Mutations in the RPE65 gene cause inherited retinal diseases such as Leber congenital amaurosis (*3*, *4*), retinitis pigmentosa (*2*), and autosomal recessive childhood-onset severe retinal dystrophy (*19*), all suggesting that RPE65 is essential for normal vision (for recent review, see ref *5*). Delivery of the RPE65 gene has been shown to restore vision in dogs with RPE65 gene mutations (*20*). The function of this protein is presently unknown. Although RPE65 is homologous to β-carotene 15,15′-monooxygenase (*21*−*23*) and related carotenoid cleavage enzymes (*24*), this protein cannot cleave β-carotene (*21*).

The RPE65 knockout (*Rpe65*$^{-/-}$) mouse, a model of extreme chromophore starvation, has been shown to have a minimal response to light by electroretinography (ERG) measurements and exhibits progressive photoreceptor degeneration (*14*). The severely depressed residual ERG signal has been shown to arise from abnormal rod function (*25*). The *Rpe65*$^{-/-}$ animals have been shown to have a disturbed RPE retinoid profile, with minimal 11-*cis* retinoid being produced in the RPE and all-*trans* retinyl esters being found substantially higher than that in age-matched wild-type (WT) mice (*14*, *26*). Administration of 9-*cis* retinal is reported to partially rescue the ERG phenotypes of the *Rpe65*$^{-/-}$ mouse and decrease the accumulation of retinyl esters in the RPE (*26*, *27*). These findings suggest that RPE65 has a critical role in retinoid processing in the visual cycle, specifically at the isomerization step (*20*).

In this study we have used LRAT inhibitors and the *Rpe65*$^{-/-}$ mouse to investigate the substrate of the isomerization step in the retinoid visual cycle.

MATERIALS AND METHODS

Animals. The generation of *Rpe65*$^{-/-}$ mouse has been previously described (*14*). C57Bl/6 and BALB/c mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Mice were maintained under a 12-h-light/12-h-dark cycle. All animal procedures followed the Association for Research in Vision and Ophthalmology and the National Institutes of Health statements regarding the Use of Animals in Ophthalmic and Vision Research.

Synthesis of 10-N-Acetamidodecyl Chloromethyl Ketone (AcDCMK). NMR spectra were obtained on a Varian Inova spectrometer operating at 400 MHz proton and 100 MHz carbon. The double quantum filtered cosy (DQ-COSY) experiment acquired in the phase sensitive mode was employed to make the proton assignments. In the DQ-COSY 2×256 fids were acquired. Digital resolution in F1 was increased by linear prediction to 1024 points, processed using the Gaussian weighting function and then Fourier transformed. The chemical shifts of unresolved multiplets were based on the chemical shifts of the cross-peaks. Carbon resonances were assigned using the heteronuclear single quantum coherence (HSQC) and heteronuclear multi bond (HMBC) experiments. In the HSQC, 128 fids were acquired, and linear prediction increased the points in F1 to 512 (Gaussian weighted, then Fourier transformed); while in the HMBC, 400 fids were acquired, and linear prediction increased the points in F1 to 1200, sinebell weighted and then Fourier transformed.

11-N-Acetamidoundecanoic Acid. 11-Aminoundecanoic acid (603 mg, 3 mmol) and 4-(dimethylamino)pyridine (100 mg, 0.82 mmol) were stirred in acetic anhydride (5 mL, 5.4 g, 53 mmol) at room temperature for 5 h. Excess anhydride was hydrolyzed by addition of 15 mL H_2O and stirring at room temperature for 1.5 h. The resulting precipitate was washed with 5 mL of 1 M HCl then H_2O and dried in vacuo over P_2O_5 to yield 720 mg (99%) of white solid, mp 77−78 °C. ^{1}H NMR ($CDCl_3$): δ1.24(m, 2, H-4), 1.25(m, 8, H-5-H-8), 1.26(m, 2, H-9), 1.46(p $J = 7.1$ Hz, 2, H-10), 1.60(p, $J = 7.4$ Hz, 2, H-3), 1.96(s, 3, CH_3), 2.31(t, $J = 7.4$ Hz, 2, H-2), 3.20(q, $J = 7.1$ Hz, 2, H-11), 5.54(bS, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 23.4(CH_3), 24.7(C-3), 27.0(C-9), 29.2(C-4), 29.2(C-5-C-8), 29.5(C-10), 34.0(C-2), 39.8(C-11), 170.6(CO), 178.9(C-1).

10-Acetamidecychloromethyl Ketone. To 11-N-acetamidoundecanoic acid (530 mg, 2.12 mmol) suspended in 12 mL CH_2Cl_2 was added 22 μL of DMF, followed by $SOCl_2$ (1.6 mL, 2.6 g, 22 mmol). After the resulting homogeneous solution was stirred at 22 °C for 2 h, the $SOCl_2$ was removed at reduced pressure. Residual $SOCl_2$ was removed by the addition of 2× 5 mL of benzene and subsequently removed at reduced pressure. Without further purification a CH_2Cl_2 solution of the acid chloride was added to an ice-cold ethanol free solution of CH_2N_2 (∼1 g). The reaction was stirred at room temperature for 2 h and cooled in an ice bath, and HCl (1 g) was added. The solvents were removed at reduced pressure to yield a waxy material. Purification was affected by chromatographing on a short column of silica gel eluting with $CHCl_3$ to yield 183 mg of product, mp 92.5−93 °C. ^{1}H NMR ($CDCl_3$): δ1.28(m, 10, H-4-H-8), 1.30(m, 2, H-4), 1.31(m, 2, H-9), 1.49(p, $J = 7.3$ Hz, H-10), 1.61(p, $J = 7.3$ Hz, 2, H-3), 1.98(s, 3, CH_3), 2.59(t, $J = 7.4$ Hz, 2, H-2), 3.23(q, $J = 7.1$ Hz, 2, H-11), 4.09(s, 3H,CH_2Cl), 5.54(bS, 1H, NH). ^{13}C NMR($CDCl_3$): δ 23.5(CH_3), 23.7(C-3), 27.0-(C-9), 29.2(C-4-C-8), 29.7(C-10), 39.8(C-2), 39.8(C-11), 170.6(CO), 203.1(C-1).

