

Preferential Release of 11-*cis*-retinol from Retinal Pigment Epithelial Cells in the Presence of Cellular Retinaldehyde-binding Protein*

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In photoreceptor cells of the retina, photoisomerization of 11-*cis*-retinal to all-*trans*-retinal triggers phototransduction. Regeneration of 11-*cis*-retinal proceeds via a complex set of reactions in photoreceptors and in adjacent retinal pigment epithelial cells where all-*trans*-retinol is isomerized to 11-*cis*-retinol. Our results show that isomerization *in vitro* only occurs in the presence of apo-cellular retinaldehyde-binding protein. This retinoid-binding protein may drive the reaction by mass action, overcoming the thermodynamically unfavorable isomerization. Furthermore, this 11-*cis*-retinol/11-*cis*-retinal-specific binding protein potently stimulates hydrolysis of endogenous 11-*cis*-retinyl esters but has no effect on hydrolysis of all-*trans*-retinyl esters. Apo-cellular retinaldehyde-binding protein probably exerts its effect by trapping the 11-*cis*-retinol product. When retinoid-depleted retinal pigment epithelial microsomes were preincubated with different amounts of all-*trans*-retinol to form all-*trans*-retinyl esters and then [³H]all-*trans*-retinol was added, as predicted, the specific radioactivity of [³H]all-*trans*-retinyl esters increased during subsequent reaction. However, the specific radioactivity of newly formed 11-*cis*-retinol stayed constant during the course of the reaction, and it was largely unaffected by expansion of the all-*trans*-retinyl ester pool during the preincubation. The absence of dilution establishes that most of the ester pool does not participate in isomerization, which in turn suggests that a retinoid intermediate other than all-*trans*-retinyl ester is on the isomerization reaction pathway.

Photoisomerization of 11-*cis*-retinal to all-*trans*-retinal is the key reaction that initiates vision (1). 11-*cis*-retinal is coupled via a Schiff base to a Lys residue located in the transmembrane portion of the rod and cone photoreceptor opsins. Photoisomerization triggers conformational changes in these receptors that lead to activation of G-proteins and subsequent initiation of the signaling cascade of reactions,

which comprise phototransduction (2).

How all-*trans*-retinal is isomerized back to 11-*cis*-retinal is one of the fundamental questions in vision. A pulse of intense light, which occasionally bleaches nearly 100% of our visual pigment, quickly generates 3 mM all-*trans*-retinal in the photoreceptor cell outer segment. Yet in a matter of minutes (the time constant in humans is 400 s for rhodopsin; Ref. 3), the entire cycle of isomerization and pigment regeneration occurs. No free retinals accumulate; all-*trans*-retinal is either complexed with opsin or reduced and esterified with fatty acids, whereas 11-*cis*-retinal combines with opsins. Analysis of the visual cycle in mice showed that the concentrations of free retinals and retinals are low compared with the ester pool and with all-*trans*-retinal and 11-*cis*-retinal bound to opsins (4). Mutations in any of the genes involved in retinoid metabolism could result in retinal dystrophies and degeneration of photoreceptors. Thus, it is important to understand this metabolic pathway at the molecular level.

Understanding of the visual cycle presents several intellectual problems. How does the stereospecific, energy-requiring isomerization occur in a two-cell system, with a substrate that is virtually insoluble in the aqueous phase? By what mechanism does the isomerization product, 11-*cis*-retinal, leave the retinal pigment epithelial (RPE)¹ cells and go to the photoreceptor cells where regeneration of visual pigments occurs? What methods are available to characterize the low abundance, membrane-bound enzymes responsible for retinoid regeneration? It is well established that all-*trans*-retinal, the product of photoisomerization, is reduced to all-*trans*-retinol in photoreceptor cells before it diffuses to RPE cells (5, 6). It is possible that esterification of all-*trans*-retinol with fatty acids, catalyzed by lecithin:retinol acyltransferase (LRAT; Fig. 1, reaction 2) in RPE cells, drives this transcellular diffusion by mass action. Rando and colleagues (7) proposed that all-*trans*-retinyl carboxylic esters are substrates for a membrane-bound isomerohydrolase that catalyzes the reaction in which all-*trans*- to 11-*cis*-isomerization is coupled to ester hydrolysis (Fig. 1, reaction 5). 11-*cis*-retinol formed by this reaction is oxidized to 11-*cis*-retinal by an NAD/NADP-dependent 11-*cis*-retinol dehydrogenase(s) present in RPE cells (8) (Fig. 1, reaction 9 and reverse reaction 10) and transported back to the photoreceptor cells where it associates with opsins to form rhodopsins (5, 9). 11-*cis*-retinal can also be esterified by LRAT to 11-*cis*-retinyl esters (Fig. 1, reaction 8). In addition to this pathway, cones may regenerate their pigments via enzymatic

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¹ The abbreviations used are: RPE, retinal pigment epithelial; LRAT, lecithin:retinol acyltransferase; apo-rCRALBP, apo-recombinant cellular retinaldehyde-binding protein; MOPS, 3-*N*-morpholinopropanesulfonic acid; HPLC, high pressure liquid chromatography; BSA, bovine serum albumin; BTP, 1,3-bis[tris(hydroxymethyl)-methylamino]propane.

