Regulation of Isomerohydrolase Activity in the Visual Cycle†

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ABSTRACT: While the overall biosynthetic pathway leading from *all-trans*-retinoids to 11-*cis*-retinoids in the visual cycle is understood, little is known about which step(s) may be rate-limiting and how control is exerted. One possible target for control is the isomerohydrolase, which processes *all-trans*-retinyl esters into 11-*cis*-retinol. The basal rate of 11-*cis*-retinol synthesis from *all-trans*-retinyl esters is extremely slow using bovine retinal pigment epithelial membranes [3.5 pmol of 11-*cis*-retinol min^{-1} (mg of protein)$^{-1}$], and only small amounts of 11-*cis*-retinyl ester are formed. However, the addition of retinol binding proteins stimulates 11-*cis*-retinol formation by a factor of approximately 13. Specific protein−protein interactions are probably unimportant because bovine serum albumin and the physiologically relevant cellular retinaldehyde binding protein (CRALBP) both stimulate 11-*cis*-retinol formation to the same extent, although CRALBP does so at much lower concentrations. The relatively rapid rate of isomerization in the presence of binding proteins [44.3 pmol of 11-*cis*-retinol min^{-1} (mg of protein)$^{-1}$] suggests that the rate-limiting enzyme in the visual cycle need not be the isomerohydrolase. Also, 11-*cis*-retinol is shown to inhibit isomerohydrolase, providing a simple mechanism for regulation of the visual cycle and the stimulating effect of binding proteins.

Upon illumination, rhodopsin photoisomerizes from a protonated 11-*cis*-retinal Schiff base configuration into its congeneric *all-trans*-retinal protonated Schiff base form in less than 200 fs (*1*), thereby triggering the conversion of rhodopsin into its physiologically active form (*2, 3*). The physiologically active form of rhodopsin, called metarhodopsin II, initiates a signal transduction cascade that eventually leads to the excitation of the photoreceptor cell (*4, 5*). To detect further light quanta, the rhodopsin form of the visual pigment has to be regenerated with 11-*cis*-retinal (*6*). In vertebrates, this regeneration of the visual chromophore is accomplished by a series of reactions that comprise the visual cycle (*7, 8*). In the first step, *all-trans*-retinal released from metarhodopsin is reduced to *all-trans*-retinol and then transported to the RPE[1] in a complex with IRBP (*9, 10*). In the RPE, *all-trans*-retinol is esterified to *all-trans*-retinyl esters in a reaction catalyzed by the enzyme LRAT (*11, 12*). In the next step(s) in the visual cycle, *all-trans*-retinyl ester is converted to 11-*cis*-retinol, in a reaction that utilizes the free energy of ester hydrolysis to drive the uphill isomerization process (*13, 14*). As this reaction may involve concerted hydrolysis and isomerization, the putative enzyme catalyzing this step is an isomerohydrolase (*15*). 11-*cis*-Retinol is then oxidized to 11-*cis*-retinal, transported back to the photoreceptor cell, and recombined with opsin to form rhodopsin (*16*−*19*).

