

Retinoid cycle in the vertebrate retina: experimental approaches and mechanisms of isomerization

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Abstract

Retinoid cycle describes a set of chemical transformations that occur in the photoreceptor and retinal pigment epithelial cells. The hydrophobic and labile nature of the retinoid substrates and the two-cell chromophore utilization-regeneration system imposes significant constraints on the experimental biochemical approaches employed to understand this process. A brief description of the recent developments in the investigation of the retinoid cycle is the current topic, which includes a review of novel results and techniques pertaining to the retinoid cycle. The chemistry of the all-*trans*-retinol to 11-*cis*-retinol isomerization is also discussed.

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Abbreviations: ABCR—a photoreceptor-specific, ATP-binding cassette transporter; COS—cone outer segments; CRALBP—cellular retinaldehyde-binding protein; CRBP1—cellular retinol-binding protein 1; IRBP—interphotoreceptor retinoid-binding protein; LCA—leber congenital amaurosis; LRAT—lecithin:retinol acyltransferase; RGR—RPE G protein-coupled receptor; ROS—rod outer segment(s); RPE—retinal pigment epithelial; RPE65—retinal pigment epithelial (cells) 65 kDa protein; RBP—retinol-binding protein; RDH—retinol dehydrogenase

Keywords: Retinol; Vitamin A; Retina; Photoreceptor cells; Retinoids; Rhodopsin; Two-photon microscopy; Retinal pigment epithelial cells

1. Introduction

The phototransduction process in the eye relies on the light-induced isomerization of the chromophore, 11-*cis*-retinal (or derivative), to all-*trans*-isomer. The regeneration of pre-illumination conditions requires reformation of 11-*cis*-retinoids. Although animals cannot synthesize vitamin A, they are capable of regenerating the active visual chromophore from vitamin A. While invertebrates rely on photochemical regenerative pathways to isomerize all-*trans*-retinal chromophore to 11-*cis*-isomer, vertebrates have an enzymatic, light-independent mechanism of chromophore regeneration, an endothermic process termed the “visual cycle” or the retinoid cycle. Therefore, aside from the mechanism of

phototransduction by the chromophore system based on opsin-vitamin A, vertebrates and invertebrates have evolved different pathways in retinoid biochemistry.

The classical vertebrate visual cycle describes the regeneration of the visual chromophore by the retinal pigment epithelial (RPE) cells independently of light conditions (Wald, 1968a, 1968b) (depicted in Fig. 1). The classical visual pathway elegantly combines the regeneration of visual chromophore by enzymatic isomerization of all-*trans*-retinol within the RPE cell and the trapping of retinoids from the choroidal circulation. The RPE is also responsible for the degradation and recycling of components of spent rod and cone outer segment disks through phagocytosis (Young & Bok, 1969) and potentially for the processing of $\beta\beta$ -carotene and other carotenoids to generate retinol (Yan et al., 2001). The conversion of all-*trans*-retinol to 11-*cis*-retinol through a yet uncharacterized isomerization process may involve either a short-lived carbocation intermediate (McBee et al., 2000), a stable intermediate (Rando, 1996), or another unidentified mechanism. Beside the

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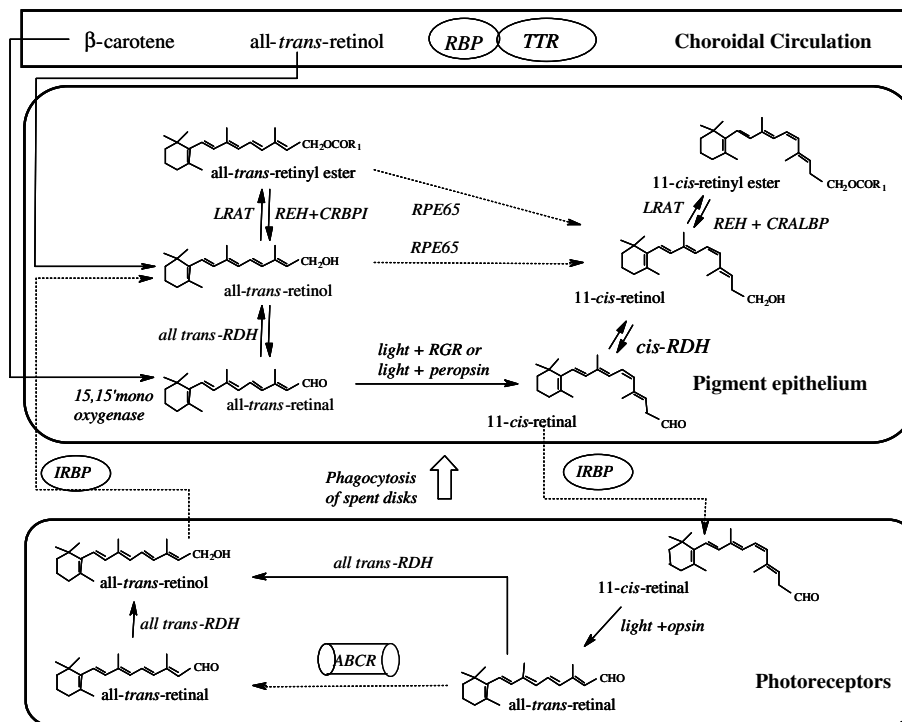


Fig. 1. Schematic representation of the visual cycle in rod-dominant animals, based on genetic and biochemical evidence. The abbreviations denote: ABCR, a photoreceptor-specific, ATP-binding cassette transporter; CRALBP, cellular retinaldehyde-binding protein; CRBP1, cellular retinol-binding protein 1; IRBP, interphotoreceptor retinoid-binding protein; LRAT, lecithin:retinol acyltransferase; RGR, RPE G protein-coupled receptor; RPE65, retinal pigment epithelial (cells) 65 kDa protein; RBP, retinol-binding protein; RDH, retinol dehydrogenase; REH, retinyl ester hydrolase; TTR, transthyretin. See text for details.

central isomerization reaction, the RPE also carries out esterification/hydrolysis as well as oxidation/reduction of retinoids, reactions that play important roles in shaping the repertoire of retinoids within the RPE (reviewed in (McBee, Palczewski, Baehr, & Pepperberg, 2001)). Production of 11-*cis*-retinal depends on the supply of substrate, all-*trans*-retinol, which is a product of the reduction of all-*trans*-retinal by rod and cone photoreceptor all-*trans*-retinol dehydrogenases (all-*trans*-RDH). In mouse retina, this reaction also constitutes the rate-limiting step of the retinoid cycle (Saari, Garwin, Van Hooser, & Palczewski, 1998). The all-*trans*-RDH of rod outer segment (ROS) and cone outer segments (COS) was shown to be an integral membrane protein of the short-chain alcohol dehydrogenase superfamily (SCAD) (Haeseleer, Huang, Lebiada, Saari, & Palczewski, 1998; Rattner, Smallwood, & Nathans, 2000).

11-*cis*-Retinol dehydrogenase (11-*cis*-RDH), another SCAD family dehydrogenase, is found in the RPE and involved in the conversion of 11-*cis*-retinol to 11-*cis*-retinal (Jang, Kuksa et al., 2001). Mutations in 11-*cis*-RDH have been identified in patients with fundus albipunctatus (Yamamoto et al., 1999). Several additional members of the SCAD superfamily, a family with over 100 members (Jornvall et al., 1995), have been shown to be expressed in other tissues besides that of

eye. Some of these dehydrogenases have been shown to oxidize retinol (Haeseleer et al., 2002). In addition to retinoids, other alcohols including ethanol, 4-hydroxynonenal, and hydroxysteroids, are potential substrates utilized by members of the SCAD family (Kasus-Jacobi et al., 2003; Kedishvili et al., 2002). Class I and IV medium chain alcohol dehydrogenases (ADH), dimers of Zn²⁺-binding metalloenzymes of 40 kDa, can also oxidize all-*trans*-retinol in vitro (Chou, Lai, Chang, Duester, & Yin, 2002) and possibly in vivo (Duester, 2001). Even though ADHs are soluble enzymes and their expression has not been demonstrated in the eye, the activity of uncharacterized members of this family in the retina or RPE cannot be ruled out.

RPE65 is another important protein of the retinoid cycle expressed primarily in the RPE (Bavik, Busch, & Eriksson, 1992; Bavik, Eriksson, Allen, & Peterson, 1991; Bavik, Levy, Hellman, Wernstedt, & Eriksson, 1993; Hamel et al., 1993) as well as possibly in cones (Znoiko, Crouch, Moiseyev, & Ma, 2002). Its expression is essential for 11-*cis*-retinol production, though its exact contribution to this process is currently unclear. RPE65 has a limited degree of similarity with $\beta\beta$ -carotene monooxygenase, and appears to bind retinyl esters (Jahng, David, Nesnas, Nakanishi, & Rando, 2003). The importance of RPE65 in the retinoid cycle is underscored by the fact that mutations within this gene cause

disease in man (Gu et al., 1997; Morimura et al., 1998), mouse (Redmond et al., 1998), and dog (Acland et al., 2001; Aguirre et al., 1998; Veske, Nilsson, Narfstrom, & Gal, 1999). Another important gene product is lecithin:retinol acyltransferase (LRAT), which synthesizes retinyl esters (MacDonald & Ong, 1988a, 1988b; Ong, MacDonald, & Gubitosi, 1988; Saari & Bredberg, 1989) by transfer of acyl moieties from phosphatidylcholine (PC). LRAT was purified by affinity labeling (Shi, Furuyoshi, Hubacek, & Rando, 1993) and then cloned from an RPE cDNA library (Ruiz et al., 1999). Retinyl esters have been proposed to be the substrates of the putative isomerohydrolase (Rando, 1991a, 1991b), an idea supported by others more recently (Moiseyev et al., 2003). Mutations in LRAT are also associated with leber congenital amaurosis (LCA) (Thompson et al., 2001).

Independent of the classical visual cycle, it has been supposed for some time that there is a retina-dependent, RPE-independent pathway that assists in the regeneration of the photopigment required by cone cells (Goldstein, 1970; Goldstein & Price, 1975; Goldstein & Wolf, 1973). A recent study has found evidence for such a pathway in cone-dominant animals, such as ground-squirrel and chicken (Mata, Radu, Clemmons, & Travis, 2002). In order for this pathway to function, the study's authors postulated the existence of a cone-specific isomerase that uses all-*trans*-retinol as a substrate to produce 11-*cis*-retinol. This isomerase is, therefore, distinct from the previously proposed isomerohydrolase, whose putative substrate is all-*trans*-retinyl ester. The existence of a retina (possibly Müller cell) specific ester synthase that converts 11-*cis*-retinol to 11-*cis*-retinyl ester was also proposed. The ester synthase activity of ground-squirrel and chicken retinas was not affected by known LRAT inhibitors (Mata et al., 2002). The proposed mechanistic argument was that endothermic isomerization would be a passive process driven by mass action. It was proposed that mass action is created through the esterification of 11-*cis*-retinol by the newly described 11-*cis*-retinyl ester synthase. No molecular characterization of these enzymes has been accomplished. Retinoid-binding proteins such as cellular retinaldehyde-binding protein (CRALBP), cellular retinol-binding protein (CRBP) and others also play an important role in controlling the flux of retinoids and driving chemical reactions uphill through capture of reaction products.