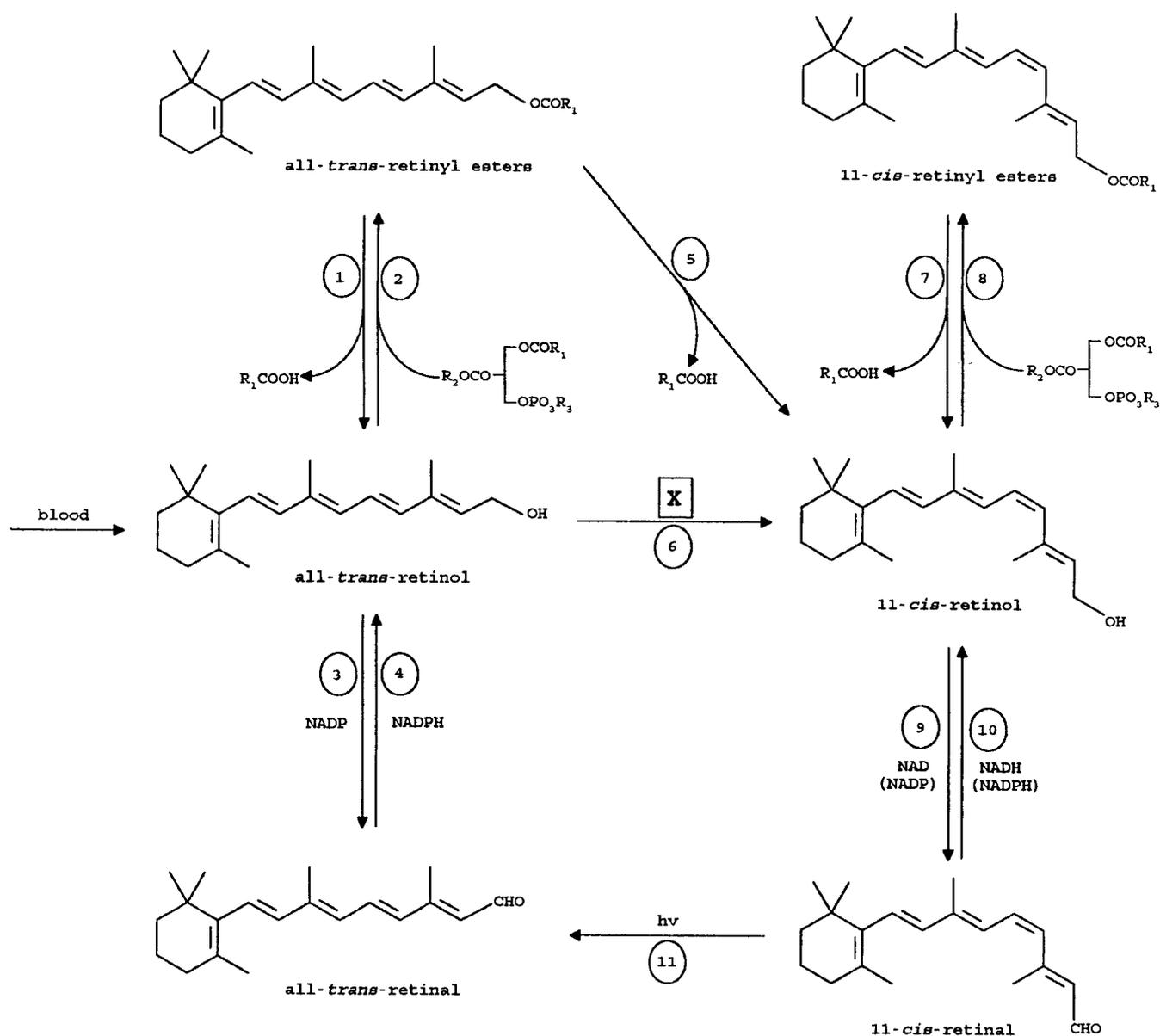


FIG. 1. **Flow of retinoids in the visual cycle.** In photoreceptors, light-sensitive visual pigments (rhodopsin in rod cells) are composed of apoprotein (opsins) and 11-*cis*-retinal coupled via protonated Schiff bases. All-*trans*-retinal is produced by photoisomerization of visual pigments (reaction 11) and reduced by retinol dehydrogenase to all-*trans*-retinol (reaction 4 and reverse reaction 3). All-*trans*-retinol diffuses to RPE cells where it is isomerized to 11-*cis*-retinol. All-*trans*-retinol is also absorbed from the blood and esterified to carboxylic esters in RPE cells by LRAT. This enzyme transfers an acyl group (2/3 palmitate and 1/3 oleate + stearate) from the *sn*-1 position of lecithin to all-*trans*-retinol (reaction 2). As proposed by Deigner *et al.* (7), the all-*trans*-retinyl carboxylic esters are substrates for an isomerohydrolase that catalyzes coupled isomerization and hydrolysis reactions (reaction 5). Alternatively, all-*trans*-retinol could be converted to 11-*cis*-retinol directly or via an unidentified, intermediate *X* (reaction 6). 11-*cis*-retinol is oxidized to 11-*cis*-retinal by NAD or NADP-dependent 11-*cis*-retinol dehydrogenase(s) present in RPE cells (reaction 9 and reverse reaction 10), and the product diffuses back to photoreceptor cells where it regenerates rhodopsins. Alternatively 11-*cis*-retinol is esterified by LRAT to 11-*cis*-retinyl esters (reaction 8). All-*trans*-retinol and 11-*cis*-retinol are also products of retinyl ester hydrolysis (reaction 1 and reaction 7). R_1 and R_2 are fatty acids, and R_3 is $-\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3^+$ and other bases.

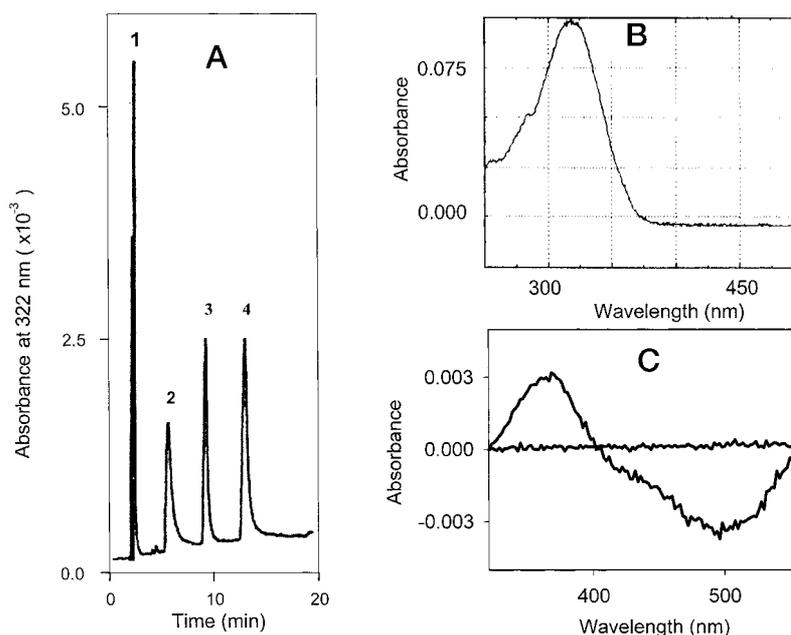
processes that also involve the neural retina (10–12).

What evidence supports the proposed isomerohydrolase reaction in RPE microsomes? 1) Isomerization occurs at the alcohol oxidation state (13, 14). 2) Isomerization occurs enzymatically without an exogenous source of energy (14, 15). Hydrolysis of all-*trans*-retinyl carboxylic ester was proposed (7) to provide the energy needed for isomerization (4 kcal/mol; Ref. 16). 3) The inhibition of all-*trans*-retinol esterification by an LRAT inhibitor prevents biosynthesis of 11-*cis*-retinol, which led to the conclusion that retinyl esters are essential intermediates in 11-*cis*-retinol formation (17). 4) During isomerization, ^{18}O -labeled all-*trans*-retinol loses its oxygen. In order for this to happen, the bond between C_{15} and oxygen must be cleaved. The

proposed mechanism of isomerization resembles a base catalyzed $\text{S}_{\text{N}}1$ alkyl cleavage. In this mechanism, double bond migration and expulsion of the carboxylate occur during addition of a base to C_{11} of the retinyl ester. Rotation about the C_{11} - C_{12} single bond is followed by attack of water at C_{15} and concomitant reshuffling of the double bonds, which locks the retinol in the 11-*cis* configuration (7).

Despite these studies supporting the isomerohydrolase reaction, there are several puzzling features. 1) Even under the best conditions, isomerization activity is too slow (1.3 pmol/min/mg protein; Ref. 15) by a factor >100 to account for the amount of 11-*cis*-retinol produced by this tissue. 2) Enzymatic production of 11-*cis*-retinol from all-*trans*-retinyl ester has not

FIG. 2. Separation and characterization of retinoids. A, HPLC separation of retinyl esters (peak 1), *syn*-11-*cis*-retinal oxime (peak 2), 11-*cis*-retinol (peak 3), and all-*trans*-retinol + *anti*-11-*cis*-retinal oxime (peak 4). B, UV spectrum of the isolated 11-*cis*-retinol peak with a maximum at 318 nm. C, bleaching difference spectrum of rhodopsin with a derived maximum at 498 nm.



been demonstrated in a chemically defined system (13, 14). 3) Enzymatic endothermic isomerization could be driven by mass action if the 11-*cis*-retinol product is selectively removed from RPE cells. 4) RPE microsomes contain a high concentration of all-*trans*-retinyl esters (>50%), which are not converted to 11-*cis*-retinol despite a favorable ΔG . 5) Evidence for participation of retinyl esters in isomerization is indirect. The LRAT inhibitor employed to block retinyl ester formation (17) could also block formation of an unidentified intermediate different from all-*trans*-retinyl ester. 6) The stoichiometry of the isomerohydrolase reaction has not been established. 7) A putative isomerohydrolase has defied purification and molecular characterization.

It was recently shown that apo-recombinant cellular retinaldehyde-binding protein (apo-rCRALBP), which binds 11-*cis*-retinol and 11-*cis*-retinal, but not all-*trans*-retinol and all-*trans*-retinal (12) or albumin, enhances production of 11-*cis*-retinol in RPE microsomes (18). This suggests that 11-*cis*-retinol may act as a potent inhibitor of the isomerase. As shown in the current study, we have also found that apo-rCRALBP greatly promotes retinoid isomerization, which allowed us to carefully study isomerization of all-*trans*-retinol in RPE microsomes. Our data reveal the complexity of the enzymatic activities in RPE microsomes that utilize retinoids. They also reveal several inconsistencies or oversimplifications in the model in which all-*trans*-retinyl ester is a substrate for a putative isomerohydrolase.