Bovine RPE Microsome Preparation. Fresh bovine eyes (from the local abattoir) were dissected to remove the anterior segment and the retina. RPE cells were brushed off the eyecup with an eyelash brush into a cold 0.1 M sodium phosphate buffer, pH 7.4. Cells were gently centrifuged (1000*g*), resuspended in 0.32 M sucrose/0.1 M sodium phosphate buffer, pH 7.4, and homogenized with a Teflon-glass homogenizer. The homogenate was centrifuged (20 min, 20 000*g*) to sediment unbroken cells, nuclei, and mitochondria. The supernatant was recentrifuged (1 h, 105 000*g*), and the microsomal pellet was resuspended in

0.1 M sodium phosphate buffer, pH 7.4, washed, and stored at −80 °C. The resultant bovine RPE microsomal fraction was a suspension of the microsomal protein without any detergent.

Purification of Recombinant CRALBP and CRBP. The CRBP expression vector was a generous gift from Dr. Kris Palczewski at the University of Washington, and the CRALBP vector was kindly provided by Dr. John Crabb at the Cleveland Clinic Foundation. Recombinant CRBP and CRALBP were purified to homogeneity by Ni^{2+}−His tag affinity chromatography as described by Crabb et al. (*28*).

LRAT Activity Assay. LRAT activity was measured as previously described (*29*). After overnight dark adaptation, mice were sacrificed and their eyes enucleated. The anterior chamber and retina were removed, and the remaining eyecup was homogenized and used for the LRAT activity assay. All-*trans* [11,12-^{3}H]-retinol (NEN Life Science Products, Boston, MA) was diluted with cold all-*trans* retinol to generate a specific radioactivity of 4×10^7 dpm/nmol. For each assay, 300 μL of eyecup homogenate (0.14 mg of protein in 10 mM BTP, pH 8.0, 0.5% BSA) was added to 20 pmol of all-*trans* [^{3}H]-retinol (dried under a stream of argon and dissolved in 5 μL of 10% BSA). The reaction mixture was incubated at 37 °C, and 50 μL aliquots were removed at 5, 10, 15, 30, and 60 min. Each aliquot was immediately quenched with ice-cold methanol (500 μL/sample) and H_2O (100 μL), and hexane (500 μL) was added for extraction of retinoids, following a documented method (*14*). The extracted retinoids were analyzed using HPLC with a normal phase Lichrosphere SI-60 (Alltech, Deerfield, IL) 5 μm column and isocratic solvent of 11.2% ethyl acetate, 2.0% dioxane, and 1.4% octanol in hexane. Elution peaks were identified by spiking with authentic standards. Radioactive HPLC fractions were calculated as a percentage of the total radioactivity, by Millenium^{32} software.

Western Blot Analysis. Mice (8 weeks of age, maintained under a 12-h-light/12-h-dark cycle) were sacrificed and eyes dissected to remove the anterior segment of the eye and the retina. The remaining eyecup with the RPE was homogenized by sonication in 200 μL PBS. In all the assays used in this study, the protein concentration was determined utilizing the Bradford assay (*30*). The same amount of protein (10 μg) was resolved in a 10% polyacrylamide gel and electrotransferred to a Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked by incubating with 10% (w/v) BLOTTO/TBST (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 1 h and separately incubated with purified polyclonal antibodies specific to RPE65 (0.2 μg/mL), anti-sera against 11-*cis* retinol dehydrogenase (RDH5) (kindly provided by Dr. John Saari at University of Washington 1:2000 dilution), LRAT (a gift from Dr. Dean Bok at UCLA, 1:1000 dilution), and CRALBP (a generous gift from Dr. John Crabb at Cleveland Clinic Foundation, 1:1000 dilution) at 4 °C overnight. The specificities of these antibodies were demonstrated previously (*11*, *29*, *31*, *32*). The membrane was washed twice with TBST and then incubated for 1 h with a horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (0.1 μg/mL) in 1% BLOTTO-TBST. The membrane was washed four times with TBST and incubated for 1 min in Renaissance Oxidizing and Enhanced Luminol developing solution (NEN Life Science Products, Boston, MA). Finally, the membrane was exposed to a Kodak Biomax MR film.

Isomerase Activity Assay. After overnight dark adaptation, mice were sacrificed and their eyes enucleated. The anterior portion of the globe and the retina were removed, and the remaining eyecup was used for the assay. Four eyecups from each strain (BALB/c, C57Bl/6 and *Rpe65*$^{-/-}$) were pooled for assay. All-*trans* [11,12-^{3}H]-retinol was diluted by unlabeled all-*trans* retinol to generate the specific radioactivity of 4×10^7 dpm/nmol. The radioactively labeled substrate was aliquoted (20 pmol/vial), dried under argon, and stored at −70 °C. For each reaction, 4 eyecups were pooled and homogenized in a glass grinder, and 0.8 mg of total protein was added into 100 μL of reaction buffer (10 mM BTP, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$) containing 0.5% BSA, 25 μM CRALBP, and 1 mM CTP to the vial containing the aliquoted retinol. After 2 h in the dark at 37 °C, the retinoids were extracted following the method described by Suzuki et al. (*33*). Utilizing 15 mL Falcon (Franklin Lake, NJ) tubes, formaldehyde (6 M, 100 μL in 0.2 M sodium phosphate buffer, pH 7.0) was added, and the reaction mixture was incubated for 2 min at 30 °C. Methylene chloride (1 mL) was added. This reaction was vortexed and kept at 30 °C for 10 min. Hexane (2 mL) was added, the reaction vortexed and centrifuged for 5 min at 3000*g*, and the upper-layer phases were collected, pooled, and dried under argon gas. Extracted retinoids were analyzed by HPLC. For bovine RPE membranes the assay was performed in the same way and 10 μg of microsomal protein was used in each experiment.

Isomerohydrolase Assay. All-*trans* retinyl palmitate [retinol-15-^{3}H] toluene solution (1 mCi/mL, 20 Ci/mmol, American Radiolabeled Chemicals, Inc, St. Louis, MO, 2 μL) was dried under argon and resuspended in the same volume of *N*,*N*-dimethyl formamide (DMF). For each reaction, 0.8 mg of mouse eyecup protein or 10 μg of bovine RPE membranes was added into 100 μL of reaction buffer (10 mM BTP, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$) containing 0.5% BSA, 25 μM CRALBP, and 1 mM CTP to the labeled retinol. Reactions were performed and products analyzed in the same way as the retinol isomerase assay.