The fourth proposed pathway of *cis*-retinoids production is light-dependent and makes use of opsin-related genes such as peropsin (Sun, Gilbert, Copeland, Jenkins, & Nathans, 1997) or RPE G protein-coupled receptor (RGR) (Shen et al., 1994) to isomerize all-*trans*-retinal directly to 11-*cis*-retinal. In the retina, all-*trans*-retinal (or all-*trans*-retinylidene), presumably bound to opsins, can also be converted by

photoisomerization to its 11-*cis* isomer (Van Hooser et al., 2000). The photoisomerization of all-*trans*-retinal to 11-*cis*-retinal through RGR and peropsin is reminiscent of the regeneration of the visual pigment in lower vertebrates such as that seen for opsin 3 of *Amphioxus* (Koyanagi, Terakita, Kubokawa, & Shichida, 2002) and squid retinochrome (Hara & Hara, 1976, 1980). RGR is expressed by RPE and Müller cells, binds all-*trans*-retinal (Hao & Fong, 1999) and converts it to 11-*cis*-retinal. Associated with RGR is *cis*-RDH, which removes 11-*cis*-retinal and converts it to 11-*cis*-retinol (Chen, Hao et al., 2001). Mutations in RGR have been associated with retinitis pigmentosa (Morimura, Saindelle-Ribeaudeau, Berson, & Dryja, 1999). Knockout mice containing a homozygous disruption of the *RGR* gene (*rgr*^{-/-}) display a slower regeneration of 11-*cis*-retinal chromophore after light-adaptation (Chen, Hao et al., 2001). The *rgr*^{-/-} mice exhibit close to normal dark-adapted kinetics of pigment regeneration, but they tend to over-accumulate retinyl ester after a bleach (Maeda et al., 2003). It is unclear why in the RPE membranes of *rpe65*^{-/-} mice, which do not display enzymatic isomerization activity, there is no significant production of 11-*cis*-retinal by the intact photoisomerase pathway (Van Hooser et al., 2000). Therefore, G protein-mediated activity of RGR cannot be excluded (Maeda et al., 2003). Peropsin, another rhodopsin-related GPCR expressed by RPE cells, has not been conclusively implicated in the retinoid cycle. A peropsin homolog found in *Amphioxus*, *Amphiop3*, was recently shown to bind all-*trans*-retinal and generate 11-*cis*-retinal upon irradiation (Koyanagi et al., 2002). Peropsin and RGR are expressed much earlier in development than rod or cone opsins (Tarttelin et al., 2003).

There are many unanswered questions in the retinoid cycle, including the identity of the enzyme complex responsible for the regeneration of the visual chromophore. We believe that integrated approaches will permit the identification of missing proteins and will support hypothetical roles of these proteins in a physiological setting. In addition, these studies would aid in the identification of novel mutations in genes of the retinoid cycle that are associated with human retinal disorders. In this study, we present some of the approaches currently used in the investigation of the biochemistry of retinoid derivatives in mammalian retinas, and discuss chemical, enzymatic and thermodynamic aspect of isomerization of all-*trans*-retinoids to 11-*cis*-retinol.

2. Materials and methods

2.1. Two-photon excitation microscopy

Two-photon excitation microscopy was performed using a Zeiss LCM 510 MP-NLO confocal microscope

(Carl Zeiss, Thornwood, NY). Briefly, 76-MHz, 100 fs pulses of 730 nm light from a mode-locked Ti:Sapphire laser (Coherent Mira, Mountain View, CA) were focused on the sample by a 40x, 1.3 NA Plan Neofluar objective lens (Carl Zeiss, Thornwood, NY). The intensity of laser was measured out of AOM (acousto-optic modulator) and kept at ~ 10 mW. Autofluorescence from the sample (390–545 nm) was collected through the objective lens, separated from the excitation light by a dichroic mirror, filtered by BG39 to remove scattered UV light, and directed to a photomultiplier tube detector. The objective lens was heated up to 37 °C by an air stream incubator (Nevtek, Bumsville, VA). A temperature-controlled microscopic stage (Heatable insert P, Carl Zeiss, Thornwood, NY) was installed on the microscope to maintain the reaction temperature at 37 °C. Fluorescent intensities reflected in pixel values were calculated by analyzing the collected images in SCION imaging software (Scion Corporation, Frederick, MD). To assay the dependency on dinucleotides for the formation of fluorescent all-*trans*-retinol we used 50 mM of each dinucleotide (NAD, NADP, NADH, or NADPH) and 0.04 mg/ml rhodopsin in 0.1 M BTP (bis-tris propane) buffer at pH 5.8 (better suited for subtle differences in fluorescence). After an intense flash, images were collected every 2 min (Fig. 4A). To evaluate the increase in fluorescence intensity in the presence or absence of prior light stimulation, images were collected every 10 min after being illuminated with a high intensity flash at pH 7.3 (Fig. 4B and C).

2.2. Chemistry

Mass-spectral analysis was performed using Kratos profile HV-3 direct probe mass-spectrometer and electron impact (EI) ionization method. NMR data was recorded on Bruker 500 MHz spectrometer using CDCl₃ or TMS as internal standard. All reagents were purchased from Aldrich, Sigma, or Fluka and were used without additional purification. Solvents were dried under standard procedures prior to use. All operations with retinoids were performed under dim red light unless otherwise specified.

2.3. Synthesis of thioretinyl acetate via palladium-catalyzed reaction

A mixture of palladium complex Pd₂dba₃·CHCl₃ tris-(dibenzylideneacetone)dipalladium(0)-chloroform adduct (23.3 mg, 22.5 μ mol) and 1,4-bis(diphenylphosphino)butane (35.9 mg, 84.2 μ mol) was stirred in THF (3 ml) at room temperature for 30 min. Potassium thiocetate (182 mg, 1.6 mmol) in 0.5 ml H₂O was added followed by a solution of all-*trans*-retinyl acetate (66.2 mg, 233 μ mol) in THF (1.5 ml). The reaction mixture

was stirred at 50 °C for 24 h. Water (5 ml) was added and the product was extracted three times with 5 ml hexane. The organic layer was washed two times with 10 ml water, dried with anhydrous MgSO₄, and evaporated. The residue was purified by column chromatography on a silica gel (hexane) to afford a mixture of 13-*cis*-, 9-*cis*-, and all-*trans*-retinyl thioacetates (Welch & Gruber, 1979) in 3:1.5:5 ratio as judged by normal-phase HPLC (Beckman Ultrasphere Si 5 μ 250 $\times$ 4.6 mm, hexane). All-*trans*-thioretinol was prepared by hydrolysis of the corresponding acetate in 80% EtOH, 100 mM NaOH, 30 min, 37 °C (Fig. 9A). All-*trans*-thioretinyl acetate: NMR (CDCl₃, 300 MHz, δ ppm): 1.02 (s, 6H, 2 $\times$ 1-CH₃), 1.43–1.50 (m, 2H), 2-CH₂ 1.57–1.66 (m, 2H, 3-CH₂), 1.71 (s, 3H, 5-CH₃), 1.88 (s, 3H, 9-CH₃), 1.95 (s, 3H, 13-CH₃), 2.01 (t J 6.0 Hz, 2H, 4-CH₂), 2.33 (s, 3H, CH₃CO), 3.69 (d J 8.3 Hz, 2H, 15-CH₂), 5.53 (t J 8.1 Hz, 1H, H14), 6.05–6.27 (m, 4H, H7, H8, H10, H12), 6.58 (dd J 10.9 Hz J 15.05 Hz, 1H, H11). MS (EI) m/z : 344 (53%), 329 (0.5%), 301(5%), 269 (100%), 255 (15%). UV spectrum had $\lambda_{\max}$ 330 nm in hexane.

2.4. Esterification, isomerization, and hydrolysis assays

A solution of the substrate (2 μ l of 1 mM in DMF) was added to a 1.5 ml Eppendorf tube containing 178 μ l of 10 mM BTP (pH 7.5) and 20 μ l of 10% BSA. For the isomerization assay, 20 μ l of CRALBP and 10 μ l of 10 mM ATP was added to the reaction mixture in place of 30 μ l BTP. The reactions were incubated at 37 °C for the indicated times. Then, 300 μ l of methanol and 300 μ l of hexane were added to the reaction. Retinoids were thoroughly extracted and 100 μ l of hexane was analyzed by normal-phase HPLC HP1100 equipped with diode-array detector using 100% hexane for thioretinyl acetate assays or 10% ethyl acetate/hexane for other substrates. For substrate specificity (Figs. 7E, and 8E and 9D), reactions were incubated for 4 min within the linear range of enzyme activity.

2.5. Pulse chase experiments

11,12-Di-[³H]-retinol (2 nmol, specific radioactivity 1200 dpm/nmol) in 2 μ l DMF was incubated for 30 min with RPE microsomes, which were treated with UV light for 5 min to destroy endogenous retinoids. After the formation of palmitate esters was completed, cold retinyl acetate (2 μ l of 1 mM DMF solution) was added to the membranes. The assay was run for the indicated times and peaks corresponding to retinol palmitate, acetate, 11-*cis*-retinol, and all-*trans*-retinol were collected, quantified, and subjected to scintillation counting. The amounts of retinoids were calculated by calibration with known amounts of standards and normalized to 100%.

2.6. Analyses of retinoids and visual pigments

All procedures were performed under dim red light as described previously (Jang et al., 2001; Palczewski et al., 1999; Van Hooser et al., 2000).

2.7. Retinoids

All reactions involving retinoids were carried out under dim red light. Retinoids were stored in *N,N*-dimethylformamide under argon at -80°C . All retinoids were purified by normal-phase HPLC (Beckman, Ultrasphere-Si, $4.6\text{ mm} \times 250\text{ mm}$) with 10% ethyl acetate/90% hexane at a flow rate of 1.4 ml/min using an HP1100 HPLC with a diode-array detector and HP Chemstation A.03.03 software. Different conditions are listed in the figure legend. The following extinction coefficients (Garwin & Saari, 2000) were used for retinoids (in $\text{M}^{-1}\text{ cm}^{-1}$): all-*trans*-retinal, $\epsilon = 48,000$ at 368 nm; 9-*cis*-retinal, $\epsilon = 36,100$ at 373 nm; 11-*cis*-retinal, $\epsilon = 36,100$ at 365 nm; 13-*cis*-retinal, $\epsilon = 38,770$ at 363 nm; all-*trans*-retinol, $\epsilon = 51,770$ at 325 nm; 9-*cis*-retinol, $\epsilon = 42,300$ at 323 nm; 11-*cis*-retinol, $\epsilon = 34,320$ at 318 nm; and 13-*cis*-retinol, $\epsilon = 48,305$ at 328 nm.

A normal-phase column (Beckman Ultrasphere Si $5\text{ }\mu\text{m}$, $4.6 \times 250\text{ mm}$) and an isocratic solvent system of 0.5% ethyl acetate in hexane (v/v) for 15 min followed by 4% ethyl acetate in hexane for 65 min at a flow rate of 1.4 ml/min at 20°C (total 80 min) with detection at 325 nm was used for separation of retinoids from mouse and bovine tissues. In addition, all of the experimental procedures related to the analysis of dissected mouse eyes, derivatization, and separation of retinoids have been described previously in detail (Kuksa et al., 2002; McBee, Van Hooser, Jang, & Palczewski, 2001; Van Hooser et al., 2000, 2002).

3. Results

3.1. Analysis of the processing of retinol at a microscopic level

Among the retinoids in the vertebrate retinoid cycle, retinol and retinyl esters exhibit the highest fluorescence (excitation λ_{ex} at $\sim 320\text{ nm}$) (Garwin & Saari, 2000). Retinals do not display significant fluorescence at room temperature (Balke & Becker, 1968; Becker, Inuzuka, & Balke, 1971; Becker, Inuzuka, King, & Balke, 1971). Early work of Sears and Kaplan suggests that the increase in the level of fluorescence after photobleaching of rod outer segment can be attributed to all-*trans*-retinol. This initial report was made using a conventional fluorescent microscope which requires UV excitation (Sears & Kaplan, 1989). Recent advances in confocal microscopy have made it possible to observe the 3D and

4D (spatial and temporal) distribution of fluorophores in complicated biological structures. Photodamage to living cells is anticipated for UV excitation, and indeed, conventional laser scanning microscopy causes massive photobleaching of fluorophores. Those problems inhibited the utilization of laser scanning microscopy (LSM) for imaging UV-excited fluorophores until the development of two-photon excitation laser scanning microscopy (TPELSM), which is a combination of mode-locked laser and LSM technology. Piston and his colleagues applied the two-photon excitation laser scanning microscopy to image the cellular redox state by using NAD(P)H as internal indicators (Bennett, Jetton, Ying, Magnuson, & Piston, 1996). The concept of two-photon microscopy is shown in Fig. 2. TPELSM does not require external fluorophores, allowing observation of fluorescent changes in the intact tissue. In ROS, the reduction of all-*trans*-retinal to all-*trans*-retinol is coupled with oxidization of NADPH (McBee, Palczewski et al., 2001).