EXPERIMENTAL PROCEDURES

Materials

Fresh bovine eyes were obtained from a local slaughterhouse (Schenk Packing Co., Inc., Stanwood, WA). Liver microsomes were isolated from fresh bovine liver employing sucrose density gradients as described for isolation of bovine rod outer segments (19).

Methods

Expression of 11-*cis*-retinol Dehydrogenase in Insect Cells—The coding sequence for 11-*cis*-retinol dehydrogenase (20) was amplified from bovine retina cDNA by polymerase chain reaction with primer FH51 (5'-CATATGTGGCTGCCTCTGCTGCTG-3'), which placed a *Nde*I restriction site at the ATG initiation site, and primer FH52 (5'-TTAGTAGACTGTCTGGCAGG-3') by 32 cycles at 94 °C for 30 s and 68 °C for 2.5 min. The polymerase chain reaction fragment was cloned into the pCR™ 2.1 vector (TA cloning kit, Invitrogen) (designated pFR425) and sequenced by dyedexy terminator sequencing (ABI-Prism, Perkin-

Elmer). The expression cassette was then transferred into the baculovirus shuttle vector (bacmid) by transposition. Sf9 insect cells were transfected with the recombinant bacmid using cationic liposome-mediated transfection (CellFECTIN reagent, Life Technologies Inc.) according to the manufacturer's protocol. For the expression of recombinant proteins, cells cultured in Sf-900 II SFM medium (Life Technologies, Inc.) at 27 °C were harvested by centrifugation at 1200 \times g, 72–96 h after infection (21).

RPE Microsomes—A microsomal membrane fraction was obtained from fresh bovine RPE cells as described previously (22). The microsomal fraction was resuspended in 10 mM MOPS, pH 7.0, containing 1 μ M leupeptin, and 1 mM dithiothreitol to a total protein concentration of 3.2 mg/ml determined according to the Bradford method (23), and stored in small aliquots at -80 °C. SDS-polyacrylamide gel electrophoresis analysis showed a typical composition of proteins as observed by others (20, 24). The protein pattern did not vary significantly between preparations. Significant variations in enzymatic activities of RPE microsome preparations were observed as a function of length of storage at -80 °C. For example, ester hydrolase and isomerase activities declined by ~50% during 3 months. Thus, <1-month-old preparations were used for all studies.

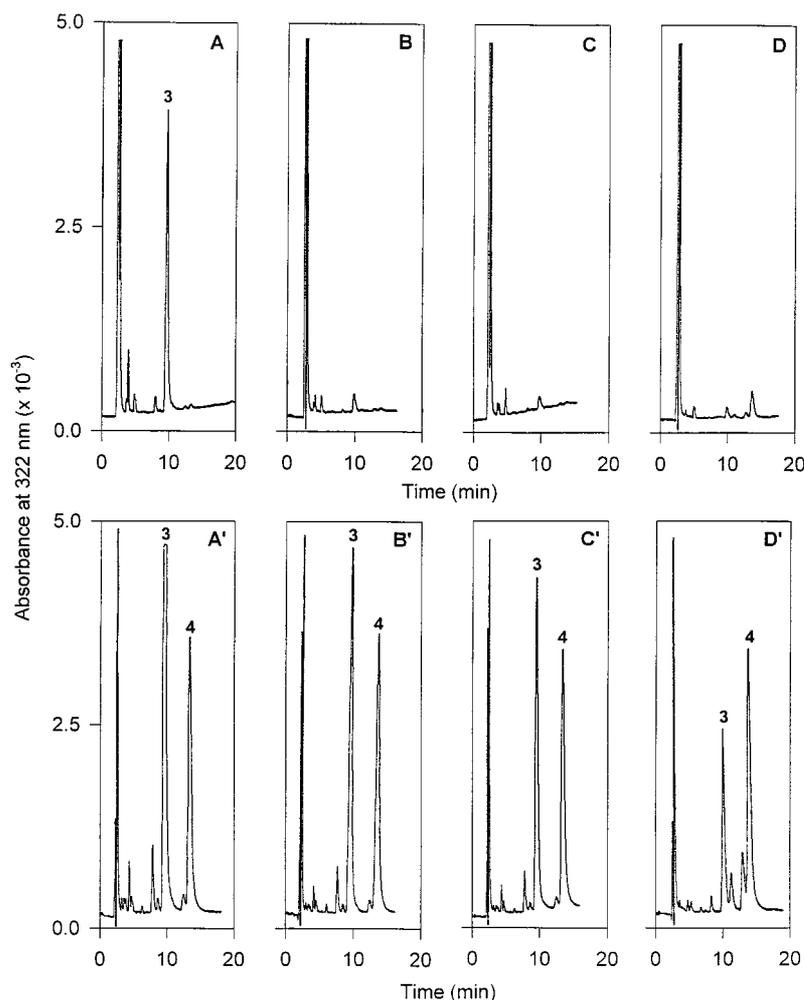
UV Treatment—To destroy endogenous retinoids, RPE microsomes (200- μ l aliquots) were irradiated in a quartz cuvette for 5 min at 0 °C using a ChromatoUVE-transilluminator (model TM-15 from UVP Inc.). UV treatment produced RPE microsomes without detectable amounts of all retinoids.

Preparation of Retinoid-binding Proteins—Interphotoreceptor retinoid-binding protein was prepared from bovine retinas as described previously (25). Apo-rCRALBP was expressed in *Escherichia coli* and purified to apparent homogeneity by Ni²⁺ affinity chromatography as described by Crabb *et al.* (26). Apo-recombinant cellular retinoid-binding protein type I was a generous gift from Dr. David Ong (Vanderbilt University, Nashville, TN).

Retinoid Preparations—To prevent isomerization and oxidation, all procedures involving retinoids were performed under dim red illumination, and the retinoids were stored under argon at -80 °C. [11,12-³H(N)]All-*trans*-retinol (NEN Life Science Products) was diluted with all-*trans*-retinol to give the desired specific radioactivity (550,000 dpm/nmol), and purified on a normal phase HPLC column (Altex, Ultrasphere-Si 5 μ ; 4.6 \times 250 mm; flow rate, 1.4 ml/min; 10% ethyl acetate in hexane; Ref. 27). Purified material was dried under argon and stored in vials (0.5–10-nmol aliquots at -80 °C) for up to 3 months. [15-³H(N)]11-*cis*-retinol (260,000 dpm/nmol) was prepared by reduction of 11-*cis*-retinal with [³H]NaBH₄ and purified by HPLC (28). Retinoid concentrations in ethanol were determined spectrophotometrically: 11-*cis*-retinal, 380 nm, ϵ = 24,935 M⁻¹ cm⁻¹; all-*trans*-retinal, 383 nm, ϵ = 42,880 M⁻¹ cm⁻¹; 11-*cis*-retinol, 319 nm, ϵ = 34,890 M⁻¹ cm⁻¹; all-*trans*-retinol, 325 nm, ϵ = 52,770 M⁻¹ cm⁻¹. Rhodopsin concentration was measured as described by McDowell (29).