Ester Quantification and Analysis. Dark-adapted mice were sacrificed and their eyes enucleated. The anterior portion of the globe and the retina were removed, and the remaining eyecups (two eyecups per sample) were homogenized in 200 μL PBS. The homogenate was extracted with 300 μL cold methanol and 300 μL hexane and centrifuged at 10 000*g* for 5 min. The upper layer was collected and used for HPLC analysis. The extracted retinoids were analyzed using HPLC with a normal phase Lichrosphere SI-60 (Alltech, Deerfield, IL) 5 μm column and isocratic solvent of 11.2% ethyl acetate, 2.0% dioxane, and 1.4% octanol in hexane. The amount of retinyl esters was quantified by comparing the area of eluted ester peak with the corresponding peak of all-*trans* retinyl palmitate of known concentration.

For ester analysis, retinyl esters were extracted and collected as described previously (*14*), from the RPE of dark-adapted animals. After saponification, the aqueous phase was acidified to pH 2−3 with HCl and extracted twice with hexane, thus yielding 95% of the nonesterified fatty acids

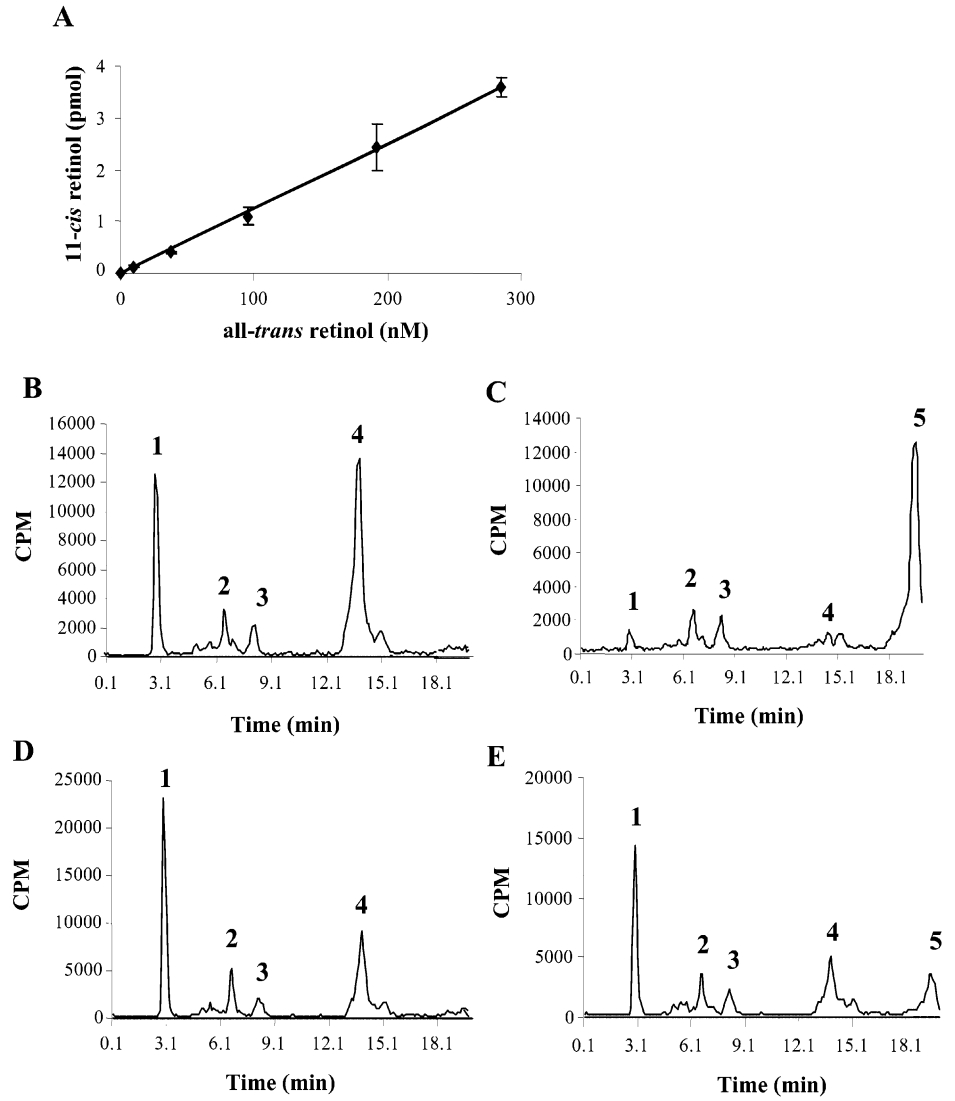

FIGURE 1: (A) Effect of all-*trans* retinol concentration on isomerization rate. Increasing amounts of all-*trans* [^{3}H]-retinol were incubated with 10 μg of bovine RPE microsomal proteins for 2 h. The 11-*cis* retinol generated was measured and expressed as mean ± SEM (n = 4). (B−E) Inhibition of retinyl ester and 11-*cis* retinol formation by apo-CRBP. All-*trans* [^{3}H]-retinol (0.2 μM) was incubated with 10 μg of bovine RPE microsomes for 2 h, and the generated retinoids were analyzed by HPLC. (B) No apo-CRBP was added to the reaction mixture; (C) 28 μM apo-CRBP was added at the beginning of the reaction; (D) the reaction mixture without CRALBP was incubated for 1.5 h to form the esters, and then 25 μM of CRALBP was added and the reaction was allowed to proceed for another 2 h; (E) the reaction mixture without CRALBP was incubated for 1.5 h, and then 25 μM CRALBP and 28 μM apo-CRBP was added and the reaction was allowed to proceed for another 2 h. Peaks were identified as follows: 1, retinyl esters; 2, all-*trans* retinal; 3, all-*trans* retinoic acid; 4, 11-*cis* retinol; and 5, all-*trans* retinol.

(*34*). Methanolic boron trifluoride (14%, 1 mL) was added and the reaction stirred (30 min, 50 °C, under argon). The resulting methyl esters were extracted with chloroform. The chloroform layer was collected, dried over $MgSO_4$ and filtered. The chloroform was evaporated and isooctane added for GC-MS analysis. Separation was achieved on a 50% cyanopropylphenyl methylpolysiloxane 30m × 0.25 mm fused silica columns. GC-MS analysis was performed with GC Hewlett-Packard 6890 instrument.

RESULTS

Dependence of Isomerase Activity on All-trans Retinol Concentration. When all-*trans* [^{3}H]-retinol was used for the isomerase activity assay, the production of 11-*cis* retinol was found to be a linear function of the all-*trans* retinol concentration within a range from 9.5 to 280 nM (Figure 1A), and therefore, all of the experiments in this study were conducted within this range.