In the eyes of dark-adapted vertebrates, most retinoids are found in the 11-*cis* form (*20*), either as 11-*cis*-retinal bound to rhodopsin or as 11-*cis*-retinyl esters in the RPE (*21*). The bleaching of rhodopsin leads to a substantial net movement of retinoids into the RPE (*22*) and to a shift of retinoids into the *trans* configuration (*23*−*25*). Under bleaching conditions, the predominant form of retinoids in the RPE is *all-trans*-retinyl ester, and in the photoreceptor cell it is *trans*-retinol (*25, 26*). When animals are placed in the dark after a thorough bleach, dark adaptation proceeds at a rate limited by rhodopsin regeneration (*22*). The half-time for rhodopsin regeneration has been measured to be 5 min for humans, 30 min for amphibians, and 40 min for rats (*20, 27*). This rate is temporally correlated with the slow in vitro rate of biosynthesis of 11-*cis*-retinoids in the dark. For frog eyes, the rate of 11-*cis* biosynthesis has been measured to be 1−2 nmol h^{-1} eye^{-1} (*23*) in vivo or 5 pmol h^{-1} (mg of protein)$^{-1}$ in vitro (*28*), and for bovine eyes, values of 1.3 pmol min^{-1} (mg of protein)$^{-1}$ (*29*) and 0.4 nmol h^{-1} (mg of protein)$^{-1}$ (*30*) have been reported in vitro. These data suggest that the isomerization rate might be rate-limiting in the visual cycle (*8, 19*). However, the rate of 11-*cis*-retinoid regeneration in a light-adapted eye, while not known, is expected to be substantially more rapid than the rate of 11-*cis*-retinoid regeneration in the dark. Otherwise, the vertebrate visual system would not be able to maintain a sufficient rate of rhodopsin synthesis. An important issue to address is how the isomerization reaction might be regulated and whether the reaction rate can be enhanced over the sluggish rate measured in vitro. In this study, we demonstrate that the rate of 11-*cis*-retinol formation in vitro can proceed much faster than previously measured. Our results also show that soluble retinoid binding proteins have a stimulating effect

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[1] Abbreviations: ADH, alcohol dehydrogenase; BSA, bovine serum albumin; CRALBP, cellular retinaldehyde binding protein; DTT, dithiothreitol; IRBP, interphotoreceptor retinoid binding protein; LRAT, lecithin:retinol acyltransferase; RPE, retinal pigment epithelium.

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on the conversion of *all-trans*-retinyl ester to 11-*cis*-retinol. This activating effect can be achieved upon addition of CRALBP, as well as BSA. A specific protein−protein interaction between a soluble retinoid binding protein and enzymes of the visual cycle is, therefore, not required for the formation and solubilization of 11-*cis*-retinol. In the absence of soluble retinol binding proteins, both 11-*cis*-retinol and 11-*cis*-retinyl ester are formed in very small amounts. These data show that soluble retinoid binding proteins increase the net formation of 11-*cis*-retinoids. A possible mechanism for this increase in isomerohydrolase activity upon addition of retinoid binding proteins is through the removal of product inhibition mediated by 11-*cis*-retinoids.

MATERIALS AND METHODS

Materials

Frozen eye cups devoid of retinas were purchased from J. A. and W. L. Lawson Co., Lincoln, NE. [11,12-$^3H_2$]-*all-trans*-Retinol (specific activity 31.4 Ci/mmol) was obtained from DuPont−New England Nuclear. His-tagged apo-CRALBP expressed in *Escherichia coli* was a generous gift from Dr. John Saari. 11-*cis*-Retinal was obtained through the National Eye Institute. *all-trans*-Retinol, 13-*cis*-retinol, DTT, and fatty acid-free BSA were purchased from Sigma. HPLC-grade solvents were from J. T. Baker.

Methods

Preparation of Isomerohydrolase-Containing Membranes. The procedure for preparation of bovine retinal pigment epithelium membranes is described elsewhere (*29*). Prior to use, the membranes were irradiated with UV light (365 nm) on ice for 5 min in order to destroy endogenous retinoids (*14*). Protein determinations were done with the amido black protein assay (*31*).

Isomerohydrolase Assay. As retinyl esters are too hydrophobic to be added to buffered membrane solutions, experiments on the isomerohydrolase reaction in RPE microsomal membranes are carried out in a two-step assay: first, ^{3}H-*all-trans*-retinol is added and converted by LRAT into ^{3}H-*all-trans*-retinyl esters. Similar to the situation in vivo, in vitro-generated retinyl esters are primarily located in membranes. In the second step, ^{3}H-*all-trans*-retinyl ester is converted into 11-*cis*-retinol. The reaction product, 11-*cis*-retinol, can be solubilized upon addition of retinoid binding proteins, and the amount of 11-*cis*-retinol formed is used as a measurement of isomerohydrolase activity.