Reaction Conditions for Isomerase, LRAT, and Hydrolase—Sub-

FIG. 3. Influence of apo-rCRALBP on the 11-cis-specific hydrolysis of endogenous retinyl esters. RPE microsomes (0.4 mg/ml) were incubated for 30 min at 37 °C in 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM CaCl₂, and 1% BSA. After quenching the reaction with methanol, retinoids were extracted into hexane, and 1/30 of the extract was analyzed by HPLC either before (A–D) or after saponification (A'–D'). A and A', RPE microsomes were incubated with 25 μM apo-rCRALBP. B and B', RPE microsomes were incubated with boiled 25 μM apo-rCRALBP. C and C', RPE microsomes were incubated alone. D and D', boiled RPE microsomes were incubated with 25 μM apo-rCRALBP. The lower amount of 11-cis-retinol in D' indicates the heat-sensitivity of 11-cis-retinol. Component 3, 11-cis-retinol; component 4, all-trans-retinol. Similar data were obtained from 17 independent experiments. Note that the numeric data are presented in Fig. 4.



strate, [³H]all-trans-retinol, or [³H]11-cis-retinol (0.5–1 nmol, 260,000–550,000 dpm/nmol), was dried from hexane/ethyl acetate under argon in a 1.5-ml polypropylene tube. 20 μl of BSA (5% solution in 10 mM BTP, pH 7.0) was added followed by 20–30 μl of apo-rCRALBP in 10 mM BTP, pH 8.0, containing 250 mM NaCl to give a final concentration of 25 μM. Next, 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂ was added to bring the total volume to 175 μl. For some experiments, additional compounds in the same buffer were added. Finally, 25 μl of RPE microsomes (80 μg of protein) were added to this mixture, and the reactions were incubated at 37 °C for the indicated times.

HPLC Separation of Retinoids—The reaction mixture (180 μl of 200 μl) was transferred to a new vial containing 300 μl of ice-cold methanol, and 300 μl of hexane was added. The sample was vortexed for 2 min and centrifuged for 4 min at 14,000 × g to separate organic and aqueous phases. 10 μl of hexane extract was injected into the HPLC column. Retinoids were separated using an HP1050 HPLC (with a single wavelength detector at 325 nm) or an HP1100 HPLC (with a diode array detector 280–400 nm) and a normal phase, narrow bore column (Alltech, Silica 5μ Solvent Miser, 2.1 × 250 mm). An isocratic solvent composed of 10% ethyl acetate in hexane at a flow rate of 0.3 ml/min was used. All-trans-retinol and 11-cis-retinol were extracted with hexane in 75–95% yield as determined using [³H]retinol tracers. To estimate the yield of retinyl ester extraction, [³H]all-trans-retinol was incubated for 1 h with RPE microsomes as a source of LRAT. Most of the [³H]all-trans-retinol was converted to hydrophobic [³H]all-trans-retinyl esters, which were extracted in ~60% yield. All-trans-retinyl esters and 11-cis-retinyl esters were eluted 0.5 min after the solvent front (Fig. 2A, peak 1), followed by 11-cis-retinol (peak 3) and all-trans-retinol (peak 4), all with a chromatographic yield of >95%. 9-cis-retinol eluted ~1 min earlier than all-trans-retinol, while 13-cis-retinol eluted on the descending side of the 11-cis-retinol peak (data not shown). Without preincubation, native RPE microsomes contained 0–0.9 nmol of all-trans-retinol/mg of protein, 0–0.3 nmol 11-cis-retinol, 8.6 ± 0.9 nmol all-trans-retinyl esters, and 9.6 ± 1.4 nmol 11-cis-retinyl esters. The

amounts of all-trans-retinyl esters and 11-cis-retinyl esters were measured after separation of a mixture of esters from polar retinoids, saponification, and another round of HPLC separation to quantify all-trans-retinol and 11-cis-retinol. The different preparations of RPE microsomes had similar amounts of retinols and retinals; however, they differed in amounts of endogenous esters (with similar ratio between 11-cis-retinyl and all-trans-retinyl esters). In some cases, the ester pool was as high as ~60 nmol/mg of protein.

Preparation of Retinal Oximes—Isomers of retinal were converted to more polar and less chemically reactive oximes before analysis (32). Reaction mixtures were stopped with the addition of an equal volume of methanol containing sufficient NH₂OH to give a final concentration of 10 mM. After 30 min at room temperature, 0.3 ml of hexane was added, and retinoids were extracted as described before. Retinals in RPE microsomes were converted to oximes with >85% yield. The *syn*-conformer of retinal oximes eluted between the retinyl ester and 11-cis-retinol peaks (Fig. 2A, peak 2), whereas smaller peaks of the *anti*-conformer eluted on the descending side of the all-trans-retinol peak (descending site of peak 4 in Fig. 2A). Retinals were present in RPE microsomes at a low level (<0.1 nmol/mg of RPE protein) and were not generated in most of our experimental conditions.

Hydrolysis of Retinyl Esters—Hexane from the extracts containing retinyl esters or from the HPLC purified retinyl ester fractions (typically 200 μl) were evaporated under argon. The residual esters were dissolved in 230 μl of absolute ethanol, and 20 μl of 6 M KOH was added. The sample was incubated for 30 min at 55 °C, diluted with 100 μl of water, chilled on ice for 2 min, and extracted with 300 μl of hexane. The retinoids in hexane were analyzed directly by HPLC.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (30) using 12% SDS acrylamide gels in a Hoefer minigel apparatus and low molecular weight markers from Amersham Pharmacia Biotech. The gels were stained with Coomassie Brilliant Blue R-250 and destained with 50% methanol and 7% acetic acid.

RESULTS

Influence of apo-rCRALBP on Formation of 11-cis-retinol—To test the influence of apo-rCRALBP on formation of 11-cis-retinol from endogenous retinoids, the binding protein was added to a suspension of bovine RPE microsomes and incubated at 37 °C. Retinoids were extracted with hexane and separated on a normal phase silica column under isocratic conditions (Fig. 2A). Addition of apo-rCRALBP to bovine RPE microsomes resulted in formation of 11-cis-retinol (Fig. 3A, peak 3). To prove that the fraction, which eluted at ~10 min, contained 11-cis-retinol and not 13-cis-retinol, which co-elutes, 1) UV spectra were recorded continuously during the chromatography (data not shown) and after the fraction was collected. The spectrum showed the smooth ascending and descending limbs (Fig. 2B) characteristic of 11-cis-retinol (31) and 2) material in the 10-min fraction was oxidized to the aldehyde by recombinant 11-cis-retinol dehydrogenase and incubated with opsin, giving the characteristic spectrum of rhodopsin (498 nm absorption maximum) with a yield of 85% (Fig. 2C). Note that 13-cis-retinol does not regenerate opsin. We conclude that the 10-min fraction contained >85% 11-cis-retinol. The retinol was bound to soluble apo-rCRALBP as determined after pelleting the RPE microsomes and analyzing the retinol content of the membrane and supernatant phases. When apo-rCRALBP was boiled or omitted, only 11-cis-retinol endogenous to RPE microsomes was found (Fig. 3, B and C). Boiling the RPE microsomes led to even smaller amounts of 11-cis-retinol and 11-cis-retinyl esters (Fig. 3D) than in unboiled samples without apo-rCRALBP, presumably due to partial destruction of 11-cis-retinoids. Ester analysis showed an apparent ~20% increase in the amount of 11-cis-retinyl ester in the sample with apo-rCRALBP as compared with the sample that lacked this retinoid-binding protein. This was attributed to higher extraction yields of polar retinoids, as compared with the more hydrophobic esters. As will be described below, most of the 11-cis-retinol formed in the presence of apo-rCRALBP is due to hydrolysis of 11-cis-retinyl esters.

To follow the fate of exogenous all-trans-retinol, RPE microsomes were incubated with 2.5 μM [^3H]all-trans-retinol. In the first 5 min of the reaction, more than 90% of [^3H]all-trans-retinol was converted to [^3H]all-trans-retinyl esters by endogenous LRAT (Fig. 4A). The amount of [^3H]all-trans-retinyl esters decreased during reaction as more [^3H]11-cis-retinol was formed in the samples containing apo-rCRALBP (Fig. 4A). During the course of the reaction, the amount of free [^3H]all-trans-retinol decreased rapidly to ~0.12 μM (or ~0.3 nmol/mg of protein). The decrease in the ester pool coincides with an increase in 11-cis-retinol, suggesting a precursor-product relationship (13, 14). However, evidence to be presented under "UV-treated RPE Microsomes" indicates that only a small fraction of the ester pool, if any, participates in the isomerization reaction. Thus, it is more likely that the decrease in the ester pool reflects conversion to all-trans-retinol, subsequent isomerization to 11-cis-retinol, and binding to apo-rCRALBP. Based on hydrolysis of the ester pool and rechromatography of retinoids, no detectable amounts of [^3H]11-cis-retinyl esters were formed during the reaction (data not shown).