Inhibition of the LRAT and Isomerase Activity by Apo-CRBP. CRALBP is known to accelerate 11-*cis* retinol generation from all-*trans* retinol by at least 13-fold (*35*, *36*), while the production of retinyl esters does not depend on CRALBP. Incubation of the RPE microsomes with all-*trans* [^{3}H]-retinol in the presence of CRALBP resulted in the formation of retinyl esters and 11-*cis* retinol (Figure 1B). To determine if isomerization of all-*trans* retinol to 11-*cis* retinol requires the formation of all-*trans* retinyl esters, two LRAT inhibitors were used. The first inhibitor was apo-CRBP, which has previously proven to be a potent LRAT inhibitor with a K_I = 0.21 μM (*37*). The inhibition is proposed to occur via direct protein−protein interactions between LRAT and CRBP (*37*). The addition of 28 μM of

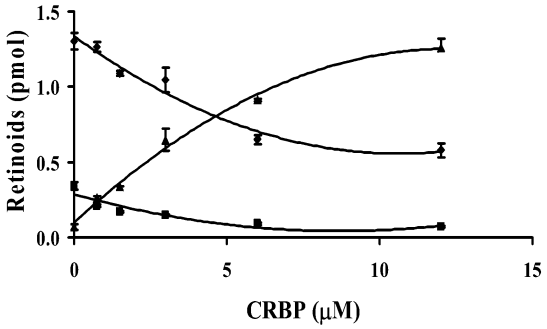

FIGURE 2: apo-CRBP concentration-dependent inhibition of LRAT and retinol isomerase activities. All-*trans* [^{3}H]-retinol (0.2 μM) was incubated with 10 μg of bovine RPE microsomal proteins for 2 h (for isomerase activity) and 15 min (for LRAT activity) in the presence of different concentrations of CRBP. The reactions were then stopped and the levels of 11-*cis* retinol (■), retinyl esters (◆), and all-*trans* retinol (▲) analyzed by HPLC at time points as indicated. Values are mean ± SEM (n = 4).

CRBP to the RPE microsomes at the beginning of the reaction almost completely blocked the formation of both retinyl esters and 11-*cis* retinol (Figure 1C). In another experiment, all-*trans* [^{3}H]-retinol was preincubated with RPE microsomes for 1.5 h without the CRALBP and CRBP to allow the complete conversion of all-*trans* retinol to retinyl esters, while not allowing the generation of 11-*cis* retinol. In the absence of CRALBP, the isomerization reaction proceeds very slowly (*36*), all-*trans* retinol was completely converted to all-*trans* retinyl esters, and no detectable level of 11-*cis* retinol was generated (data not shown). CRALBP (25 μM) (Figure 1D) or CRALBP (25 μM) plus CRBP (28 μM) (Figure 1E) was then added to and incubated with the reaction mixture for an additional 2 h. In both cases, significant amounts of 11-*cis* retinol were generated (Figure 1D,E), suggesting that CRBP does not inhibit isomerization once the ester is formed. This result supports the conclusion that the formation of retinyl esters is essential for the isomerization of all-*trans* retinol to 11-*cis* retinol.

To determine whether the inhibition of LRAT and isomerase activity is dependent on CRBP concentration, different concentrations of CRBP were added to the reaction mixture at the beginning of the reaction, and the production of retinyl esters and 11-*cis* retinol was measured. The RPE microsomes and all-*trans* retinol concentrations were kept constant for all measurements. As shown in Figure 2, CRBP displayed a concentration-dependent inhibition of both LRAT and isomerase activities. The concentration-dependent inhibition of isomerase activity correlated with the decreased retinyl ester levels but not with all-*trans* retinol levels, providing further evidence that retinyl ester formation is required for the isomerase activity (Figure 2).

Inhibition of the Formation of Retinyl Esters and 11-cis Retinol by AcDCMK (for Structure, See Figure 3, Inset). Dodecyl chloromethyl ketone (DCMK) has been previously shown to be a specific inhibitor of LRAT. The biocytinyl analogue of DCMK has been used for specific affinity-labeling of LRAT and characterization of the enzyme (*29*). This study used AcDCMK, an analogue of DCMK to inhibit LRAT activity.

When bovine RPE microsomes were incubated with 4 μM AcDCMK for 15 min prior to the addition of all-*trans* [^{3}H]-retinol, the production of retinyl esters and 11-*cis* retinol was significantly inhibited (Figure 3A). In the second series of experiments, the RPE microsomes were incubated with all-*trans* [^{3}H]-retinol for 15 min in the absence of CRALBP. After the 15 min incubation, all-*trans* retinol was completely converted to retinyl esters, but no 11-*cis* retinol was detected (data not shown). AcDCMK and CRALBP were then added into the mixture and incubated for an additional 2 h. When retinyl esters formed prior to the addition of AcDCMK

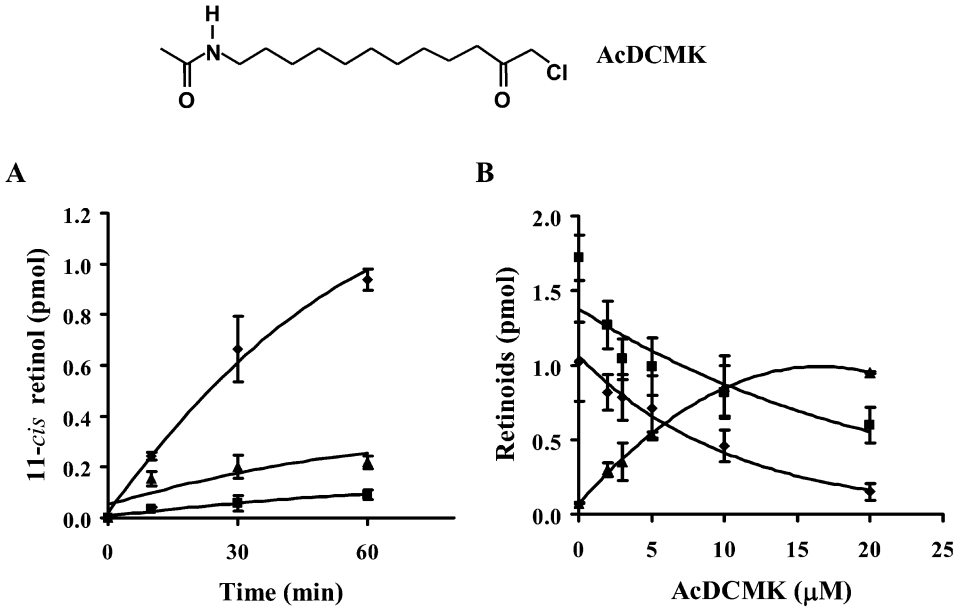

FIGURE 3: Inhibition of LRAT and retinol isomerase activities by AcDCMK. (A) Time course of 11-*cis* retinol formation. (■) All-*trans* retinol was incubated with 10 μg of bovine RPE microsomes pretreated with 4 μM AcDCMK; (▲) 4 μM AcDCMK was added into RPE microsomes after the conversion of all-*trans* retinol into retinyl esters (after 15-min incubation in the absence of CRALBP); (◆) no AcDCMK was added. (B) The AcDCMK concentration-dependent inhibition of LRAT and isomerase activity. All-*trans* [^{3}H]-retinol (0.2 μM) was incubated with 10 μg of bovine RPE microsomal proteins for 2 h (for isomerase activity) and 15 min (for LRAT activity) in the presence of different concentrations of AcDCMK. The reactions were then stopped and the products analyzed to measure the levels of 11-*cis* retinol (◆), retinyl esters (■), and all-*trans* retinol (▲). Data points represent mean ± SEM (n = 3).