For the future application of two-photon excitation as a non-invasive technique to image the all-*trans*-retinol in the eye, *in vitro* experiments were carried out to monitor formation of all-*trans*-retinol in a mixture of bovine ROS and dinucleotides. At a concentration of $50\text{ }\mu\text{M}$, fluorescence of soluble NAD(P)H was negligible (Fig. 3A and C). When bovine ROS and NADH were mixed and rhodopsin was bleached by an intense flash of light, no increase in fluorescence intensity was observed (Fig. 3A and B). As expected, ROS membranes became highly fluorescent after incubation with NADPH and after the sample was exposed to an intense flash of light (Fig. 3D). The fluorescence signal can be quantified and, as shown in Fig. 4A, this signal is strictly NADPH-dependent. Additionally, this increase in fluorescence was only observed when the samples were exposed to flash stimulation (Fig. 4B and C). Excellent agreement was observed between the time course of the increase in all-*trans*-retinol quantified by retinoid analysis (Fig. 4B) and by the fluorescence method (Fig. 4C). This observation confirms the earlier work of Sears and Kaplan, in which they attributed the fluorescence signal to all-*trans*-retinol (Sears & Kaplan, 1989). Thus, the amount of all-*trans*-retinol produced in ROS yields enough fluorescence to image by TPELSM and will be useful for monitoring the retinols and retinyl esters in the eye.

3.2. Analysis of retinoids in large animal models

Analysis of retinoids is critical for understanding the metabolically relevant pathway in the eye. Modern separation techniques require only a few hundred fmol of material to be reproducibly detected in a routine assay. Therefore, large eyes, such as those of human, bovine, pig, or dog, can be further subdivided for analysis using small biopsies and the retina and RPE can be

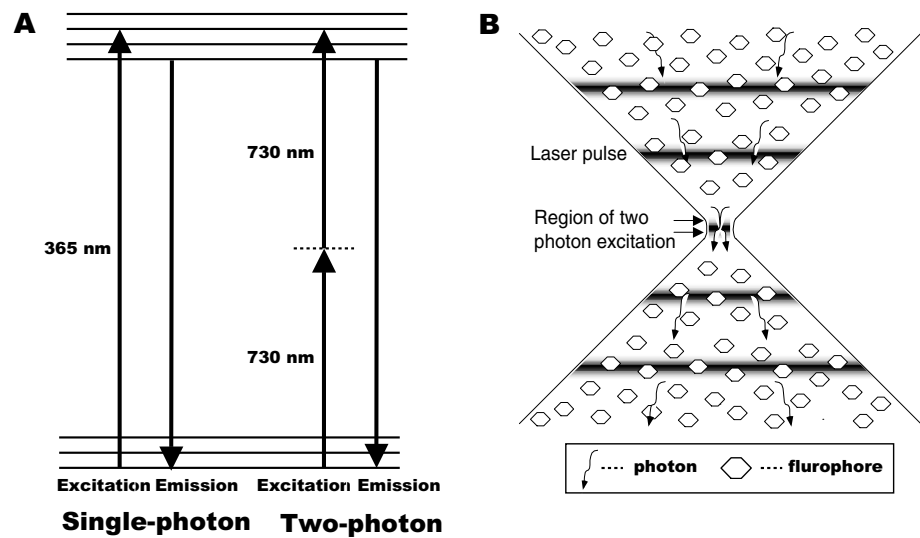


Fig. 2. Illustration of the theoretical basis of two-photon microscopy. (A) Jablonski diagram of the single-photon versus two-photon process. Note the short-lived intermediate shown in the two-photon process. (B) Demonstration of photon crowding, which can lead to simultaneous interaction with the fluorophore. Modified from (Piston, 1999; Piston & Knobel, 1999).

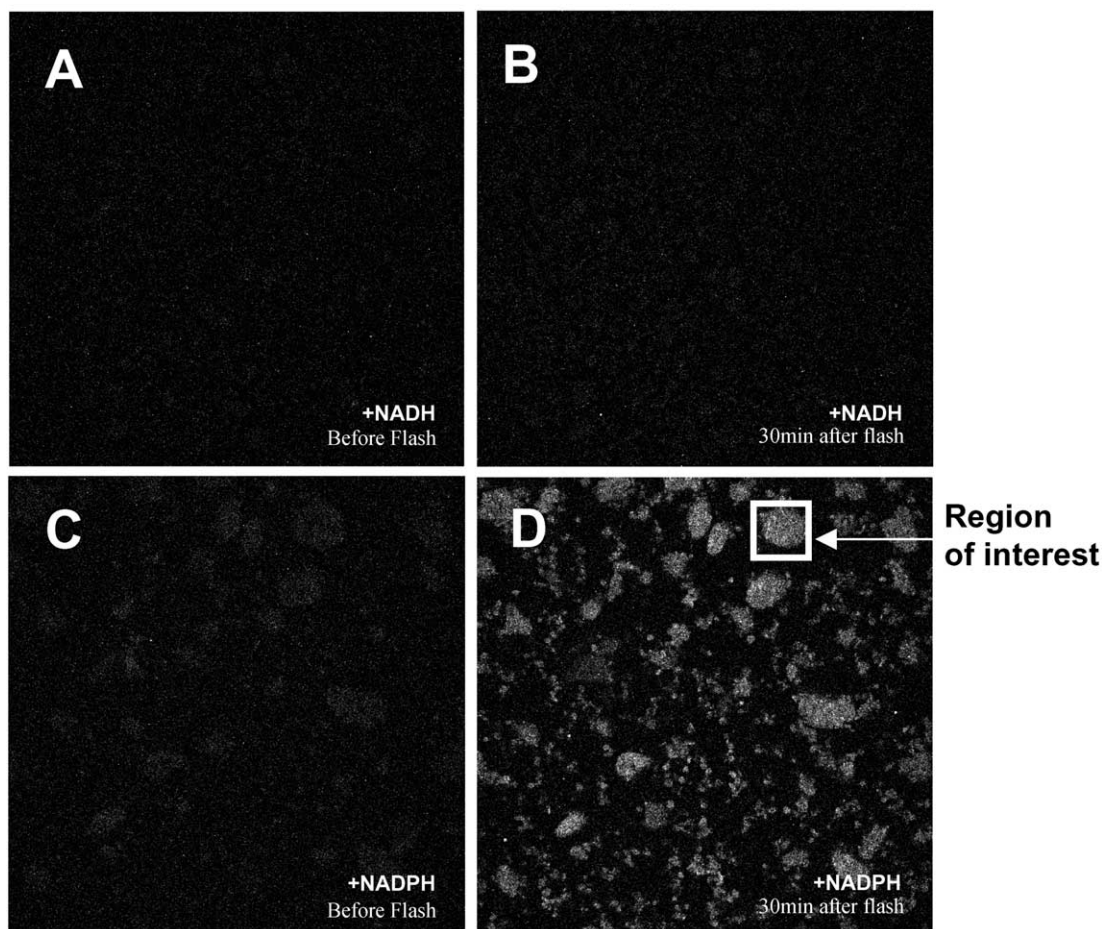


Fig. 3. Fluorescence from all-*trans*-retinol imaged directly by two-photon microscopy. Dinucleotide dependency on the formation of all-*trans*-retinol was examined. An image before flash in the presence of 50 μ M NADH (A) or NADPH (C), and 30 min after flash (B, D). Significant change in fluorescent intensity was observed only in the sample containing NADPH. The experimental setting is described in Section 2.

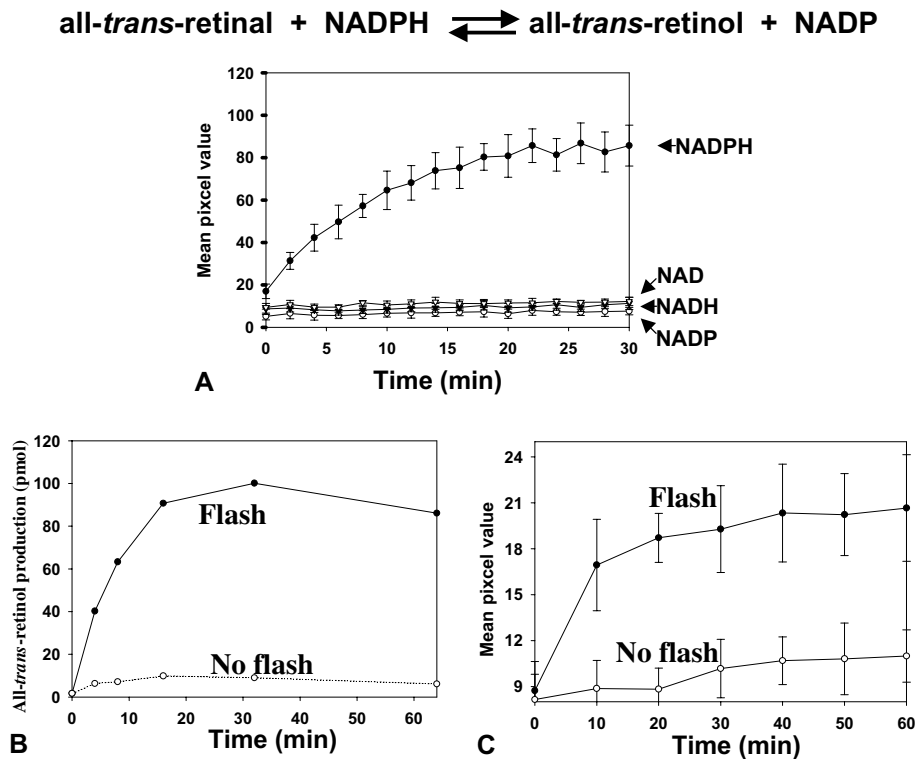


Fig. 4. Time-lapse quantification of fluorescent intensity by two-photon microscopy. (A) Dinucleotide dependency of increase in fluorescent intensity. Each dinucleotide was added at the concentration of 50 μ M. Fluorescence level increased in the presence of NADPH. An increase in all-trans-retinol measured by retinoid analysis (B) or fluorescent intensity (C) was observed after flash stimulation. See Section 2 for details.

separated for individual analyses. As shown in this example of retinoid analysis there is a large difference in the retinoid levels depending on where in the eye the sample is taken (Fig. 5A). 11-*cis*-Retinal was predominantly found in the retina (Fig. 5B), and only low levels are detected in the RPE (Fig. 5C). Retinyl esters are prominent in the RPE (Fig. 5C). Low levels of esters were also detected in the retina (Fig. 5B), but this may be due to cross contamination of the retina by RPE. Bovine eyes, as well as those of rodent, have low levels of *cis*-retinyl esters. This type of analysis utilizing small retinal punches is particularly useful in detecting retinoid formation in a large animal model of LCA with a defect in RPE65 that was treated locally with the AAV virus carrying RPE65 gene to identify the region of 11-*cis*-retinal formation that supports vision (unpublished).

3.3. Analysis of mouse models with disrupted genes encoding proteins involved in the retinoid cycle

Analysis of retinoids in mice offers advantages due to the possibility of genetic manipulation in this animal. To eliminate any interference from background light on the isomeric composition of retinoids in the eye, all mice were raised in the dark. All retinoids were identified by co-elution with authentic retinoid standards, on-line UV spectroscopy, and, in some cases, by mass spectrometry.