Isomerization was measured as the formation of [^3H]11-cis-retinol. Based on the initial rate, the isomerization proceeded with formation of 0.09 nmol of 11-cis-retinol/min/mg of protein (Fig. 4A).² The amount of [^3H]all-trans-retinol converted to

² Because RPE microsomes contain endogenous retinoids, the specific radioactivity of the labeled pool is lower than that of the [^3H]all-trans-retinol added. The mol amounts given for each retinoid species are calculated from the cpm data using the specific radioactivity of the added [^3H]all-trans-retinol and are lower limit values.

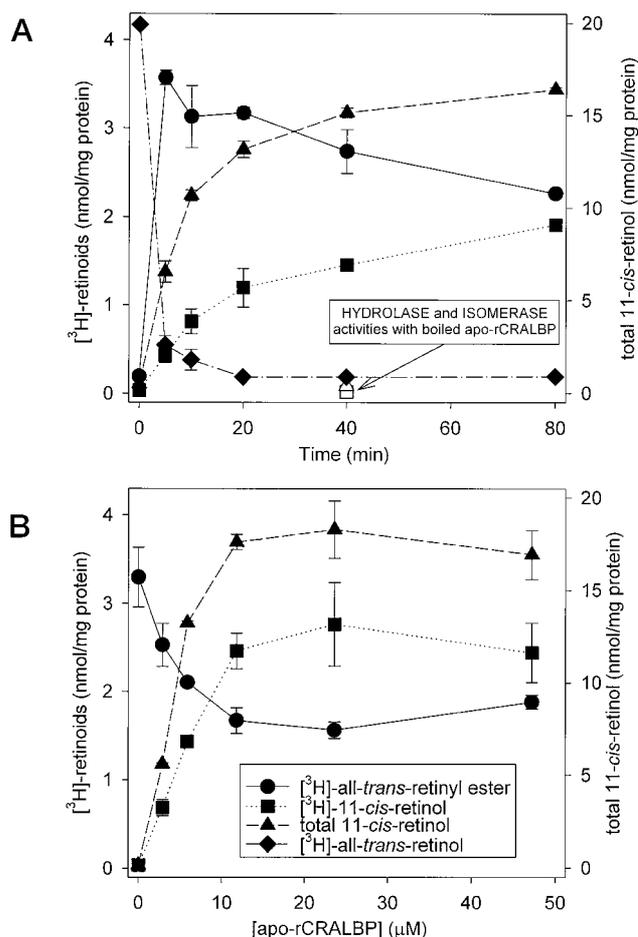


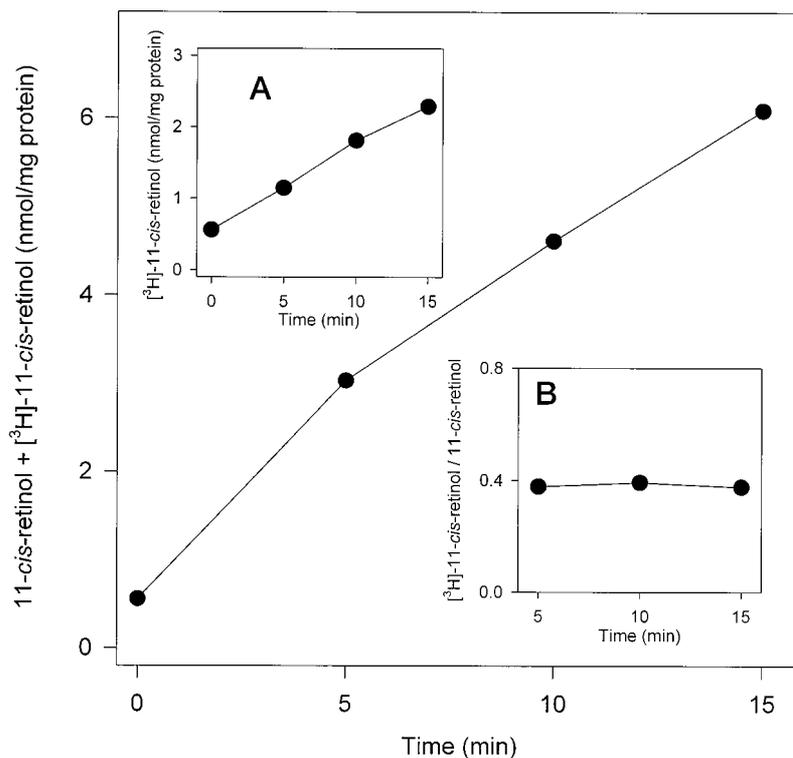
FIG. 4. Retinyl ester hydrolase, 11-cis-retinol isomerase, and LRAT activities in the presence of apo-rCRALBP. The reaction was carried out in the presence of RPE microsomes (0.4 mg/ml of protein), 2.5 μM [^3H]all-trans-retinol (550,000 dpm/nmol) in 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM CaCl_2 , and 1% BSA at 37 °C. After quenching the reaction with methanol, retinoids were extracted into hexane and 1/30 of the extract was analyzed by HPLC. A, time courses for formation of 11-cis-retinol (triangles, dashed line); [^3H]all-trans-retinyl esters (circles, solid line), and [^3H]11-cis-retinol (squares, dotted line) in the presence of 25 μM apo-rCRALBP. Changes in [^3H]all-trans-retinol are shown by diamonds on a dashed and dotted line. B, the effect of apo-rCRALBP concentrations on 11-cis-retinol (total and ^3H -labeled) and [^3H]retinyl ester formation. The reaction was carried out in the presence of the indicated concentration of apo-rCRALBP for 80 min.

[^3H]13-cis-retinol was estimated as ~5 pmol/mg of protein by quantifying the amount of [^3H]13-cis-retinol formed at time 0 in samples with boiled apo-rCRALBP. 13-cis-retinol was identified by its specific retention time (0.2 min after 11-cis-retinol) and characteristic UV spectrum (data not shown) (31). No additional [^3H]13-cis-retinol was formed during incubation with RPE microsomes (data not shown). Thus, 13-cis-retinol was only a small fraction (~1/200) of 11-cis-retinol and did not significantly affect interpretation of our data.

In addition to isomerization, 11-cis-retinol was also produced as a result of hydrolysis of endogenous 11-cis-retinyl esters (described below in Fig. 5). The isomerization plus hydrolysis reactions proceeded with an initial rate of 1.4 nmol/min/mg of protein, which is faster than the rate of isomerization (0.09 nmol of 11-cis-retinol/min/mg of protein), and reached a plateau in ~60 min to form ~16 nmol of 11-cis-retinol/mg of RPE protein (Fig. 4A).

As shown in Fig. 4B, the concentration of apo-rCRALBP used in the above experiments (25 μM) was sufficient to give maximal effects on 11-cis-retinol and [^3H]11-cis-retinol formation.

FIG. 5. Release of 11-cis-retinol from RPE microsomes. Release of 11-cis-retinol and [^3H]11-cis-retinol from the endogenous 11-cis-retinyl ester pool and preformed [^3H]11-cis-retinyl esters, respectively. RPE microsomes (80 μg of protein in 200 μl) were incubated at 37 $^{\circ}\text{C}$ in the presence of 2.5 μM [^3H]11-cis-retinol (260,000 dpm/nmol) in 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM CaCl_2 , and 1% BSA. After 45 min, 25 μM apo-rCRALBP was added, and the release of 11-cis-retinol was measured by integration of HPLC peak; [^3H]11-cis-retinol scintillation counting followed with time. After quenching the reaction with methanol, retinoids were extracted into hexane, and 1/30 of the extract was analyzed by HPLC. *Inset A*, release of [^3H]11-cis-retinol from preformed [^3H]11-cis-retinyl ester. The pre-existing amount of ~ 0.4 nmol [^3H]11-cis-retinol at time 0 in both figures is due to incomplete esterification of [^3H]11-cis-retinol by LRAT during the 45-min preincubation. *Inset B*, ratio of amounts of 11-cis-retinol and [^3H]11-cis-retinol released. Here, a ratio of 1 is defined as the specific radioactivity of the [^3H]11-cis-retinol added to RPE microsomes. Similar results were obtained in two independent experiments.