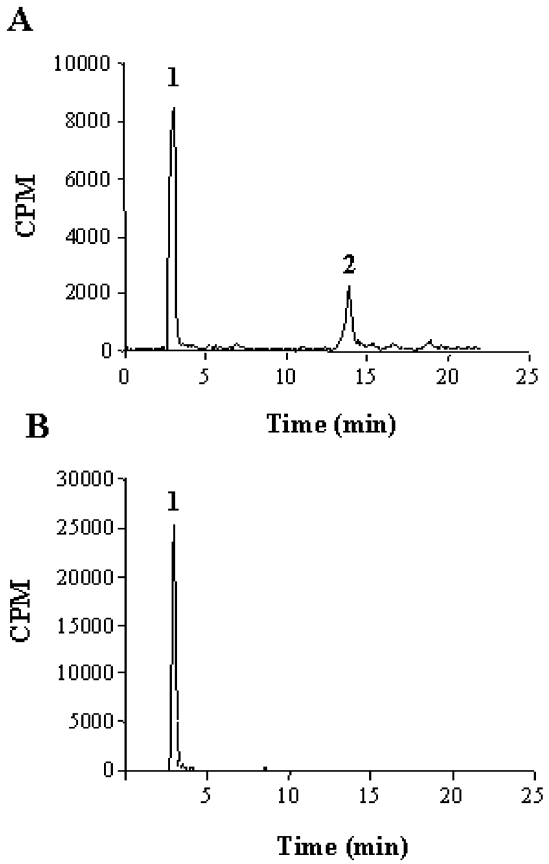

FIGURE 4: Detection of isomerohydrolase activity in RPE microsomes using all-*trans* retinyl palmitate as a substrate. (A) all-*trans* [^{3}H]-retinyl palmitate (0.2 μM) was incubated with 10 μg of bovine RPE microsomal proteins for 2 h and retinoids analyzed by HPLC. (B) The same amount of all-*trans* [^{3}H]-retinyl palmitate was incubated in the absence of microsomal proteins. Peaks correspond to the following: 1, retinyl esters; and 2, 11-*cis* retinol.

inhibitor, a significant amount of 11-*cis* retinol was produced (Figure 3A).

When the RPE microsomes were preincubated with different concentrations of AcDCMK concentrations, isomerase and LRAT activities were inhibited in an AcDCMK concentration-dependent manner. The curves were drawn using the Microsoft Excel program, and best fitting was achieved using single phase exponents for the decrease of both retinyl ester and 11-*cis* retinol (Figure 3). To determine the correlation between the decrease in retinyl ester and the decrease in 11-*cis* retinol, we have calculated the linear correlation coefficient of these two curves. The calculated correlation coefficient is 0.92 which is statistically significant ($P < 0.001$, $n = 6$), demonstrating that the decrease in isomerase activity parallels the decrease of the LRAT activity (Figure 3B).

Generation of 11-cis Retinol from All-trans Retinyl Palmitate. To further prove that RPE microsomes generate 11-*cis* retinol directly from all-*trans* retinyl esters, dried all-*trans* [^{3}H]-retinyl palmitate was dissolved in DMF and added to the RPE microsomes in a small volume (2 μL). After 2 h incubation, 0.22 pmole of 11-*cis* retinol (peak 2) was generated (Figure 4A). This peak was confirmed by spiking with an 11-*cis* retinol standard. In the reaction mixture, no all-*trans* retinol was detected (Figure 4). In the control, no 11-*cis* retinoid was detected in the absence of the RPE microsomes (Figure 4B). Similarly, incubation of all-*trans* [^{3}H]-retinyl palmitate with eyecup homogenates from BALB/c and C57Bl/6 mice also generated significant amounts of 11-*cis* retinol (Table 1). These experiments provided direct evidence that isomerase can directly convert all-*trans* retinyl esters into 11-*cis* retinol.

Table 1: 11-*cis* Retinol Generated in Mouse Eyecup Homogenates[a]

	mouse strains		
substrates used	BALB/c	C57B1/6	*Rpe65*$^{-/-}$
all-*trans* retinol	635 ± 125	140 ± 19	0
all-*trans* retinyl palmitate	33 ± 7	10 ± 2	0

[a] Values represent 11-*cis* [H^3]-retinal (fmoles) generated from these two substrates in mouse eyecup homogenates (mean ± SEM; $n = 4$).

Retinol Isomerase Activity Correlates with RPE65 Levels. The conversion of all-*trans* retinol into 11-*cis* retinol was analyzed in eyecup homogenates from BALB/c, C57Bl/6 and *Rpe65*$^{-/-}$ mice at 8 weeks of age. Significant amounts of 11-*cis* retinol were generated after incubation of all-*trans* retinol with BALB/c and C57Bl/6 eyecup homogenates (Table 1). The average levels of 11-*cis* retinol formed in the reactions with BALB/c and C57Bl/6 mice are presented in Table 1. In all isomerization reactions, the BALB/c eyecups generated approximately 4-fold more 11-*cis* retinol than that of C57Bl/6 eyecups (see Table 1). In contrast, incubation of *Rpe65*$^{-/-}$ eyecup homogenates with all-*trans* [^{3}H]-retinol resulted in formation of retinyl esters as has been reported previously (*14*). No traces of 11-*cis* retinoids were found in the *Rpe65*$^{-/-}$ eyecups (Table 1).