Retinoid analyses of dark-adapted WT mice showed 536 ± 87 pmol/eye of 11-*cis*-retinal (Fig. 6A). The amount of all-trans-retinal was only $\sim 1/10$ of that of 11-*cis*-retinal, while retinols were present only in a trace amounts. 11-*cis*-retinal is mostly present in the retina, bound to opsin to form rhodopsin. The second biggest component is all-trans-retinyl esters (mostly palmitoyl and steroyl derivatives). There are low (11-*cis*- and 13-*cis*-) or undetectable (9-*cis*-) levels of the *cis*-retinyl esters in these wild type C57BL/6 mice. To illustrate the usefulness of these retinoid analyses, we analyzed the retinoid makeup of several lines of transgenic knockout mice with disruptions in genes involved in the retinoid cycle. First, *rhodopsin-knockout* (*rho*^{-/-}) mice have low amounts of 11-*cis*-retinal, which are most likely bound with CRALBP in the retina (unpublished). Also much lower amounts of the esters are observed (Fig. 6B). Dark raised *rpe65*^{-/-} mice do not have *cis*-retinoids (McBee, Van Hooser et al., 2001; Van Hooser et al., 2000, 2002), but acquire some when exposed to light (Van Hooser et al., 2002). Moreover, they show large accumulations of all-trans-retinyl esters (Fig. 6C) (Redmond et al., 1998; Van Hooser et al., 2000). 11-*cis*-RDH (*rdh5*^{-/-}) mice appear to accumulate 11-*cis*- and 13-*cis*-retinyl esters (Fig. 6D) (Driessen et al., 2000; Jang et al., 2001). These examples illustrate how genetic changes lead to a modified balance of retinoids in the eye, hinting to the

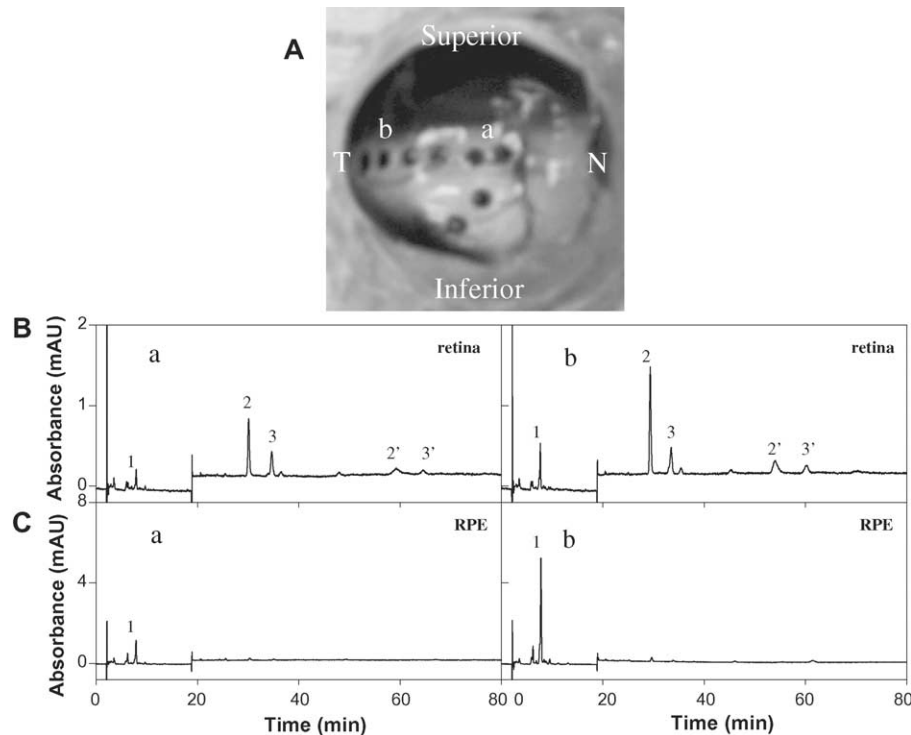


Fig. 5. HPLC of biopsy samples from bovine eye retina and RPE. (A) Bovine eye showing locations of the 3 mm biopsies. (B) HPLC of retina biopsy sample from center of eye (a) and temporal region (b) shows all-*trans*-retinyl esters (peak 1), *syn*-11-*cis*-retinal oxime (peak 2), *syn*-all-*trans*-retinal oxime (peak 3), *anti*-11-*cis*-retinal oxime (peak 2'), and *anti*-all-*trans*-retinal oxime (peak 3'). Levels of these retinoids are shown to increase from the central region toward the temporal side. (C) RPE shows all-*trans*-retinyl esters (peak 1), but no *syn*-11-*cis*-retinal oxime, *syn*-all-*trans*-retinal oxime, *anti*-11-*cis*-retinal oxime, or *anti*-all-*trans*-retinal oxime (peak 3'). T and N denote temporal and nasal parts of the eye, respectively. Retinoid separation was carried out as described in Section 2.

roles of these genes in the normal flow of retinoids in the vertebrate eye.

Other excellent examples of the use of mice to investigate the retinoid cycle and potential pharmacological therapy include studies on the ABCR transporter expressed exclusively in photoreceptor cells (Mata et al., 2001; Mata, Weng, & Travis, 2000; Radu et al., 2003; Weng et al., 1999).

In spite of the redundant biochemical pathways, the use of knockout transgenic animals still plays an important role in the study of the retinoid cycle due to the absence of a cellular system that consistently and accurately resembles the RPE. In addition to the labile and insoluble nature of retinoids and the fact that most retinoid processing enzymes are membrane-associated, the components of these biochemical pathways cannot yet be reconstituted *in vitro* from purified components. The most reduced system for studying the retinoid pathway is based on bovine RPE microsomes. In Table 1, we illustrate the current progress in elucidating the roles of different genes involved in the processing of retinoids and we correlate their roles with the pathological manifestations of mutations in these genes. There are, however, enzyme activities for which the enzyme or enzyme complex involved has not been biochemically defined, these are listed in Table 2.

3.4. *In vitro* analysis of the biochemical pathways of the retinoid cycle

Freshly isolated bovine RPE microsomes have been used to study the isomerization and esterification reactions of the retinoid cycle. While these studies have provided interesting findings regarding the kinetics and possible substrates of reactions, they have not led to the identification of any new players in this pathway. The reason for this difficulty is attributed to the fact that bovine RPE microsomes contain a full, functional complement of enzymes and that these enzymes have not been amenable to separation and reconstitution experiments.

LRAT, isomerase and hydrolase specificities. Here, we synthesized and tested different retinoids for LRAT and isomerase substrate specificity by employing truncated/extended retinol analogs together with different isomeric retinols. LRAT has fewer requirements concerning the structure of its substrate compared to the isomerase. LRAT was found to be sensitive to retinol length and to primary or secondary substitution of a hydroxy group of the retinoid (Fig. 7). While little esterification of C-17 primary alcohol occurred (Fig. 7A, dotted line, peak a), secondary C-18 and primary C-22 alcohols were not esterified (Fig. 7B and D, dotted lines). In control

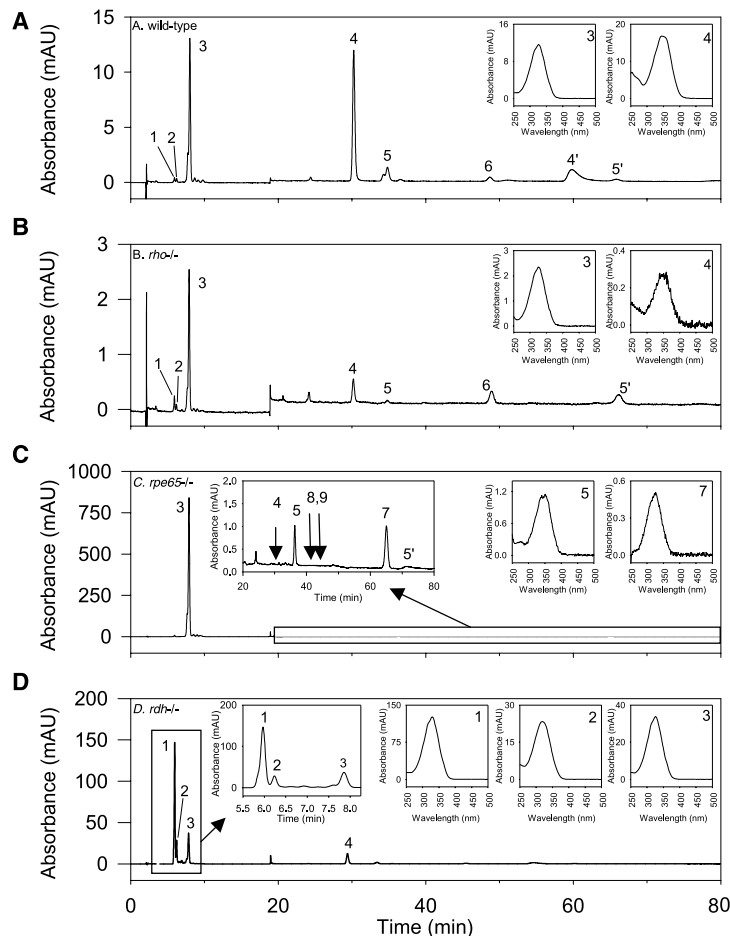


Fig. 6. HPLC traces of eye retinoids from wild type and genetically modified mice. (A) Eyes from an adult wild type C57BL/6J mouse. Normal levels of all-*trans*-retinyl esters (peak 3) and 11-*cis*-retinal oxime (*syn*-) (peak 4) are confirmed by spectra (inset 3 and 4, respectively). Also present are 13-*cis*-retinyl esters (peak 1), 11-*cis*-retinyl esters (peak 2), all-*trans*-retinal oxime (*syn*-) (peak 5), 11-*cis*-retinol (peak 6), 11-*cis*-retinal oxime (*anti*-) (peak 4'), and all-*trans*-retinal oxime (*anti*-) (peak 5'). (B) Eyes from an adult *rho*^{-/-} mouse. Note the difference in absorbance scale from the wild type. All-*trans*-retinyl esters and 11-*cis*-retinal are present but in much lower levels. (C) Eyes from an adult *rpe65*^{-/-} mouse. The level of all-*trans*-retinyl ester is much greater than that of the wild type. The HPLC signal from 20 to 80 min is magnified to show the levels of all-*trans*-retinal oxime (*syn*-) (peak 5), all-*trans*-retinal oxime (*anti*-) (peak 5') and all-*trans*-retinol (peak 7). Note lack of 11-*cis*-retinal oxime in the sample (arrow 4), 13-*cis*-retinal oxime (arrow 8) and 9-*cis*-retinal oxime (arrow 9). (D) Eyes from an adult *rdh*^{-/-} mouse. High levels of 13-*cis*-retinyl ester (peak 1), 11-*cis*-retinyl ester (peak 2), and all-*trans*-retinyl ester (peak 3) are present. Also present are 11-*cis*-retinal oxime (*syn*-) (peak 4), all-*trans*-retinal oxime (*syn*-) (not shown due to scale compression), 11-*cis*-retinol (not shown due to scale compression), 11-*cis*-retinal oxime (*anti*-) (not shown due to scale compression), and all-*trans*-retinal oxime (*anti*-) (not shown due to scale compression). Retinoid separation was carried out as described in Section 2.

samples, boiled RPE membranes showed no reaction (dashed lines). There was also no isomerization of the synthetic retinoids since no *cis*-retinoids peaks were detected on the chromatogram (Fig. 7A, B and D, solid lines). In a positive control experiment, C20 (all-*trans*-retinol), the native substrate of LRAT and isomerase (Fig. 7C, dotted and solid lines respectively), showed the highest activity for both proteins. Without the presence of CRALBP, all-*trans*-retinol was completely esterified by LRAT and when CRALBP was added, almost 70% of the total retinoid content was 11-*cis*-retinol, the product of the isomerization reaction (Fig. 7C, solid line, peak c). HPLC traces were recorded at $\lambda_{\max}$ of each retinoid (Fig. 7F). To summarize, LRAT shows substrate specificity with regards to retinoid substrate chain

length with the most active being C20, all-*trans*-retinol (Fig. 7E).