Note that the concentration of CRALBP used in this study is within the *in vivo* concentration predicted to be ~ 25 – 50 μM (12). The initial formation of [^3H]all-trans-retinyl esters was unaffected by the presence of active apo-rCRALBP or boiled apo-rCRALBP.

To measure retinyl ester hydrolase activity directly without isomerization, exogenously added [^3H]11-cis-retinol was converted to 11-cis-retinyl esters, by LRAT present in RPE microsomes, before apo-rCRALBP was added. Most of the exogenously added 11-cis-retinol ($\sim 90\%$) was converted to the esters. Addition of apo-rCRALBP led to the release of 11-cis-retinol and [^3H]11-cis-retinol (Fig. 5, *inset A*) with an initial rate of 0.7 nmol/min/mg of protein. If isomerization would contribute significantly to the total amounts of 11-cis-retinol formed, it is expected that with time, more 11-cis-retinol would be converted, diluting [^3H]11-cis-retinol formed from the [^3H]11-cis-retinyl ester pool. The ratio of [^3H]11-cis-retinol to total 11-cis-retinol, however, remained constant during the reaction (Fig. 5, *inset B*), suggesting only a small contribution of the isomerization of endogenous all-trans-retinol to 11-cis-retinol formation. No radioactivity was detected in the all-trans-retinol peak in the presence or absence of apo-rCRALBP. All together, the results show that isomerization of all-trans-retinol to 11-cis-retinol is essentially an irreversible reaction in RPE microsomes under the conditions studied.

In control experiments, liver microsomes were used to assay isomerase activity in the presence of apo-rCRALBP and exogenously added [^3H]all-trans-retinol. Liver microsomes had high LRAT/ acyl-CoA:retinol acyltransferase activities; however, no detectable isomerization to [^3H]11-cis-retinol was observed above nonenzymatic formation of [^3H]13-cis-retinol (data not shown).

Specificity of Retinoid-binding Proteins in the Hydrolysis of Endogenous Retinyl Esters—To explore whether formation of 11-cis-retinol was due to apo-rCRALBP forming a complex with hydrolase/isomerase, retinoid-binding proteins with different specificities were employed. Interphotoreceptor retinoid-binding protein and cellular retinol-binding protein type I, which

TABLE I
Influence of different retinoid-binding proteins on all-trans-retinol and 11-cis-retinol formation

RPE microsomes (80 μg of protein in 200 μl), which served as the source of endogenous retinyl esters and retinyl ester hydrolase and isomerase, were incubated at 37 $^{\circ}\text{C}$ in 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM CaCl_2 , and 1% BSA for 40 min in the presence of the indicated concentration of a bovine interphotoreceptor retinoid-binding protein (IRBP) or bovine cellular retinol-binding protein (CRBP). The reaction was quenched with methanol. Retinoids were extracted into hexane and were analyzed by HPLC as described under "Experimental Procedures."

Concentration	IRBP		CRBP	
	11-cis-retinol	all-trans-retinol	11-cis-retinol	all-trans-retinol
μM	nmol			
5			1.40 \pm 0.03	1.97 \pm 0.06
5	0.73 \pm 0.01	0.15 \pm 0.01		
10	1.26 \pm 0.03	0.28 \pm 0.01		
20			4.18 \pm 0.01	4.02 \pm 0.01
40			8.10 \pm 0.25	6.56 \pm 0.20

bind both 11-cis-retinol and all-trans-retinol, accelerated release of 11-cis-retinol and all-trans-retinol from RPE microsomes (Table I). The effect was specific for native forms of the proteins; heat denaturation destroyed their stimulation properties (Fig. 6). These results suggest that retinoid-binding proteins promote release of retinols from retinyl esters by trapping retinols released by the hydrolase. Thus, retinoid-binding proteins probably do not act by forming specific complexes with enzymes producing 11-cis-retinol. However, we cannot exclude the possibility that each retinoid-binding protein exerts its effect by different molecular mechanisms. Similar results were obtained for release of all-trans-retinol by apo-recombinant cellular retinol-binding protein type I from liver microsomes (33).

Oxidation of 11-cis-retinol Does Not Enhance Isomerization—To test whether oxidation of 11-cis-retinol to 11-cis-retinal affects conversion of all-trans-retinol to 11-cis-retinol, NAD and NADP, cofactors of 11-cis-retinol dehydrogenase, were

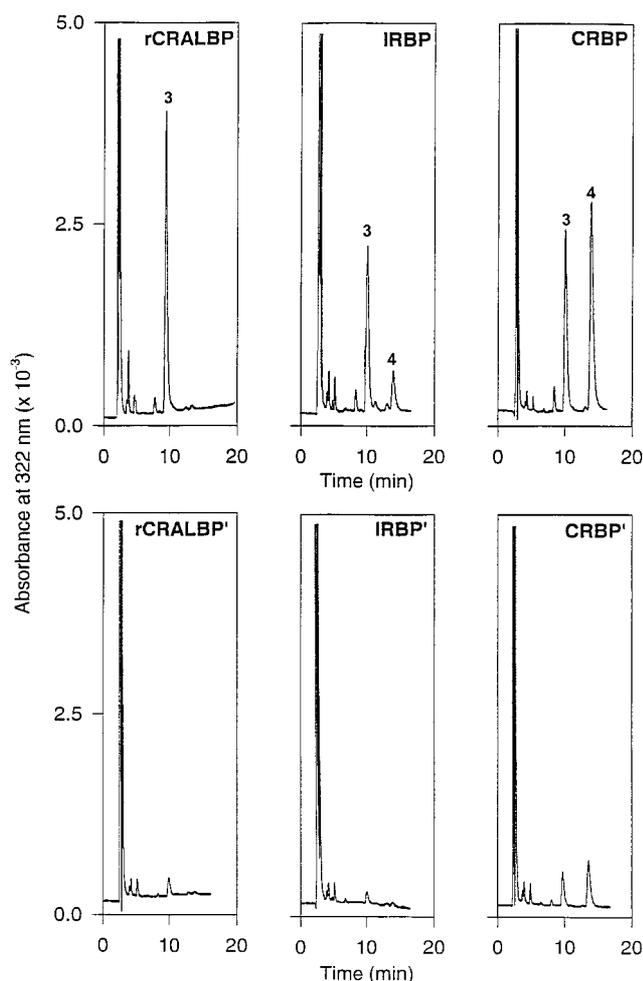


FIG. 6. Influence of different retinoid-binding proteins on 11-*cis*-retinol and all-*trans*-retinol formation. RPE microsomes (80 μ g of protein in 200 μ l) were incubated for 30 min at 37 $^{\circ}$ C in 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM CaCl₂, and 1% BSA with 25 μ M native retinoid-binding proteins (upper panels) or with boiled binding proteins (lower panels). After quenching the reaction with methanol, retinoids were extracted into hexane, and 1/30 of the extract was analyzed by HPLC. Component 3, 11-*cis*-retinol; component 4, all-*trans*-retinol; IRBP, bovine interphotoreceptor retinoid-binding protein; CRBP, bovine cellular retinoid-binding protein. Similar results were obtained in two independent experiments.

added. Note that RPE microsomes contain endogenous 11-*cis*-retinol dehydrogenase (8). In the presence of apo-rCRALBP and NAD or NADP, efficient production of 11-*cis*-retinal was observed (as its oxime derivative, mixture of *syn*- and *anti*-isomers), whereas NAD alone had almost no effect (data not shown). It should be noted that CRALBP has a higher affinity for 11-*cis*-retinal than for 11-*cis*-retinol and that 11-*cis*-retinol bound to CRALBP is a good substrate of 11-*cis*-retinol dehydrogenase (34). The initial rate of 11-*cis*-retinoid formation was only minimally affected when 11-*cis*-retinol was oxidized to 11-*cis*-retinal (Table II), suggesting that the dehydrogenase was not able to further drive the isomerization reaction in our experimental conditions.