The labeled all-*trans* retinyl palmitate was also used as a substrate to compare isomerohydrolase activities in *Rpe65*$^{-/-}$, BALB/c, and C57Bl/6 mouse eyecups. Again, a significant difference in the amount of 11-*cis* retinol produced was observed between the BALB/c and C57Bl/6 strains, with the BALB/c eyecups generating more than 3-fold more 11-*cis* retinol than the C57Bl/6 (Table 1). No 11-*cis* retinol was generated from the all-*trans* retinyl ester by the *Rpe65*$^{-/-}$ eyecups.

To determine if there is a correlation between the levels of isomerase activity with certain retinoid processing proteins, the protein levels of RPE65, 11-*cis* RDH, LRAT, and CRALBP were compared in age-matched BALB/c, C57Bl/6 and *Rpe65*$^{-/-}$ mice (Figure 5). Equal amounts of total eyecup protein were used for Western blot analyses using specific antibodies. The expression levels of each protein were quantified by densitometry and normalized by β-actin levels. The RPE65 protein level in BALB/c eyecups is approximately 5-fold higher than that in C57Bl/6 (Figure 5A), correlating with the difference in their isomerase activities (Table 1). No trace of RPE65 was found in *Rpe65*$^{-/-}$ mice. Thus, the protein levels of RPE65 correlate with the retinol isomerase and isomerohydrolase activities in these three strains of mice.

The levels of CRALBP were decreased in the *Rpe65*$^{-/-}$ mice, when compared with the BALB/c and C57Bl/6 strains (Figure 5A). In contrast, levels of 11-*cis* RDH, which converts 11-*cis* retinol to 11-*cis* retinal (*11*), were similar in all three strains of mice. Likewise, the protein levels of LRAT were similar in all of the three mouse strains (Figure 5A).

Analysis of Retinyl Esters. Previously, it was shown in several species that endogenous retinyl esters in the RPE

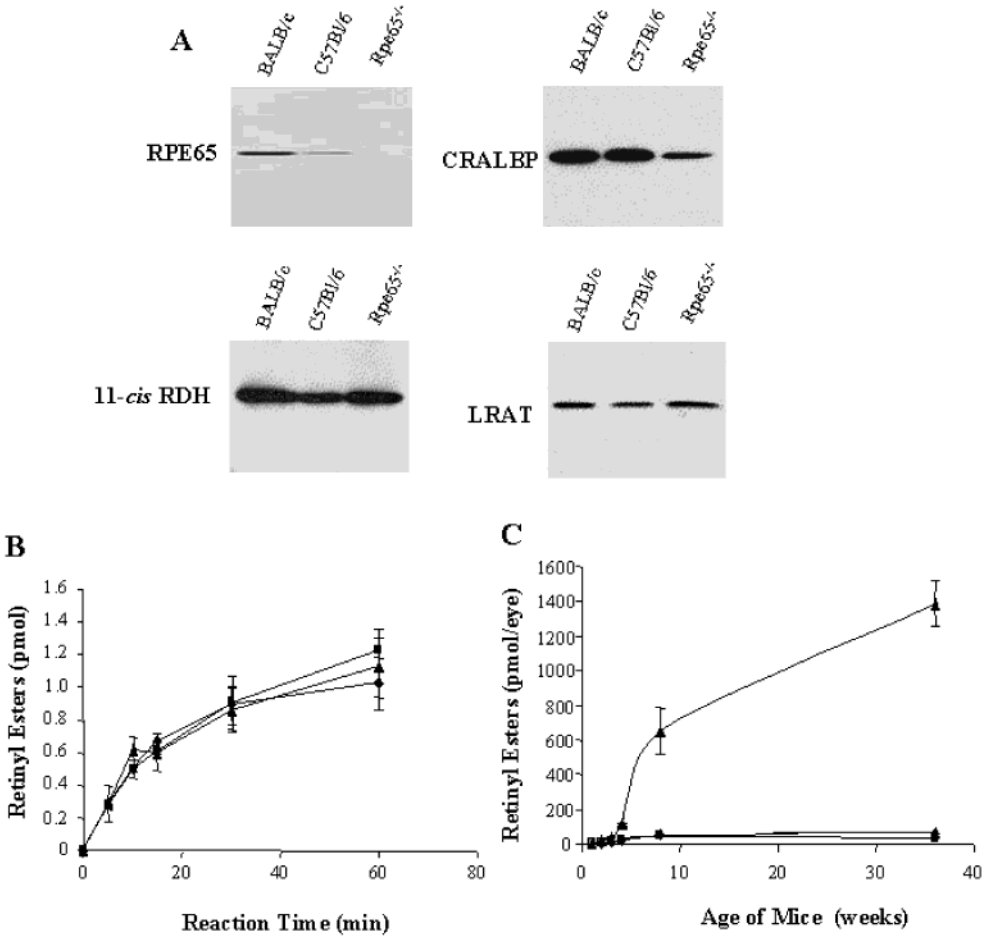

FIGURE 5: (A) Protein levels of RPE65, CRALBP, 11-*cis* RDH and LRAT in the *Rpe65*$^{-/-}$ and WT mouse eyecups. Equal amounts (10 μg) of total protein from eyecups of BALB/c, C57Bl/6, and *Rpe65*$^{-/-}$ mice were subjected to Western blot analysis separately using antibodies specific to RPE65, CRALBP, 11-*cis* RDH, and LRAT. (B) LRAT activity in *Rpe65*$^{-/-}$ and WT mouse eyecups. The homogenates from eyecups of BALB/c, C57Bl/6, and *Rpe65*$^{-/-}$ mice (0.8 mg of protein each) were used for LRAT activity assay with 0.07 μM all-*trans* [^{3}H]-retinol as a substrate. The levels of generated retinyl esters are expressed as percentages of the total [^{3}H] labeled retinoids (mean ± SEM, $n = 4$). (C) Time course of retinyl ester accumulation in *Rpe65*$^{-/-}$ mice. Endogenous retinyl esters were extracted from eyecups of BALB/c, C57Bl/6, and *Rpe65*$^{-/-}$ mice at ages as indicated. Retinyl ester levels were analyzed by HPLC and calculated based on the standard. Values represent mean ± SEM ($n = 4$). (◆) BALB/c, (■) C57Bl/6, and (▲) *Rpe65*$^{-/-}$ mouse eyecups.

consist of both the all-*trans* and 11-*cis* retinol (*38*, *39*), with their ratio depending on their exposure to light. We wished to determine the isomeric composition of the esters obtained in mouse eyecups upon incubation with all-*trans* retinol. The eyecups of all three strains were incubated with all-*trans* [^{3}H]-retinol and the esters isolated by HPLC and then saponified (*40*). Analysis of the saponified retinols showed only all-*trans* retinol, with no 11-*cis* isomer in the eyecups of all the three strains tested (data not shown).