To test LRAT substrate specificity to geometrical retinol isomers, different isomers were incubated with RPE membranes (Fig. 8A–D solid lines). As a control for the non-enzymatic factors that affect this process, we employed boiled RPE (Fig. 8A–D dotted lines). The highest activity of LRAT was towards its native substrate, all-*trans*-retinol. 11-*cis*-Retinol was also a relatively efficient substrate compared to all-*trans*-retinol, while 9-*cis*- and 13-*cis*-retinols were poorer substrates for LRAT (Fig. 8E). Other esterifying enzymes in RPE such as ARAT (Saari & Bredberg, 1990) can be responsible for this minor esterification of retinoids.

Table 1
Genes involved in supply and enzymatic processing of visual chromophore

Gene name	Human chromosomal location	Function	Disease phenotype and animal models
RPE65	1p31.2	Retinal pigment epithelium-specific protein (65 kDa) is necessary for production of 11- <i>cis</i> -retinal (Redmond et al., 1998). Has similarity to BCMO	<ul style="list-style-type: none"> • LCA2 recessive, 10–15% of all cases (Gu et al., 1997) • RP20 recessive RP cloned gene; accounts for 2% of recessive RP (Morimura et al., 1998) • <i>RPE65</i>^{-/-} Swedish Briard-Beagle dog (Veske et al., 1999)
ABCA4, ABCR	1p22.1	ATP-binding cassette transporter-proposed to work as an <i>N</i> -retinylidene-PE flippase, transporting <i>N</i> -retinylidene-PE from the luminal to cytoplasmic leaflet of the membrane of ROS discs (Sun, Smallwood, & Nathans, 2000; Weng et al., 1999)	<ul style="list-style-type: none"> • STGD1 recessive Stargardt disease (Allikmets et al., 1997; Nasonkin et al., 1998) • juvenile and late onset; recessive MD (Allikmets, 2000) • RP19 recessive RP (Martinez-Mir et al., 1998) • recessive fundus flavimaculatus (Rozet et al., 1998) • recessive cone-rod dystrophy (Ducroq et al., 2002) • <i>abcr</i>^{-/-} mice show high levels of lipofuscin accumulation and delayed dark adaptation (Weng et al., 1999)
RDH14	2p24.3	Oxidation and reduction of 11- <i>cis</i> - and all- <i>trans</i> -retinal and retinol (pro- <i>R</i> retinol, pro- <i>S</i> NADPH) (Belyaeva & Kedishvili, 2002; Haseleer et al., 2002)	
CRBP-I	3q23	Cellular retinol-binding protein 1 associates with all- <i>trans</i> -retinol and affects its transport, esterification and oxidation within cells (Saari et al., 2002)	<i>crbpl</i> ^{-/-} mice have normal ERG (Ghyselinck et al., 1999) yet all- <i>trans</i> -retinol accumulates transiently in the neural retina, and there are reduced amounts of retinyl esters in the RPE (Saari et al., 2002)
CRBP-II	3q23	Cellular-enterocyte retinol-binding protein 2 is important in the uptake and metabolism of dietary retinol by the small intestine (E et al., 2002)	<i>crbp-II</i> ^{-/-} mice show increased neonatal mortality; both fetal and maternal CRBP II are needed for adequate delivery of vitamin A to the developing fetus (E et al., 2002)
RHR, peropsin	4q25	May be involved in the photoisomerization of all- <i>trans</i> -retinal to 11- <i>cis</i> -retinal in the RPE (Sun et al., 1997)	
LRAT	4q32.1	Involved in the esterification of all- <i>trans</i> - and 11- <i>cis</i> -retinol (Saari, Bredberg, & Farrell, 1993)	Early onset severe recessive RP (Thompson et al., 2001)
RDH10	8q13.2	Oxidation/reduction of all- <i>trans</i> -retinol and retinal in the RPE using NADPH/NADP as a cofactor (Wu et al., 2002)	
RBP3, IRBP	10q11.2	Interphotoreceptor retinol-binding protein is involved in the shuttling of retinol to and from the RPE and the ROS (Liou, Bridges, Fong, Alvarez, & Gonzalez-Fernandez, 1982)	<i>irbp</i> ^{-/-} mice shown modest reduction in pigment regeneration compared to wild-type (Palczewski et al., 1999)
RGR	10q23	Involved in photoisomerization of all- <i>trans</i> - to 11- <i>cis</i> -retinal (Chen, Lee, & Fong, 2001)	RP recessive (S66R) and dominant (+1frameshift at G275) (Morimura, Saindelle-Ribeaudau et al., 1999); <i>rgr</i> ^{-/-} mice show impaired pigment regeneration and all- <i>trans</i> -retinyl ester accumulation after light-adaptation, dark-adapted state is normal (Chen, Hao et al., 2001)
RBP4	10q23.33	Serum retinol-binding protein, in combination with transthyretin, carries retinol from liver stores and digestive tract throughout the body (Quadro et al., 1999)	recessive RPE degeneration; night blindness, reduced visual acuity (Seeliger et al., 1999); <i>rbp4</i> ^{-/-} mice display impaired vision at weaning due to lack of postprandial retinol uptake by eye in the absence of RBP-4 (Quadro et al., 1999)

Table 1 (continued)

Gene name	Human chromosomal location	Function	Disease phenotype and animal models
RDH5, 11- <i>cis</i> -RDH	12q13.2	Oxidation/reduction of 11- <i>cis</i> -retinol and retinal in the RPE using NADH/NAD as a cofactor (pro- <i>S</i> retinol, pro- <i>S</i> NADH) (Jang, Kuksa et al., 2001) also found associated with RGR (Chen, Lee et al., 2001)	<ul style="list-style-type: none"> • recessive fundus albipunctatus; stationary night blindness with sub-retinal spots and delayed dark adaptation, recessive cone dystrophy (Yamamoto et al., 1999) • <i>rdh5</i>^{-/-} mice show accumulation of 11-<i>cis</i>- and 13-<i>cis</i>-retinol and retinyl esters isomers in the RPE (Cideciyan et al., 2000; Driessen et al., 2000)
RDH11	14q23.3	Oxidation/reduction of 11- <i>cis</i> - and all- <i>trans</i> -retinal and retinol (pro- <i>R</i> retinol, pro- <i>S</i> NADPH) (Haeseleer et al., 2002; Kedishvili et al., 2002)	
RDH12	14q23.3	Oxidation/reduction of 11- <i>cis</i> - and all- <i>trans</i> -retinal and retinol (pro- <i>R</i> all- <i>trans</i> -retinol, pro- <i>S</i> NADPH) (Haeseleer et al., 2002)	
RLBP1, CRALBP	15q26.1	Cellular retinaldehyde-binding protein binds 11- <i>cis</i> -retinal preferentially assisting the enzymatic photoisomerization process (Crabb, Goldflam, Harris, & Saari, 1988)	<ul style="list-style-type: none"> • recessive RP (Golovleva et al., 2003; Maw et al., 1997) • recessive bothnia dystrophy (Burstedt et al., 2001) • recessive retinitis punctata albescens (Morimura, Berson, & Dryja, 1999) • recessive Newfoundland rod-cone dystrophy (Eichers et al., 2002). <i>cralbp</i>^{-/-} mice show delayed rhodopsin regeneration and delayed dark adaptation and accumulate all-<i>trans</i>-retinyl esters (Saari et al., 2001)
BCMO, beta-carotene 15,15' monooxygenase	16q21–q23	Cleaves β-carotene to generate two molecules of retinal (Redmond et al., 2001; Yan et al., 2001)	
TTR transthyretin	18q11.2–q12.1	Transthyretin, in association with RBP4, carries retinol from liver stores and digestive tract throughout the body (Episkopou et al., 1993)	TTR mutations can cause familial amyloidotic polyneuropathy, through amyloid deposition (Saraiva, 1995); I84S mutation causes low serum RBP levels (Benson et al., 1993); <i>ttr</i> ^{-/-} mice have normal retinal function in spite of low serum levels of RBP and retinol (Bui et al., 2001; Episkopou et al., 1993)
prRDH, also known as RDH8	19p13.2–p13.3	Oxidation/reduction of all- <i>trans</i> -retinal and all- <i>trans</i> -retinol specifically in the photoreceptor outer segment (pro- <i>R</i> retinol, pro- <i>S</i> NADPH) (Rattner et al., 2000)	

Table 2
Additional enzymatic activities awaiting biochemical characterization

Observed activity	Substrate	Product	Cell type where activity was reported	Reference
Isomerohydrolase	All- <i>trans</i> -retinyl ester	11- <i>cis</i> -retinol	RPE	(Rando, 1991b)
Isomerase	All- <i>trans</i> -retinol or all- <i>trans</i> -retinyl ester	11- <i>cis</i> -retinol through carbocation intermediate	RPE	(McBee et al. (2000))
Retinyl ester hydrolase	11- <i>cis</i> -retinyl and much less so all- <i>trans</i> -retinyl esters	All- <i>trans</i> -or 11- <i>cis</i> -retinol	RPE	(Blaner, Das, Gouras, & Flood (1987); Mata & Tsien (1998))
Acyl CoA:retinol acyl-transferase (ARAT)	Fatty-acyl CoA and free retinol (in the absence of CRBP-I)	Retinyl ester	RPE	(Saari & Bredberg (1990))
Isomerase	All- <i>trans</i> -retinol	11- <i>cis</i> -retinol through mass action due to esterification	Cone-dominant chicken and ground-squirrel retinas	(Mata et al. (2002))
Retinyl ester synthase 11- <i>cis</i> -RDH	11- <i>cis</i> -retinol 11- <i>cis</i> -retinol + NADP (pro- <i>S</i> retinol)	11- <i>cis</i> -retinyl ester 11- <i>cis</i> -retinal + NADPH (pro- <i>S</i> NADPH)		

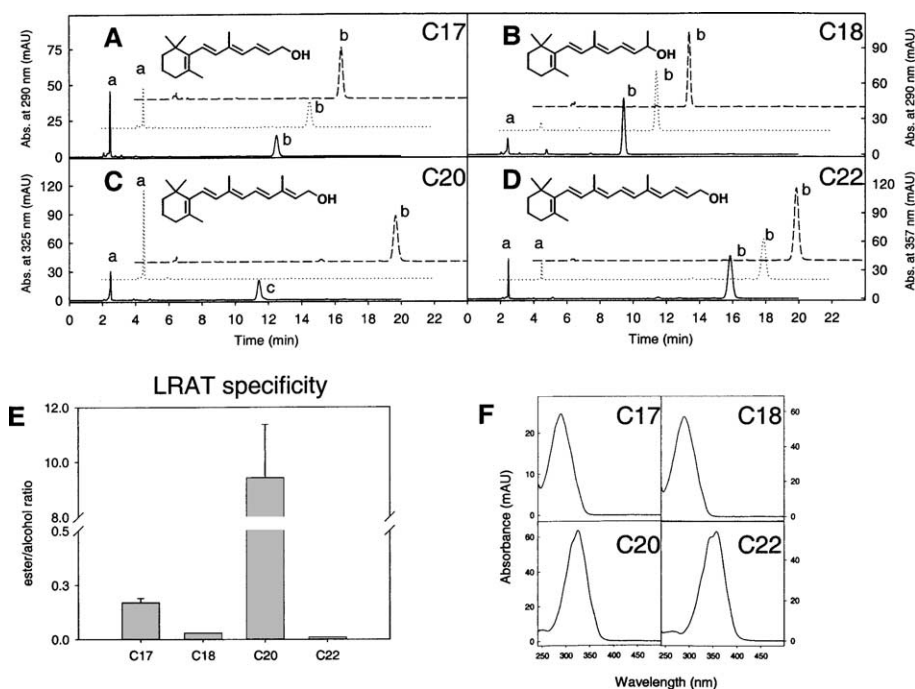


Fig. 7. LRAT and isomerase activity employing retinol homologs of different chain lengths. (A–D). HPLC traces for each homolog. Absorbance was recorded at the corresponding λ_{max} of each compound. Solid line—incubation of homolog in the presence of CRALBP. Dotted line—no CRALBP is added. Dashed line—incubation with boiled RPE. Peak (a)—esters, peak (b)—alcohol, peak (c)—11-*cis*-retinol, formed in the isomerization reaction. Insets are the corresponding structures of retinol homologs and their short names. (E) LRAT substrate specificity with retinoids of different lengths. For simplicity, the ratio of areas of the ester peak to alcohol peak was calculated for each isomer. (F) On-line UV-Vis spectra of retinol homologs in 10% ethyl acetate/hexane solution.