Unless otherwise mentioned, all procedures were performed under dim red light with samples kept on ice. In a typical assay, 3.6 nmol [11,12-$^3H_2$]-*all-trans*-retinol (solvent dried under N_2 stream and retinol redissolved in 15 μL of methanol) were added to 1425 μL of the pigment epithelium membranes (180 μg of protein, 100 mM Tris/HCl, pH 9.0, 2.5 μM final retinol concentration). The membranes were incubated on a shaker for 60 min at room temperature, allowing the formation of [11,12-$^3H_2$] retinyl ester. After the addition of BSA [final concentrated 1% (w/v), to bind unconverted *all-trans*-retinol], the retinyl ester-containing membranes were collected by centrifugation (100000*g* for 30 min at 4 °C), washed twice, and redissolved in 100 mM Tris/HCl, pH 8.0. The solution was sonicated briefly in ice water. At this point, the membranes usually contained between 5 and 6 nmol of retinoids/mg of protein, the majority of which is found in the form of *trans*-retinyl esters (70% of total retinoids). The membranes also contained a substantial amount of retinol isomers that dissolved in the membranes and, therefore, were not completely removed by centrifugation. *trans*-Retinol accounted for 15%, 11-*cis*-retinol for 10%, and 13-*cis*-retinol for 5% of total retinoids.

The membrane solution was divided into aliquots (5 μg of protein/sample) and then further incubated for 2 h in a shaking water bath at 37 °C (100 mM Tris/HCl, pH 8.0, 100 μL total volume, BSA or other protein added as indicated). The reactions were quenched with methanol (500 μL/sample), 100 μL of H_2O was added, and 500 μL of hexane (containing butylated hydroxytoluene at 1 mg/mL) was used for extraction of the retinoids. The isomeric retinols were analyzed on a 5-μm PVA-Sil column (250 × 4.00 mm, YMC); the eluant was 7% dioxane in hexane at a flow rate of 1.5 mL/min. Retinyl esters were separated on a 5-μm maxisil column (250 × 4.00 mm, Phenomenex) with 0.4% ether (preservative-free) in hexane at 0.8 mL/min. The isomers were identified through coelution of standard mixtures of isomeric retinols (monitored at 325 nm) and retinyl esters (313 nm) prepared as described previously (*32*). Radioactivity was counted with an on-line Bertold LB 506-C HPLC radioactive monitor interfaced with an IBM 386 computer. Calibration of the system was done by extracting and injecting defined amounts of [11,12-$^3H_2$]-*all-trans*-retinol. In a typical isomerohydrolase assay, control samples optimized for 11-*cis*-retinol formation (5% BSA) contained on average 32% 11-*cis*-retinol, 11% 13-*cis*-retinol, 24% *trans*-retinol, and 33% retinyl ester. Control samples were taken in triplicate before the reaction and analyzed for their retinoid content. The amount of 11-*cis*-retinol formed during the second reaction step was determined by subtracting the average amount of 11-*cis*-retinol found in these three aliquots analyzed after the first reaction step (retinyl ester formation) from the amount of 11-*cis*-retinol found at the end of the isomerohydrolase reaction. Unless indicated otherwise, the amounts of 11-*cis*-retinol obtained are given as percent of total retinoids or as picomoles per milligram of microsomal protein.

RESULTS

Initial experiments were aimed at exploring the effects of BSA concentration on isomerohydrolase activity in isolated bovine RPE membranes. Increasing the amount of BSA relative to the amount of membranes had a drastic effect on 11-*cis*-retinol formation. Assays conducted with 50 μg of microsomal protein and 0.5% BSA (= 75 μM) yielded about 10−15% 11-*cis*-retinol (*29*). In the experiments described below, a decrease in the amount of microsomal membranes used (to 5 μg) combined with an increase in BSA concentration (up to 5%) increased 11-*cis*-retinol formation to 32% of total retinoids. It was of interest to determine whether the observed stimulation of 11-*cis*-retinol formation was due to an enhanced reaction rate and to determine what mechanism would account for this effect. The experiments described below address this issue.