UV-treated RPE Microsomes—Analysis of retinoid conversions in RPE microsomes is complicated by the presence of endogenous retinoids. Thus, RPE microsomes were exposed to UV radiation for 5 min to destroy all of the endogenous retinoids (7), as determined by the HPLC retinoid analysis. Formation of 11-*cis*-retinol from exogenously added all-*trans*-retinol is not detectable without apo-rCRALBP, but all-*trans*-retinyl esters are formed (Fig. 7). To examine the possibility

TABLE II
Oxidation of 11-*cis*-retinol in RPE microsomes in the presence of apo-rCRALBP

RPE microsomes (80 μ g of protein in 200 μ l), which served as the source of endogenous retinyl esters, retinyl ester hydrolase, isomerase and 11-*cis*-retinol dehydrogenase, were incubated at 37 $^{\circ}$ C in 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM CaCl₂, and 1% BSA with and without 0.5 mM NAD/0.5 mM NADP. At indicated times, the reaction was quenched with methanol and NH₂OH (final concentration, 5 mM) to form retinal oximes. Retinoids were extracted into hexane and were analyzed by HPLC as described under "Experimental Procedures."

Time	Conditions 1 (-NAD/NADP)		Conditions 2 (+NAD/NADP)	
	11- <i>cis</i> -retinol	11- <i>cis</i> -retinal	11- <i>cis</i> -retinal oximes	total 11- <i>cis</i> -retinoids
min	nmol			
0	0.49 \pm 0.03	0.41 \pm 0.03	0.38 \pm 0.02	0.79
5	6.54 \pm 0.58	2.23 \pm 0.20	2.76 \pm 0.18	4.99
10	10.68 \pm 0.29	3.08 \pm 0.20	4.39 \pm 0.18	7.47
20	13.14 \pm 0.44	3.93 \pm 0.07	6.52 \pm 0.01	10.45

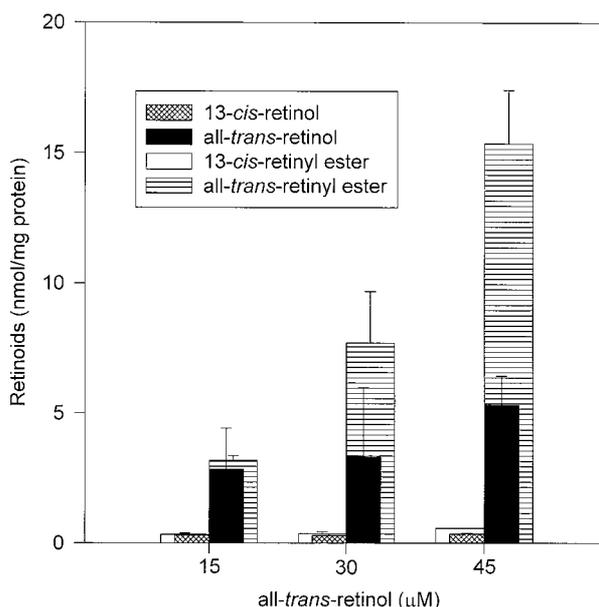


FIG. 7. Lack of isomerase activity in UV-treated RPE microsomes in the absence of apo-rCRALBP. UV-treated RPE microsomes (80 μ g of protein in 200 μ l) were incubated with 15 μ M all-*trans*-retinol, 30 μ M nmol all-*trans*-retinol, and 45 μ M all-*trans*-retinol for 60 min at 37 $^{\circ}$ C in 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, and 1% BSA. After quenching the reaction with methanol, retinoids were extracted into hexane and analyzed by HPLC both before and after saponification. 11-*cis*-retinol was not detected.

that 11-*cis*-retinol is formed and then converted to 11-*cis*-retinyl esters in the absence of apo-rCRALBP, the HPLC ester fraction was collected and saponified. Analysis of retinols revealed the presence of all-*trans*-retinol and small amounts of 13-*cis*-retinol, the latter formed nonenzymatically, but no 11-*cis*-retinol was detected.

To directly test whether the pre-existing pool of retinyl esters has an effect on isomerization, unlabeled all-*trans*-retinol was incubated for 1 h with UV-treated RPE microsomes to produce unlabeled retinyl esters, before 2.5 μ M [³H]all-*trans*-retinol (550,000 dpm/nmol) and apo-rCRALBP were added (Fig. 8). As expected, for the control sample without the addition of unlabeled all-*trans*-retinol during preincubation, the specific radioactivity of [³H]all-*trans*-retinyl esters and all-*trans*-retinol remained constant over 60 min (Fig. 8, A and B). For comparison purposes, this constant specific radioactivity is set to 1 (i.e. the ratio of [³H]all-*trans*-retinoid/total all-*trans*-retinoid = 1). For the samples preincubated with 30 and 45 μ M of unlabeled