An important observation was that isomerization occurs at the alcohol oxidation stage, and not at the retinal oxidation stage (Bernstein, Law, & Rando, 1987). The two processes, esterification by LRAT and isomerization, were shown to be closely intertwined; however, the isomerase showed more restricted substrate specificity. First, non-hydrolyzable retinyl methyl ether is not a substrate for isomerization (Canada et al., 1990). Secondly, no conjugation breakage can be allowed in the polyene chain, therefore retinol analogs such as 5,6-, 7,8-, 9,10-, 11,12-dihydroretinols are not substrates for the isomerization (Canada et al., 1990). Additional conjugation is allowed and it was shown that 3,4-didehydroretinol (Vitamin A₂) is a substrate for isomerization. However, none of the aromatic analogs were substrates for isomerization (Canada et al., 1990). Modifications of the methyl groups of analogs were performed and it was shown that 5- and 13-demethyl-retinols were substrates for isomerization. Meanwhile, 9-demethyl-retinal, initially thought to be a poor substrate for isomerization (Canada et al., 1990), was later shown to be isomerized in the presence of CRALBP (Stecher, Prezhdho, Das, Crouch, & Palczewski, 1999). Addition of CRALBP increases the production of 11-*cis*-retinol about 15–20-fold (McBee et al., 2000).

Bulkiness at C-15 also affects the isomerization reaction. Hence, 15-methylretinol was not isomerized.

Similarly, the presence of electron-withdrawing groups on the retinyl also affects the reaction, and 9-trifluoromethyl- and 11-fluoro-retinol were not substrates for isomerization. Some analogs that were freely isomerized were 10-Me-, 11-Me-, and 11,19-ethanoretinols, thus bulky groups at this part of the retinol molecule were tolerated by the isomerase (Canada et al., 1990). This interesting observation can be explained by the fact that substitution at position 10 or 11 twists the C-11=C-12 double bond, thus facilitating isomerization. Also, a high degree of spontaneous isomerization was observed with these analogs. From these studies, it was deduced that the only mechanism by which retinol could be isomerized was via nucleophilic addition of enzyme to the C-11 position. None of the methyl groups of retinol were involved in the isomerization. This conflicts, however, with the fact that bridged 11,19-ethanoretinol, which has a sterically hindered C-11 atom, was a good substrate (Canada et al., 1990).

It is unclear if the substrate for isomerization is all-*trans*-retinol, all-*trans*-retinyl esters, or another unidentified analog. This ambiguity is a result of the many competing enzymatic reactions in RPE, most notably the powerful LRAT activity. To illustrate that the hydrolase reaction is taking place in the RPE, here we used a retinol analog that is not a substrate for LRAT, namely *thioretinol*. For the first time for studies on the

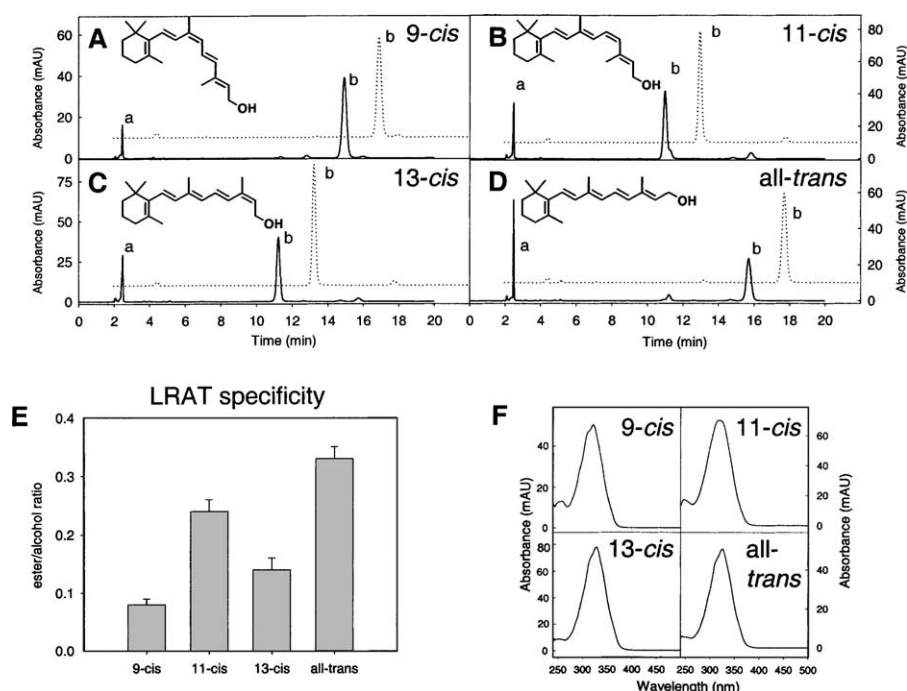


Fig. 8. LRAT activity with four geometric isomers of retinol. (A–D) HPLC traces for each isomer. Absorbance was recorded at 325 nm. Solid line—reaction in native UV-bleached RPE. Dotted line—boiled RPE. In each trace, the insets are the corresponding structures of retinol isomers and their names. (E) Comparison of LRAT activity for different isomers. For simplicity, the ratio of the area of the ester peak to retinol peak area was calculated for each isomer. (F) On-line UV-Vis spectra of retinol isomers in 10% ethyl acetate/hexane solution. The RPE reaction was carried out within the linear range of enzymatic activity (up to 4 min). Peaks a represent esters, peaks b represent alcohols. All retinoids were purified by normal-phase HPLC (Beckman, Ultrasphere-Si, 4.6 mm $\times$ 250 mm) with 10% ethyl acetate/90% hexane at a flow rate of 1.4 ml/min using an HP1100 HPLC with a diode-array detector and HP Chemstation A.03.03 software.

retinoid cycle, all-*trans*-thioretinyl acetate was prepared by two different methods, palladium-catalyzed reaction of all-*trans*-retinyl acetate and potassium thioacetate (Fig. 9A) and the Wittig reaction of 7-(2,6,6-trimethylcyclohex-1-en-1-yl)-1,5-dimethyl-2,4,6-heptatrienyl triphenylphosphonium bromide and *S*-acetyl-2-thioacetaldehyde (data not shown). All-*trans*-isomer was purified by normal-phase HPLC and showed identical UV and NMR spectra to those described in literature (Welch & Gruber, 1979) (Section 2). All-*trans*-thioretinyl acetate was incubated with RPE microsomes under conditions well established in our laboratory. It was shown that it is hydrolyzed by the hydrolases present in RPE to give rise to a peak eluting at 3.2 min. No hydrolysis reaction occurred in the presence of boiled RPE membranes proving that hydrolysis is an enzymatic reaction (Fig. 9B). All-*trans*-thioretinyl acetate is hydrolyzed at a catalytic rate and within 2 h, more than 90% of the acetate was hydrolyzed by the RPE microsomes (Fig. 9C). Isomers were separated by HPLC and tested for hydrolase substrate specificity. Incubation time for this experiment was 10 min and was within the linear range of the product formation. Compared to all-*trans*-isomer, 9-*cis*- showed about 20% of the activity while 13-*cis*- showed only 10%. Furthermore, thioretinyl acetate and products of its hydrolysis were analyzed by mass

spectrometry and the spectra showed the corresponding molecular ion peaks at 344 *m/z* (thioacetate) and 302 *m/z* (thioretinol). Fragmentation patterns of thioretinyl acetate and thioretinol were similar to those of oxyretinols. However, instead of a peak at 268 *m/z* (anhydroretinol), as in case of retinols, a peak at 269 ([*M*-SH]⁺ or [*M*-CH₃COS]⁺) *m/z* had formed, confirming that thioretinol is more stable and does not dehydrate as easily as retinol. Therefore, even if the majority of retinol is converted to ester, retinol can be liberated by the hydrolase and subjected to further transformation.

Therefore, thioretinyl acetate was found to be a substrate for the hydrolases present in the RPE and was hydrolyzed to all-*trans*-thioretinol, as confirmed by mass spectral analysis. Thioretinyl acetate was hydrolyzed at a slower rate than all-*trans*-retinyl acetate. However, the formed all-*trans*-thioretinol was not a substrate for LRAT. No 11-*cis*-isomer (either thio or oxy) was formed when incubated with RPE membranes in the presence of CRALBP (data not shown). This can be explained first by the fact that the transfer of fatty acid residue from the thiol group of LRAT to the thiol group of thioretinol is not thermodynamically favored and as a result, the thioacylation reaction does not proceed. The second reason why thioretinol was not isomerized is that upon protonation, the thiol group forms a stable sulphonium

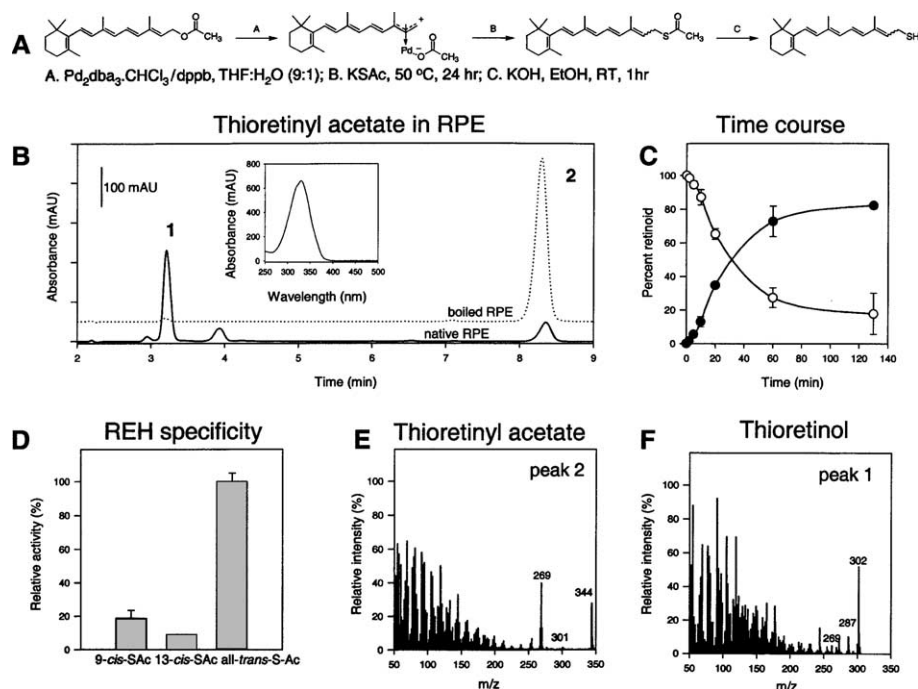


Fig. 9. Synthesis and biochemical properties of all-*trans*-thioretinyl acetate. (A) Synthesis of all-*trans*-thioretinyl acetate via palladium-catalyzed reaction. (B) HPLC traces after incubation of all-*trans*-thioretinol with RPE membranes. Top trace—boiled RPE, showing no activity. Bottom trace—native UV-treated RPE showing hydrolysis of acetate to produce thioretinol. Peak 1—thioretinol. Peak 2—thioretinyl acetate. Inset—UV spectrum of all-*trans*-thioretinyl acetate in hexane, $\lambda_{\max}$ 330 nm. (C) Normalized time course for the hydrolysis of all-*trans*-thioretinyl acetate. Opened circles—thioretinyl acetate, solid circles—thioretinol. (D) Substrate specificity of thioretinyl acetate hydrolysis. Activity toward all-*trans*-isomer was taken as 100%. (E) and (F) EI mass-spectra of thioretinyl acetate with molecular ion at 344 *m/z* (2) and peak 1 from enzymatic reaction (B) with 302 *m/z* corresponding to thioretinol. All retinoids were purified by normal phase HPLC (Beckman, Ultrasphere-Si, 4.6 mm $\times$ 250 mm) with 10% ethyl acetate/90% hexane at a flow rate of 1.4 ml/min using an HP1100 HPLC with a diode-array detector and HP Chemstation A.03.03 software. Retinoids were analyzed by mass spectrometry as described in Section 2.

ion (RSH_2^+) that is not as prone to an elimination reaction compared to the protonated OH group.