Time Course of 11-cis-Retinol Formation. Two experiments were carried out with different retinoid concentrations

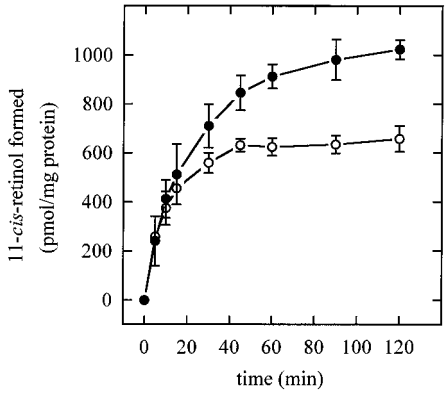

FIGURE 1: Time-dependent formation of 11-*cis*-retinol from *all-trans*-retinyl ester in bovine RPE microsomal membranes. The experiment was conducted with two different retinoid concentrations, one set of samples containing 6.8 nmol of total retinoids/mg of protein (SD = 0.70, n = 27, ●), the other set 3.1 nmol per mg protein (SD = 0.16, n = 18, ○). For each curve, three (●) or two (○) identical samples containing 45 μg of microsomal protein (100 mM Tris/HCl, pH 8.0, 5% (w/v) BSA final concentration, 900 μL of total volume) were incubated at 37 °C. Of each sample, an aliquot of 5 μg of protein was taken before the reaction and after 5, 10, 15, 30, 45, 60, 90, and 120 min of incubation.

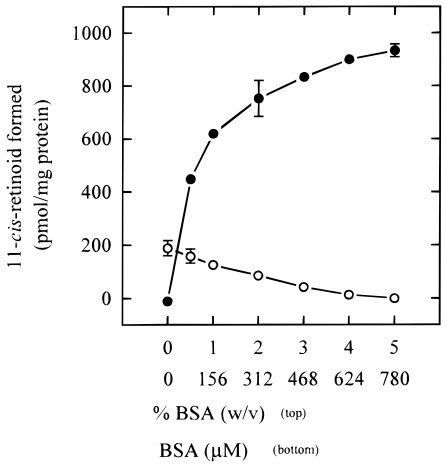

FIGURE 2: Formation of 11-*cis*-retinoids as a function of BSA concentration. A preparation of microsomal membranes (120 μg of protein) containing [11,12-$^3H_2$]-*all-trans*-retinyl ester was prepared as described in Materials and Methods. For each measured point, three identical samples (5 μg of protein, 100 mM Tris/HCl, pH 8.0, 100 μL of volume) were incubated for 2 h at 37 °C at the same final BSA concentrations indicated in Figure 1 and then analyzed for their content of 11-*cis*-retinol (●) and 11-*cis*-retinyl ester (○). In the absence of BSA, some of the 11-*cis*-retinol present before the isomerohydrolase reaction (see Materials and Methods) is converted to 11-*cis*-retinyl ester; the amount of 11-*cis*-retinol formed without BSA is therefore negative (−10.7 pmol/mg of protein, SD = 12.4, n = 3). The membranes contained 3.9 nmol of total [11,12-$^3H_2$]retinoids/mg of protein (SD = 0.4, n = 24). Where no error bars are indicated, the error bars were smaller than the circles.

(Figure 1). The membranes contained 3.1 nmol (SD = 0.16, n = 18) and 6.8 nmol (SD = 0.70, n = 27) retinoids/mg of protein, which is equivalent to 0.70 and 1.53 nmol of retinoids/eye, respectively. At lower retinoid concentrations, the formation of 11-*cis*-retinol levels off after 45 min with a maximum amount of 11-*cis*-retinol formed of about 650 pmol/mg of protein (Figure 1, ○). At higher retinoid concentrations (6.9 nmol/mg of protein), 11-*cis*-retinol formation reaches a maximum of about 1000 pmol/mg of protein after 2 h (Figure 1, ●). The membranes formed 221 pmol (SD = 4, n = 3) of 11-*cis*-retinol within the first 5 min of incubation. Bovine microsomal membranes are, therefore, able to form about 44 pmol (± 0.8) of 11-*cis*-retinol (mg of protein)$^{-1}$ min^{-1} under these in vitro conditions. It should be noted that this apparent V_{max} underestimates the true V_{max} of the isomerizing system for two reasons. First, the amount of substrate may have been rate-limiting in the formation of 11-*cis*-retinol. This could not be directly tested because it was not possible to generate microsomal membranes containing higher concentrations of retinyl esters with the methods used. Second, the stated value is clearly a minimal one and does not take the known instability of 11-*cis*-retinol with respect to nonenzymatic isomerization into its 13-*cis* and *all-trans* congeners into account (*14*).