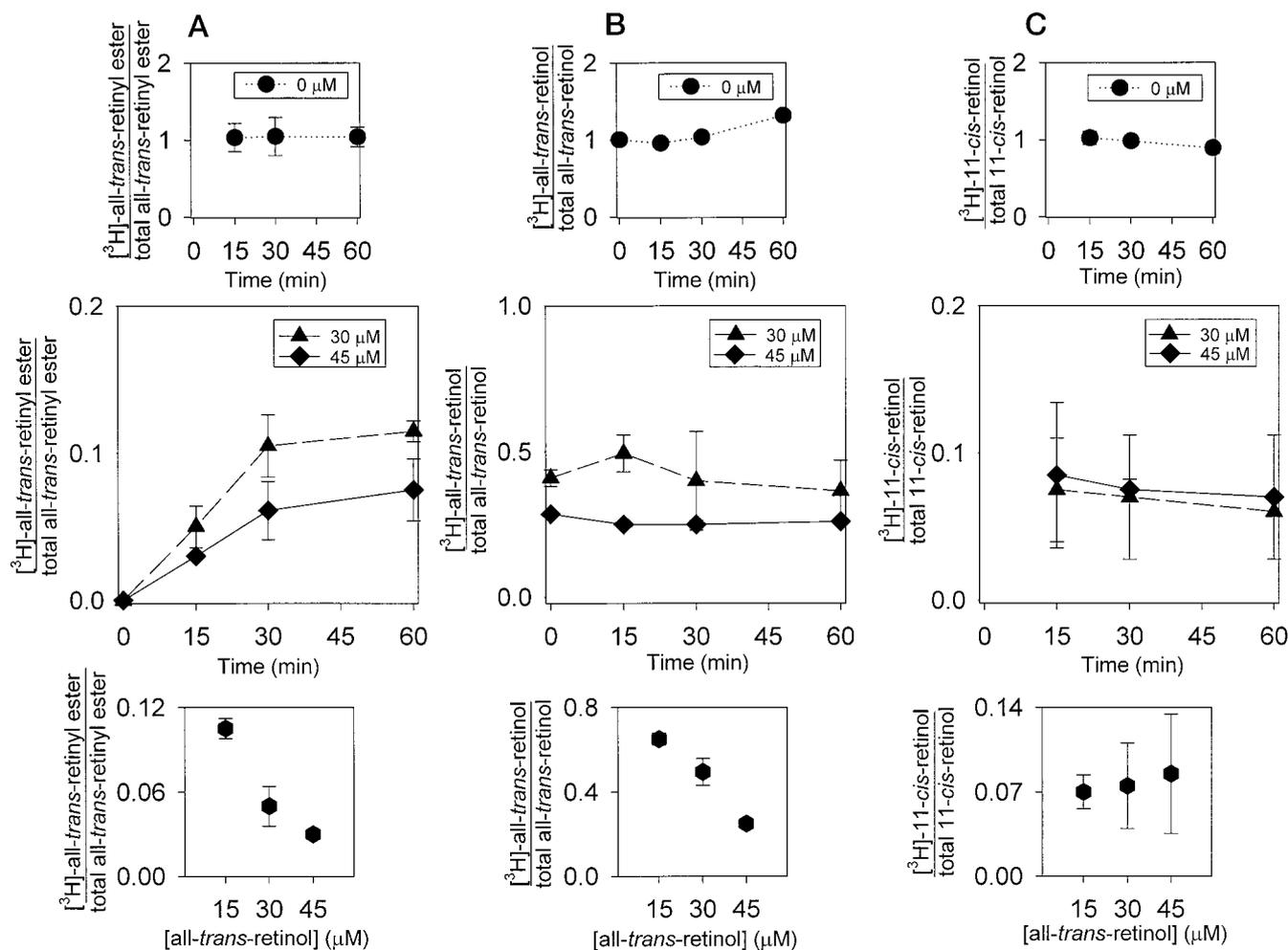


FIG. 8. The effect of preformation of all-trans-retinyl esters on the isomerization. UV-treated RPE microsomes (80 μg of protein in 200 μl) were incubated without all-trans-retinol (circles, dotted lines), with 30 μM all-trans-retinol (triangles, dashed lines), or with 45 μM all-trans-retinol (diamonds, solid lines) for 60 min at 37 $^{\circ}\text{C}$ in 10 mM BTP, pH 8.0, containing 100 mM NaCl, 2 mM MgCl_2 , 2 mM CaCl_2 , and 1% BSA. Next, 2 μM [^3H]all-trans-retinol and 25 μM apo-rCRALBP were added, and the radioactive and total amounts of all-trans-retinyl ester (A), all-trans-retinol (B), and 11-cis-retinol (C) were measured at the indicated times. Panels at the bottom represent the dilution effects at 15 min. Error bars indicate standard deviations.

all-trans-retinol, the specific radioactivity of [^3H]all-trans-retinyl esters increased with time of subsequent incubation (Fig. 8A). Because unlabeled esters formed during the preincubation are not hydrolyzed in the absence of apo-rCRALBP (Fig. 3) and because >85% of the unlabeled all-trans-retinol is esterified during the 60-min preincubation, the specific radioactivity of the all-trans-retinyl esters should increase with time from 0 to ~ 0.12 (30 μM unlabeled retinol added) or to ~ 0.05 (45 μM unlabeled retinol added), and this was observed (Fig. 8A). These data also indicate that a small fraction (2.5–7.5 μM) of unlabeled all-trans-retinol remained unesterified after the preincubation. These results also show that in the presence of apo-rCRALBP, no hydrolysis of all-trans-retinyl esters occurred because the specific radioactivity of [^3H]all-trans-retinol remained constant during the second incubation period (Fig. 8B). The specific radioactivity of free [^3H]11-cis-retinol formed after the preincubation period was ~ 10 -fold lower than the initial specific radioactivity of the [^3H]all-trans-retinol added (Fig. 8C). This result is compared with a ~ 3 -fold dilution for [^3H]all-trans-retinol (Fig. 8B). Importantly, the specific radioactivity of 11-cis-retinol was unchanged during the reaction period with apo-rCRALBP and was independent of the amount of unlabeled retinyl esters formed during the preincubation with unlabeled all-trans-retinol (Fig. 8C). This lack of dilution proves that most of the ester pool does not participate in

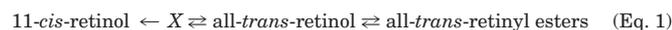
isomerization. Had 11-cis-retinol come from the ester pool, its specific radioactivity would have increased over time as in Fig. 8A. It may be noted that because only trace amounts of [^3H]all-trans-retinol were added, conversion of this radiolabel to all-trans-retinyl ester does not significantly change the concentration of esters in the microsomes and therefore does not cause a change in the rate of ester consumption.

DISCUSSION

Apo-retinoid-binding Proteins Are Necessary in Vitro to Monitor Isomerase and Hydrolase Activities—Our results show that the isomerase activity is reliably assayed in RPE microsomes only in the presence of apo-rCRALBP, in contrast to earlier reports (Refs. 13–17; but see more recent studies, such as Ref. 18). Furthermore, apo-rCRALBP also promotes the hydrolysis of 11-cis-retinyl esters in RPE microsomes, which can also be easily measured using [^3H]11-cis-retinol-labeled native and UV-treated RPE microsomes. Hydrolysis of all-trans-retinyl esters was not affected by apo-rCRALBP. The enzymatic activity of retinyl ester hydrolase in RPE microsomes is ~ 15 -fold higher than the isomerase activity in the presence of apo-rCRALBP (Figs. 4 and 5). In contrast, apo-rCRALBP had no effect on esterification of all-trans-retinol, probably because it does not bind all-trans-retinol. A high affinity binding site for 11-cis-retinol appears to be sufficient to drive isomerization

and hydrolysis (Fig. 6). Our data point to the possibility that *in vivo* apo-CRALBP or another 11-*cis*-retinol withdrawing system would efficiently drive the isomerization reaction. NAD/NADP in the absence of apo-rCRALBP does not significantly drive isomerization *in vitro*. However, *in vivo*, in a highly compartmentalized cell, oxidation and rhodopsin regeneration may be more efficient in driving this reaction. The consequences of this feature of the visual cycle would be that 11-*cis*-retinol/11-*cis*-retinal would not accumulate to any appreciable amount in RPE cells or retina, and 11-*cis*-retinal would be formed on demand, as observed *in vivo* (4).

Isomerization of All-trans-retinol to 11-cis-retinol—Two lines of evidences suggest that isomerization may not proceed through carboxylic retinyl esters. 1) The necessity of retinoid-binding proteins for assaying isomerase activity *in vitro* suggests that these proteins may be a driving force for the reaction. Without apo-CRALBP, we found no evidence of enzymatic isomerization even when the concentrations of endogenous retinyl esters (Fig. 3) or those formed *in situ* were high (Fig. 7). Winston and Rando (18) proposed that 11-*cis*-retinol is a potent product inhibitor of isomerization. However, with the conditions of the experiment presented in Fig. 8, this explanation seems unlikely because 11-*cis*-retinol or its esters were not present in detectable amounts in the RPE microsomes. According to the current model of endothermic isomerization coupled to exothermic ester hydrolysis, it is difficult to understand why all-*trans*-retinyl esters are not largely converted to 11-*cis*-retinol (13–15). 2) The results presented in Fig. 8 are inconsistent with the entire pool of retinyl esters being the substrate for isomerization. If the reaction would proceed through retinyl esters, the specific radioactivity of 11-*cis*-retinol should increase with the time of reaction, as the all-*trans*-retinyl ester pools becomes labeled, and should be inversely proportional to the size of the unlabeled retinyl ester pool. These results were not observed. The fact that the specific radioactivity of 11-*cis*-retinol is ~3-fold lower than that of all-*trans*-retinol (Fig. 8, B and C) suggests that the isomerization proceeds through a reaction similar to that shown in Equation 1 (Fig. 1, reaction 6).



X could be an unidentified intermediate, an enzyme-retinol intermediate, or a specific subpopulation of retinyl esters with properties that are distinct from the bulk of retinyl esters. Although, ATP stimulated hydrolase activity 4-fold and isomerase activity 1.5–2-fold, X may not be a high energy intermediate generated from triphosphate nucleotides because or-

thophosphate alone stimulates the activities.³ In addition, isomerization is observed in the presence of apo-rCRALBP without an exogenous energy source.

With these findings in hand, the precise mechanism of isomerization can be analyzed in the future, including the identity of the putative intermediate X . These studies are expected to yield the tools needed to identify the enzyme responsible for isomerization of all-*trans*-retinol to 11-*cis*-retinol.

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