To determine if the fatty acid portion of the retinyl esters differed between the *Rpe65*$^{-/-}$ and WT animals, the fatty acids were analyzed. The fatty acid composition of the retinyl esters in ocular tissue is known to be species-specific (*38*). When analyzed by GC-MS, these three mouse strains demonstrated no significant difference in the fatty acid composition of the retinyl esters (C14, 4.7−6.8%; C16, 34.7−38.1%; C18, 57.2−58.5%). Therefore, even though the *Rpe65*$^{-/-}$ animals had substantially higher quantities of retinyl esters, the composition of these esters is the same as that found in animals having the RPE65 protein.

LRAT Activity is Unchanged in the Rpe65$^{-/-}$ *Mouse*. To elucidate the mechanism of retinyl ester overaccumulation in the RPE of the *Rpe65*$^{-/-}$ mouse, we measured LRAT ester synthesis activity using all-*trans* [^{3}H]-retinol as a substrate. The results showed that all three strains of mice have equivalent rates of the esterification reaction (Figure 5B). No significant difference was detected between the *Rpe65*$^{-/-}$ and WT mice at any of the assayed time-points, suggesting that the ester overaccumulation in the *Rpe65*$^{-/-}$ mouse did not arise from an alteration in LRAT activity.

Time Course of Retinyl Ester Accumulation in the RPE of the Rpe65$^{-/-}$ *Mouse*. Levels of retinyl esters in the RPE were quantified in *Rpe65*$^{-/-}$ mice at ages of 1, 2, 3, 4, 8, and 36 weeks and compared with those in age-matched BALB/c and C57Bl/6 mice. As shown in Figure 5C, starting at 4 weeks of age, the retinyl ester levels were significantly higher in the RPE of the *Rpe65*$^{-/-}$ mice than in the WT mice. The ester accumulation reached a plateau at 8 weeks of age in the WT mice. In contrast, the accumulation continued in the *Rpe65*$^{-/-}$ mice, reaching a level greater than 20-fold higher than in the WT animals at age of 36 weeks (Figure 5C).

DISCUSSION

The RPE is the site of the esterification, isomerization, and oxidation reactions of the visual cycle, as well as being the storage site of the esters (*1*, *41*, *42*). The esterification

of retinol is catalyzed by LRAT (*43*). Presumably, ester formation is used as a storage mechanism, and retinyl esters can be hydrolyzed and converted to 11-*cis* retinol as needed for regeneration of visual pigments. Two routes are possible: (1) retinyl esters can either be hydrolyzed by the retinyl ester hydrolase to all-*trans* retinol which is then isomerized to 11-*cis* retinol, or (2) it can be a direct substrate for a putative isomerohydrolase, as proposed by Rando (*44*). Recently, McBee et al. (*12*) suggested that all-*trans* retinol may be directly isomerized into 11-*cis* retinol by a carbocation mechanism. The purpose of this study was to explore these two possibilities by comparing the two potential substrates.

As the putative retinol isomerase has not been purified, most studies of the isomerase have used RPE microsomes (*12*, *44*). Due to the existence of a high-level of LRAT activity in the RPE microsomes, retinyl esters are always formed when all-*trans* retinol is incubated with RPE microsomes. This makes it difficult to determine if the all-*trans* retinol is isomerized directly to the 11-*cis* isomer, or if it is first converted to esters that are then isomerized-hydrolyzed to 11-*cis* retinol. To overcome this difficulty, this study utilized two inhibitors of LRAT.

It has been shown that apo-CRBP specifically inhibits LRAT activity through direct protein−protein interactions rather than via sequestration of all-*trans* retinol (*37*). It should be mentioned that the 1:1 complex of all-*trans* retinol and CRBP enhances the LRAT activity. A recent paper on CRBP knockout mice showed that in the absence of CRBP, the rate of retinyl esters formation is significantly lower than in the WT mice (*45*). In contrast, when apo-CRBP is in excess over all-*trans* retinol, CRBP has inhibitory effect on LRAT. In the presence of apo-CRBP, the formation of retinyl esters is blocked and there is no 11-*cis* retinol generation. The inhibition of isomerase activity correlated with the decreased ester levels in a CRBP concentration-dependent manner. To exclude the possibility that the inhibition of isomerase activity by CRBP could occur through sequestration of all-*trans* retinol, we have calculated the free retinol concentration in the reaction mixture. In this study, all of the isomerase activity assays were done with all-*trans* retinol concentration in the linear range, and thus, the 11-*cis* retinol generated should be proportional to the free all-*trans* retinol concentration. On the basis of the known dissociation constant of the retinol-CRBP complex ($K_d \sim 10$ nM) (*46*), we calculated that in the presence of 0.75 μM CRBP, the free all-*trans* retinol should only constitute less than 2% of the total added all-*trans* retinol, while 98% of the all-*trans* retinol should be bound by CRBP in the reaction mixture. If the sequestration of retinol were directly responsible for the inhibition of isomerization, then isomerase activity would be inhibited by 50-fold because only 2% free all-*trans* retinol is available. However, the results demonstrated that 0.75 μM CRBP only inhibits the isomerase activity by less than 2-fold. Therefore, the inhibition of isomerase by CRBP is unlikely to be through sequestration of all-*trans* retinol or the unavailability of free all-*trans* retinol. The parallel inhibition curves of LRAT and isomerase activities suggest that inhibited isomerase activity is a result of the decreased retinyl esters available for the isomerase.

As shown in Figure 2, with the increase of CRBP concentration, the concentration of all-*trans* retinol bound to CRBP is also increased, while the production of 11-*cis*-retinol is decreased, i.e., the isomerization is negatively correlated with the all-*trans* retinol concentration. Therefore, it is less likely that the retinol bound to CRBP is the substrate for the isomerase.

CRALBP is critical for the isomerization of all-*trans* retinol to generate 11-*cis* retinol, as it binds 11-*cis* retinol and, thus, possibly drives the reaction by mass action (*35*, *36*). In the absence of CRALBP, RPE microsomes generate only trace amounts of 11-*cis* retinoids from all-*trans* retinol (*35*). Utilizing this feature, we incubated RPE microsomes with all-*trans* retinol for 1.5 h in the absence of CRALBP. Almost all of the all-*trans* retinol was converted to retinyl esters, while no 11-*cis* retinol was detected at the end of this incubation. CRALBP and CRBP were then added to the mixture, with the latter at a concentration that can completely block LRAT activity. With preformed retinyl esters, a significant amount of 11-*cis* retinol was generated even though CRBP was present. This experiment indicates that as long as esters are available, isomerization can occur. This result further confirms that the inhibition of isomerase seen when CRBP was added at the beginning of the incubation is due to blockage of ester production.