Formation of 11-cis-Retinoids Is Dependent on the Concentration of Soluble Retinoid Binding Proteins. As shown in Figure 2, the amount of BSA has a significant effect on the formation of 11-*cis*-retinol (●). With no BSA added, the membranes convert a small proportion of 11-*cis*-retinol left over after the first reaction step (conversion of *trans*-retinol to *trans*-retinyl ester and subsequent centrifugation) into 11-*cis*-retinyl ester. The amount of newly formed 11-*cis*-retinol in the absence of BSA is therefore negative (−11 pmol/mg of protein, SD = 12, n = 3). Addition of BSA at low concentration (0.5% = 78 μM) increases the amount of 11-*cis*-retinol newly formed significantly ($p < 0.05$) to 448 pmol/mg of protein (SD = 13, n = 3). The 11-*cis*-retinol content increases further with increasing BSA concentrations. The maximum value measured in this experiment was 933 pmol/mg of protein (SD = 24, n = 3) at 5% BSA.

Analysis of the retinyl esters showed that in all samples most of the retinyl esters are in the *all-trans* form. 13-*cis*-Retinyl esters are only found in trace amounts (below 100 pmol/mg of protein). A maximum of 189 pmol of 11-*cis*-retinyl ester (SD = 29, n = 3) is formed in samples that contain no BSA. Upon addition of BSA, the amount of 11-*cis*-retinyl ester decreases (Figure 2, ○). The amount of 11-*cis*-retinol formed at higher BSA concentrations (933 pmol/mg of protein at 5% BSA) significantly ($p < 0.05$) exceeds the amount of 11-*cis*-retinyl ester formed without BSA (189 pmol/mg of protein). Therefore, BSA does not simply lead to a shift in the equilibrium between 11-*cis*-retinyl ester and 11-*cis*-retinol but, rather, stimulates the net formation of 11-*cis*-retinoids.

The results depicted in Figure 3 demonstrate that BSA not only affects the overall formation of 11-*cis*-retinol, but actually increases the rate of 11-*cis*-retinol biosynthesis. The amount of 11-*cis*-retinol formed was determined after 5 and 10 min, respectively, with varying BSA concentrations. As the BSA content of the samples increases from 0 to 5% BSA, 11-*cis*-retinol formation increases significantly ($p < 0.05$) from 17 pmol/mg of protein (SD = 8, n = 3) without added protein to 223 pmol/mg of protein (SD = 10, n = 3) after 5 min reaction time, and from 46 pmol/mg of protein (SD = 12, n = 3) at 0% BSA to 379 pmol/mg protein (SD = 10, n = 3) at 5% BSA after 10 min. From the amount of 11-*cis*-retinol formed within the first 5 min of the reaction, the rate of 11-*cis*-retinol formation can be calculated to be 3.5 pmol min^{-1} (mg of protein)$^{-1}$ without BSA, and 44.7 pmol min^{-1} (mg of protein)$^{-1}$ at 5% BSA. With 5% BSA,

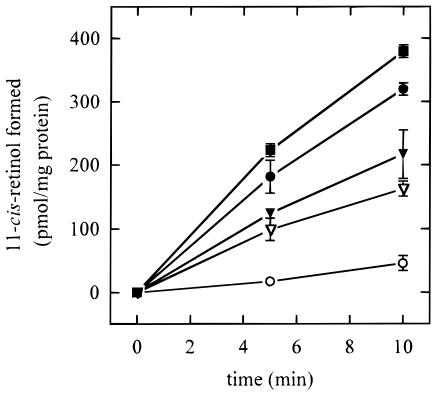

FIGURE 3: Rate of 11-*cis*-retinol formation as a function of BSA concentrations. Microsomal membranes containing 5.1 mg of [11,12-$^3H_2$] retinoids/mg of protein (SD = 0.2, n = 48) were prepared as described in Materials and Methods. Aliquots were incubated at 37 °C (5 μg of protein/sample, 100 mM Tris/HCl, pH 8.0, 100 μL total volume) for 5 or 10 min, respectively, with varying BSA concentration as indicated. Results of triplicate measurements are shown; where no error bars are indicated, the error bar was smaller than the symbol used. (■) 5% BSA; (●) 2% BSA; (▲) 0.5% BSA; (△) 0.25% BSA; (○) no BSA.