This hydrolase activity could also be illustrated using retinyl acetate and RPE membranes (Fig. 10A and B). UV-treated RPE (without endogenous retinoids) was incubated with all-*trans*-retinyl acetate, which was rapidly hydrolyzed to all-*trans*-retinol by hydrolases present in RPE. Without CRALBP (Fig. 10B), retinol was re-esterified by LRAT to form fatty acid esters. More than 90% conversion of acetate to palmitate took place within 30 min, after which equilibrium was reached and 11-*cis*-retinol was slowly formed, reaching 10% of total retinoids after 2 h incubation. A small amount of unesterified all-*trans*-retinol was present. However, when CRALBP was introduced into the assay (Fig. 10A), slower hydrolysis and slower palmitate ester formation were observed. Higher amounts of free all-*trans*-retinol were present in this assay and the formation of 11-*cis*-retinol was considerable, reaching as much as 60% of total retinoid content after 2 h. It is important to note that the hydrolase activity also showed significant substrate specificity. To test the substrate specificity of the hydrolases, three different retinyl acetates were incubated with RPE. The highest activity was observed with all-*trans*-retinyl acetate and the activities of 9-*cis*- and

13-*cis*-acetates were about 5–10% compared to all-*trans* isomer (Fig. 10D).

The isomerization assay reported by Winston and Rando (Winston & Rando (1998, 2000)) and refined by us (Stecher, Gelb, Saari, & Palczewski, 1999; Stecher & Palczewski, 2000; Stecher, Gelb et al., 1999) run in the presence of CRALBP is a significant step forward toward the complete characterization of the enzyme responsible for the isomerization as compared to the earlier assays carried out in the absence of this retinal-binding protein. In order to identify the substrate for isomerization, RPE was incubated with radioactive all-*trans*-retinol (1200 dpm/nmol). After the formation of palmitate was completed, cold all-*trans*-retinyl acetate was introduced into the assay together with CRALBP. Since acetate is rapidly hydrolyzed, specific radioactivity of the forming palmitate decreased rapidly. In a parallel process during the first 5 min, the radioactivity of retinol also decreased. After 10 min, the specific radioactivity of all-*trans*-retinol started to increase while that of palmitate and 11-*cis*-retinol were decreasing in a parallel manner (Fig. 10C). These results strongly suggest a dynamic exchange of retinol with all-*trans*-retinyl esters and powerful LRAT and hydrolase activities. More complete studies using similar types of experiments

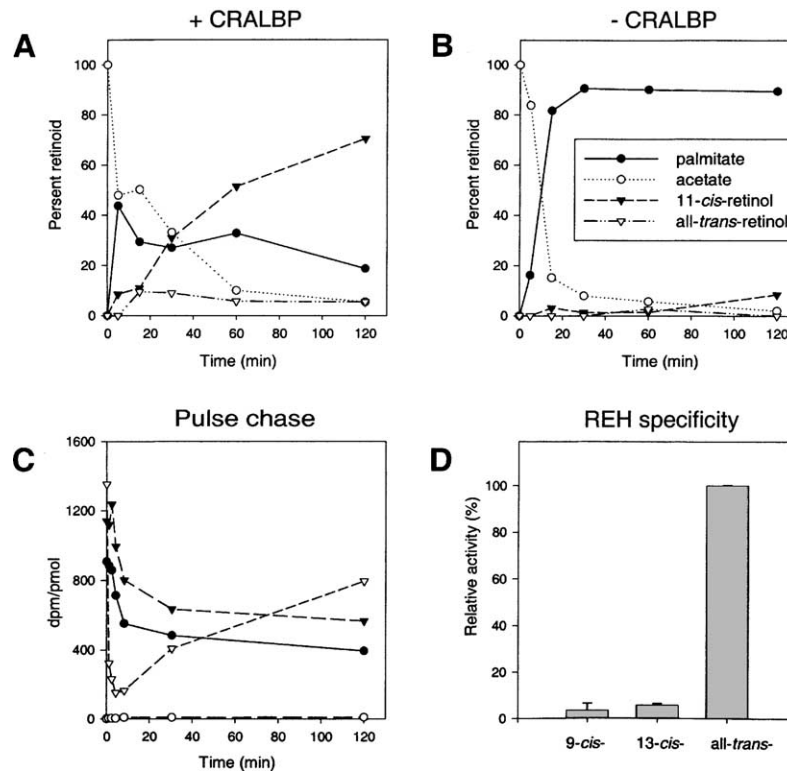


Fig. 10. Isomerization reaction using all-trans-retinyl acetate as a substrate. (A) Time course of the RPE reaction in the presence of CRALBP showing large production of 11-cis-retinol (solid triangles). (B) Reaction in the absence of CRALBP showing very small production of 11-cis-retinol. Retinoid amounts are normalized. (C) Radioactive time course for isomerization reaction in the presence of CRALBP. Solid circles—retinyl esters. Open circles—all-trans-retinyl acetate. Solid triangles—11-cis-retinol. Open triangles—all-trans-retinol. (D) Substrate specificity of retinyl acetate hydrolysis. Activity toward all-trans-isomer was taken as 100%. All retinoids were purified by normal-phase HPLC (Beckman, Ultrasphere-Si, 4.6 mm $\times$ 250 mm) with 10% ethyl acetate/90% hexane at a flow rate of 1.4 ml/min using an HP1100 HPLC with a diode-array detector and HP Chemstation A.03.03 software.

(Stecher, Gelb et al., 1999) led us to conclude that most of the ester pool does not participate in isomerization. The potential isomerization substrate could be retinol, a sub-pool of retinyl esters, or an as yet unidentified intermediate.

4. Discussion

Several useful methods were presented in this study. Laser scanning microscopy, especially two-photon microscopy, provides novel ways to investigate the flow of retinoids truly in vivo. We are positive that this technique will have a dramatic impact on our understanding of the compartmentalization and flow of retinoids in the eye. In combination with retinoid analyses from large animal models or WT and genetically altered rodent models, new information on the kinetics and distribution of retinoids can be obtained. In addition, numerous biochemical and chemical methods are important to understanding at the molecular level, how the complex of isomerization reactions assemble in normal and disease states. Understanding the retinoid cycle on the

molecular/chemical level carries a promise that many inherited and acquired human diseases could be potentially treated with simple pharmacological interventions.

4.1. Flow of retinoids in the eye

The physical and chemical properties of retinoids affect their distribution and availability. Due to their amphipathic nature imparted by the hydrophobic ionone ring, isoprenoid chain, and the polar terminus, retinoids are found in both aqueous and membrane compartments. Both the lipid composition and the membrane curvature influence the partition constant of retinol between membranes and an aqueous buffer. Retinol and retinal display modest water solubility, determined to be 60 nM and 110 nM, respectively (Szuts & Harosi, 1991). The formation of micellar complexes through self-association enhances the uptake and solubility of retinoids (Li, Zimmerman, & Wiedmann, 1996). The diffusion of retinoids in water is, however, relatively slow, corresponding to approximately 2.5×10^{-6} cm²/s (Dodson, Peresypkin, Rengarajan, Wu, & Nickerson, 2002). However, they partition to the hydrophobic

phase. As a consequence, retinoids are found in association with either cellular membranes or proteins. The transport of retinoids through aqueous environments is often in association with proteins that have various degrees of affinity for retinoid substrates. The most important role of retinoid-binding proteins is the protection of the soluble pools of retinoids from chemical degradation and isomeric rearrangements (Crouch et al., 1992; McBee, Van Hooser et al., 2001). The flow of soluble retinoids in either free or protein-bound form is governed by the gradient set up through the conversion of 11-*cis*- and all-*trans*-retinol to insoluble esters in the RPE and the conversion of 11-*cis*-retinal to 11-*cis*-retinylidene-opsin in rod photoreceptor cells.

The retinyl esters are both more stable and insoluble than their retinol or retinal counterparts. For this reason, retinyl esters constitute storage forms and may also represent reaction intermediates in the isomerization of all-*trans*- to 11-*cis*-isomers (reviewed in (Rando, 1992, 1996) and supported recently by similar and new experiments (Moiseyev et al., 2003)). Many of the enzymes involved in the processing and synthesis of retinyl esters are either transmembrane proteins (LRAT, RDH5) or associated with lipid compartments (RPE65, (Tsilou, Hamel, Yu, & Redmond, 1997)). This is due to the insolubility of their substrates and may also be due to the possible role for compartmentalization in driving unfavorable chemical reactions through mass action. Both the RPE plasma membrane and the endoplasmic reticulum have been shown to be involved in retinoid processing (Mata & Tsin, 1998).

4.2. Redundancy of retinoid enzyme activities

The use of transgenic mouse models containing homozygous disruptions of various genes involved in the retinoid cycle has well illustrated the redundancy of retinoid enzyme activities, transport, and supply systems. Beneficially, this redundancy improves the pathological outcome of mutations in enzymes that process the visual chromophore. For example, mutations in the serum retinol-binding protein (RBP4) lead to a visual impairment in *rpb4*^{-/-} mice at the time of weaning, which can be ameliorated through a vitamin A-supplemented diet (Quadro et al., 1999; Vogel et al., 2002). The transient visual impairment of the *rpb4*^{-/-} mice was attributed to the emerging role of RBP4 in retinol uptake by the RPE (Vogel et al., 2002). Similarly, patients affected by mutations in RBP4 exhibited minor clinical symptoms (Biesalski et al., 1999; Seeliger et al., 1999). The lack of RBP4 was circumvented through the delivery of retinol through chylomicrons or by conversion from carotene or retinyl esters. Vision was restored as localized stores of retinoids in the retina are established. Mutations in several proteins involved in chromophore transport such as, interphotoreceptor

retinoid-binding protein (IRBP), TTR and RBP-1 were associated with only minor impairments of the retinoid cycle.

Biochemical studies in conjunction with the studies on the genetically engineered mice are very powerful. For example, biochemical studies of the retinol dehydrogenase activity in the isolated membranes in combination with the analysis of the RPE from genetically engineered mice provide proof of the involvement of the gene of interest in the retinoid cycle. An example of such an approach was used to infer the existence of additional oxidation pathways in vertebrates (Jang, McBee, Alekseev, Haeseleer, & Palczewski, 2000; Jang et al., 2001). In these studies, we analyzed the stereospecificity of RDH5 and compared it to the stereospecificities of the different RDH activities present in the RPE and then combined this knowledge with the retinoid profile in the RPE from *rdh5*^{-/-} mice.

4.3. Enzymatic isomerization of the chromophore

Isomerization of all-*trans*-retinol to 11-*cis*-retinol in RPE cells is an important step in the retinoid cycle. This reaction provides opsin with its chromophore after the oxidation of 11-*cis*-retinol to the aldehyde form by 11-*cis*- RDH present in the RPE. The precise mechanism of this 'reverse,' energetically uphill isomerization is not known. Two distinct mechanisms for the isomerization reaction have been proposed (Fig. 11) (McBee et al., 2000; Rando, 1991a).

After the reduction of all-*trans*-retinal formed in ROS as a consequence of photoisomerization of 11-*cis*-retinal bound to opsin, all-*trans*-retinol is transported into RPE cells, where it is esterified to mostly palmitoyl-retinol in a reaction catalyzed by a highly active LRAT. These insoluble esters are a part of RPE membranes and are thought to be primarily a form of retinol storage and secondarily as a substrate for the isomerization reaction.