The second LRAT inhibitor used in this study is AcDCMK, as its analogue DCMK has been shown to specifically bind to and inhibit LRAT (*29*). Previously, it was shown (*47*) that all-*trans* retinyl α-bromoacetate inhibits both the ester synthetase and the isomerase activities in parallel. However, as the chemical structure of this inhibitor is similar to both all-*trans* retinol and retinyl ester, it is hard to exclude the possibility that this inhibitor may interact with both LRAT and isomerase, thus inhibiting both activities. In addition, it has been observed that this inhibitor can be hydrolyzed by bovine RPE membranes, generating retinol and α-bromoacetate, and the products of this hydrolysis are not inhibitory (*47*). Unlike all-*trans* retinyl α-bromoacetate, the AcDCMK inhibitor lacks the β-ionone ring. As its structure differs significantly from that of retinol, AcDCMK is less likely to have a direct interaction with retinol isomerase. Our results showed that LRAT activity is inhibited by AcDCMK in a dose-dependent manner, while all-*trans* retinol levels increased with the AcDCMK concentrations. Isomerase activities decreased in parallel with the decreasing ester levels but not with the all-*trans* retinol levels. Moreover, if AcDCMK was added after the esters had formed, a significant amount of 11-*cis* retinol was generated. These observations suggest that the inhibition of isomerization by AcDCMK can be ascribed to blocked ester production, and thus, retinyl esters are essential for the isomerization to generate 11-*cis* retinol.

Although retinyl esters have been proposed to be a substrate of isomerohydrolase (*44*), direct evidence that 11-*cis* retinol can be generated from the all-*trans* retinyl esters has not been documented. The major difficulty in detecting isomerase activity using the all-*trans* retinyl esters as a substrate is the insolubility of the retinyl esters in the reaction mixture. In this study, we mixed a labeled retinyl ester (retinyl palmitate) dissolved in a small volume of DMF with RPE microsomes. This delivered a small fraction of retinyl palmitate into the microsomes which became accessible by isomerase. As a result, a significant amount of 11-*cis* retinol was formed. No all-*trans* retinol was detected in the mixture,

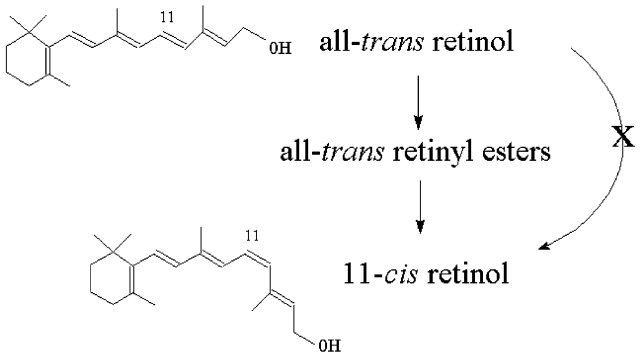

FIGURE 6: Isomerization step of the visual cycle.

suggesting that the generation of 11-*cis* retinol is not via the all-*trans* retinol. Mouse eyecup proteins also generated 11-*cis* retinol from the all-*trans* retinyl ester (Table 1). These results provide direct evidence for the first time that 11-*cis* retinol can be generated from all-*trans* retinyl esters. Taken together, these experiments all support that retinyl esters are the substrate of the isomerohydrolase.

The enzyme, or enzymatic complex, which catalyzes the isomerization reaction, has not yet been identified. On the basis of the absence of 11-*cis* retinal in *Rpe65*$^{-/-}$ mice, RPE65 has been proposed as a possible candidate for the isomerase (*14*). Though the role of RPE65 in this process has been questioned (*13*), several laboratories have confirmed that indeed the generation of 11-*cis* retinal is strikingly reduced in the absence of RPE65 (*20*, *27*, *48*). Our results have demonstrated that isomerase activity correlated with the RPE65 protein levels. In agreement with the results of Wenzel et al. (*48*), our Western blot analyses show that RPE65 protein levels are higher in BALB/c than in C57Bl/6 mice. Moreover, the isomerase activity is also higher in BALB/c than in C57Bl/6 (Table 1). The *Rpe65*$^{-/-}$ mice have no RPE65 expression and lack isomerase activity. The correlation of RPE65 protein levels with isomerase activities suggests an essential role for RPE65 in the retinol isomerization reaction.

In the *Rpe65*$^{-/-}$ mice, all-*trans* retinyl esters continuously increased during the entire age range examined, while ester levels in the WT mice plateaued after 8 weeks of age. This accumulation of retinyl esters is probably responsible for the oil droplet accumulation observed in the RPE of *Rpe65*$^{-/-}$ mice (*14*, *49*). In this respect, the *Rpe65*$^{-/-}$ mouse differs from diet-induced vitamin A deficient animals which exhibit decreased RPE retinyl ester levels with time (*50*). Likewise, in the retinol binding protein (RBP) knockout model of vitamin A deficiency, there is an impaired transport of retinol into the RPE, reducing the formation of retinyl esters (*51*). RBP knockout mice at 8 months of age have ester levels 10-fold lower than the WT animals (*51*). Therefore, the *Rpe65*$^{-/-}$ mouse is a particularly interesting model, as it has a chromophore deprived retina but can be considered a model of hypervitaminosis for the RPE.

Either increased ester synthesis or decreased ester utilization can contribute to the ester overaccumulation. Our results showed that LRAT protein levels and activity are unchanged in *Rpe65*$^{-/-}$ mice, compared to age-matched WT mice, suggesting that the overaccumulation of retinyl esters in *Rpe65*$^{-/-}$ mice is not ascribed to increased ester synthesis. The decreased utilization of all-*trans* retinyl esters due to deficient isomerization contributes, in whole or in part, to the overaccumulation of retinyl esters.

The impact of the continuous retinyl ester accumulation on the physiological function of the RPE and its role in the photoreceptor degeneration in the aged *Rpe65*$^{-/-}$ animals remain to be determined. A summary of the isomerization reaction as proposed by this study is shown in Figure 6. Further study is required to determine if the decrease in CRALBP is a result of the ester accumulation, or is due to the lack of RPE65. Likewise, the mechanism of the isomerization remains to be determined. However, the data presented here indicate that retinyl esters are the substrate for this reaction and that in the absence of an isomerization reaction, the esters are not further processed and, thus, continue to accumulate over the life of the animal.

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