One proposed mechanism of isomerization involves the addition of nucleophilic anion to C-11 of all-*trans*-retinyl palmitate, followed by elimination of the palmitate anion (Rando, 1991a). The adduct formed then rotates around the C-11–C-12 single bond to 11-*cis*-conformation. Then, nucleophilic attack of a water molecule coupled with the leaving of the protein active site leads to the stabilization of 11-*cis*-conformation and release of 11-*cis*-retinol, which can also be esterified by LRAT to the corresponding 11-*cis*-retinyl palmitate. In the proposed mechanism, the energy of hydrolysis is used to drive the energetically uphill isomerization. The enzyme thus was called 'isomerohydrolase' (Fig. 11).

The second possible mechanism of isomerization could be through the protonation reaction by the alkyl cleavage into an all-*trans*-retinyl carbocation. The all-*trans*-retinyl palmitate or some other 'active' intermediate formed by transesterification of palmitate could be

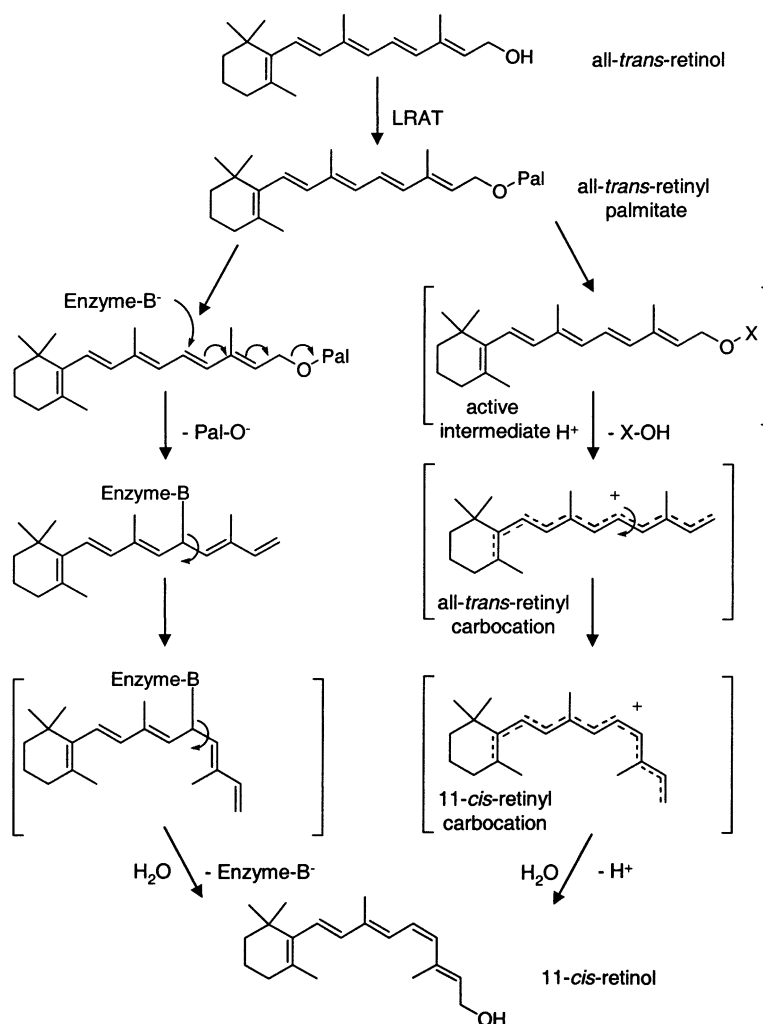


Fig. 11. Proposed mechanisms of 11-*cis*-retinol formation in RPE from all-*trans*-retinol. *Left panels.* The isomerization mechanism involving the isomerohydrolase reaction (Rando, 1991b, 1996). *Right panels.* The mechanism that involves a carbocation intermediate (McBee et al., 2000).

the substrate of the protonation reaction. In such a polyene carbocation, the positive charge is distributed along the polyene chain, resulting in a reduced bond order. Due to this delocalization of the double bond, the rotation of the C-11–C-12 bond is energetically more feasible compared to the rotation of a true double bond. Subsequently, the ‘isomerase’ enzyme stabilizes the all-*trans*-retinyl carbocation and hydration of the former with deprotonation leads to 11-*cis*-retinol (Fig. 11).

During isomerization there is an inversion of configuration at C-15, thus confirming the enzymatic reaction (Jang et al., 2000; Law & Rando, 1988). In addition, using stable-isotope labeling experiments, it was shown that the formation of 11-*cis*-retinol from all-*trans*-retinol leads to C-15–O bond cleavage (Deigner, Law, Canada, & Rando, 1989; McBee et al., 2000).

The fact that the energetically unfavorable isomerization reaction does not require an exogenous source of energy begs the question of where the energy comes from. The all-*trans*- to 11-*cis*-isomerization was calcu-

lated to require 4 kcal/mol for the process (Rosenfeld, Hong, Ottolenghi, Hurley, & Ebery, 1977). In the isomerohydrolase model it was proposed that this energy comes from the hydrolysis of palmitate (Rando, 1992). Addition of the enzyme active-site nucleophile to the C-11 of polyene chain is connected with elimination of a palmitate anion, thus forming 11-hydro-intermediate.

In the carbocation mechanism, protonation of the retinyl derivative (retinol or retinyl palmitate) or another intermediate followed by alkyl cleavage of the positively charged active intermediate group is the key step. It was shown chemically that protonation of retinyl ester leads to the formation of *retro*-retinoid, while protonation of retinol itself gives anhydroretinol. In our laboratory attempts to prepare retinyl ester with a strong electron-withdrawing group, such as tosylate and trifluoroacetate have failed due to the exclusive formation of anhydroretinol during these reactions (unpublished). Retinol was dehydrated as the unstable product

was formed. Thus, it is most likely that the transesterification reaction (which does not require a source of energy) occurs prior to retinyl carbocation formation. The active intermediate is susceptible to an alkyl cleavage elimination reaction and retinyl carbocation is, thus, formed. The carbocation is stabilized by the putative isomerase and it rotates easily around the C-11–C-12 bond. Hydration of the carbocation occurs by a water molecule. The resulting retinol isomer is released and picked up by a retinol-binding protein. The substrate specificity of the isomerization is correlated with the substrate specificity of the retinoid-binding protein (McBee et al., 2000). If CRALBP is used for the isomerization reaction, most of the product is 11-*cis*-retinol without O¹⁸-label. If CRBP (higher affinity for all-*trans*- or 13-*cis*-retinol, than for 11-*cis*-retinol) is used, the all-*trans*-retinol produced still has the O¹⁸ label (it is most likely formed by the action of REH), but formed 13-*cis*-retinol loses its O¹⁸ label. This fact cannot be explained by the isomerohydrolase mechanism. Also, an Arrhenius plot showed that the calculated energy of isomerization reaction *via* carbocation has approximately the same value as that of the experimental, thus suggesting that the mechanism is *via* carbocation. Moreover, the presence of an electron-withdrawing group on the retinyl chain had a negative effect on the isomerization. Groups such as 11-fluoro- or 9-trifluoromethyl destabilized the carbocation and, as a consequence, the isomerization did not occur (Law & Rando, 1988; McBee et al., 2000). Another idea that this isomerization *in vivo* is non-enzymatic (Bernstein, Lichtman, & Rando, 1985) appears also to be incorrect.

We propose a general mechanism of retinoid isomerization. All-*trans*-retinol is transported to RPE, where it is esterified by LRAT to form insoluble esters in RPE cell membranes. Then, two possibilities should be considered. First, a subset of these esters (Stecher, Gelb et al., 1999) or the hydrolysis product all-*trans*-retinol can be directly protonated and alkyl cleavage of positively charged retinyl residues occurs to form fatty acid and a retinyl carbocation. Second, the active intermediate can be formed from palmitate by a transesterification reaction. This intermediate (having a strong electron-withdrawing acid group) undergoes elimination induced by a negatively charged acid residue to form the retinyl carbocation. The carbocation is stabilized by the protein and rotates freely around its bonds, since the positive charge is distributed along the polyene chain and reduces the bond order. Thermodynamic equilibrium for the carbocation is reached and then, depending on the specificity of the retinoid-binding protein (CRALBP or CRBP), it is shifted towards the better substrate. Withdrawal of the product leads to a shift of equilibrium towards the withdrawn product accelerating the reaction greatly. This model is not substantiated by direct evidence.

4.4. Important considerations regarding the two models of the isomerization

Given the two models proposed in the literature, the carbocation and the putative isomerohydrolase, the debate will continue until this process is revealed at the molecular and cellular levels. It is important, however, at this stage of our understanding of the process to compare conditions of the RPE reconstitution assays as used by the various research groups, as these can affect the interpretation of the results and the validity of the experimental approaches. Recently, Gollapalli and Rando (Gollapalli & Rando (2003)) questioned our results pertaining to the pulse chase experiment (Stecher, Gelb et al., 1999). To directly test whether the pre-existing pool of retinyl esters has an effect on isomerization, we incubated unlabeled all-*trans*-retinol with UV-treated RPE microsomes to produce unlabeled retinyl esters, before 2.5 μM [³H]all-*trans*-retinol (550,000 dpm/nmol) and apo-rCRALBP were added (Stecher, Gelb et al., 1999). 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. This lack of dilution proves that most of the ester pool does not participate in isomerization.

Why is there difference between these results obtained in different laboratories? In our experimental conditions, we use 10–20 μM all-*trans*-retinol (comparable amounts to those found in the bovine eyes) that result in reproducible and robust production of 11-*cis*-retinol. Up to 50% of the product is generated within the first 10 min of the assay in the presence of 11-*cis*-retinoid-binding protein, 25 μM CRALBP, that specifically extracts 11-*cis*-retinol product from the RPE microsomal membranes (McBee et al., 2000; Stecher, Gelb et al., 1999). In contrast, results in question were re-examined by Gollapalli and Rando (Gollapalli & Rando, 2003) using comparable amounts of RPE membranes to our study, but employed 0.09 or 0.85 μM all-*trans*-retinol as the substrate and used 1 mM BSA. In these conditions, there was ~11,000-fold molar excess of BSA over all-*trans*-retinol and ~20 times over proteins of RPE microsomes used in the assay. Not surprisingly, low amounts of the substrate and vast excess of BSA leads to massive extraction, ~50%, of the retinoids from the membrane into the aqueous phase, and therefore low amounts of *cis*-product even after a 2 h incubation (Gollapalli & Rando, 2003). Thus small amount of 11-*cis*-retinol product, equal to production of 13-*cis*-retinol, also poses difficulty in terms of the uniformity and background signals in study's published chromatographic separations as evident in their Fig. 3B and C, bottom panels. The study also uses generic pharmacological inhibitors in concentrations up to 0.1 mM (one

of which can hydrolyze to all-*trans*-retinol). In addition, the majority of retinoids and lipids are extracted by the vast amounts of BSA, which has high affinity for many hydrophobic substrates. Therefore, the assays described by Rando's laboratory (Gollapalli & Rando, 2003) display a low isomerase specific activity (about 100 times lower than measured in our experimental condition approaching 16 nmol/min/mg).

One of many critical arguments that needs to be addressed in order for the isomerohydrolase model to gain credibility is the lack of isomerization of all-*trans*-retinol by the hydrolysis-driven reaction ($\Delta G^\circ < 0$) in the absence of binding proteins. Previously, it was reported that this isomerization reaction is inhibited by its products (11-*cis*-retinol and 13-*cis*-retinol) with IC_{50} about 1 μ M and >10 μ M, respectively (Winston & Rando, 1998). Interestingly, the IC_{50} is higher than the TOTAL concentration of retinoids used in Rando's study. Therefore, the isomerohydrolase model is not substantiated by direct evidence, but derived based on a minor enzymatic activity that may or may not reflect physiological processes (reviewed in (McBee, Palczewski et al., 2001)).

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