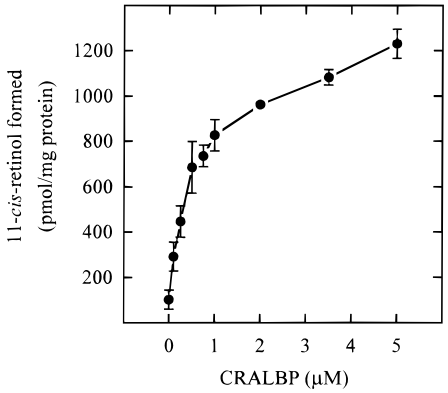

FIGURE 4: Formation of 11-*cis*-retinol as a function of CRALBP concentrations. Samples containing 5 μg of microsomal protein (6.1 nmol of total retinoids/mg of protein, SD = 0.37, n = 30) were incubated at 37 °C for 2 h (50 mM Tris/HCl, pH 7.5, 0.2% BSA, and 1 mM DTT, 100 μL total volume) with 0, 0.1, 0.25, 0.5, 0.75, 1.0. 2.0, 3.5, or 5.0 μM CRALBP, respectively. Each measured point represents the results of experiments performed in triplicate. Where no error bars are given, the error bars were smaller than the circle.

therefore, the production of 11-*cis*-retinol proceeds at a rate about 13 times faster than in the absence of BSA. The rate of 11-*cis*-retinol formation of 44.7 pmol/min at 5% BSA measured in this experiment is in good agreement with the value of 44.3 pmol/min found in Figure 1.

Control samples extracted after 2 h of incubation time gave the following results: 59 pmol of 11-*cis*-retinol/mg of protein (SD = 16) at 0% BSA, 337 pmol (SD = 41) at 0.25% BSA, 472 pmol (SD = 58) at 0.5% BSA, 925 pmol (SD = 37) at 2% BSA, and 1061 pmol (SD = 108) at 5% BSA (n = 3 for all values). The amounts of 11-*cis*-retinol formed after 2 h are thus in good agreement with the results shown in Figure 2.

Effects of Other Binding Proteins on 11-cis-Retinoid Formation. Further experiments were conducted to reveal the possible mechanism of the stimulation of 11-*cis*-retinoid formation by BSA. When BSA is replaced by ovalbumin [same concentration, 5% (w/v) = 780 μM], a soluble protein that does not bind retinoids, 11-*cis*-retinol formation was minimal. In the presence of 5% BSA, 11-*cis*-retinol was formed at a rate of approximately 1100 pmol min^{-1} (mg of protein)$^{-1}$. In the presence of 5% ovalbumin, or with no added protein at all, the rates are less than 140 pmol min^{-1} (mg of protein)$^{-1}$. In fact, the rate with ovalbumin was actually lower than the rate in the absence of added binding protein. This shows that the affinity of BSA for retinoids is crucial for the formation of 11-*cis*-retinol in our assay.

The natural binding protein for 11-*cis*-retinal and 11-*cis*-retinol in RPE cells is cellular retinaldehyde binding protein (CRALBP) (*33*, *34*). CRALBP has the same stimulating effect on the formation of 11-*cis*-retinoids as BSA does. Under conditions optimized for the isomerohydrolase reaction (pH 8.0, 5% BSA = 780 μM), the membranes formed about 1000 pmol/mg of protein 11-*cis*-retinol (SD = 88.7, n = 3). Altering the conditions to those optimized for binding of 11-*cis*-retinoids by CRALBP [pH 7.5, 1 mM DTT (*35*)] slightly decreases isomerohydrolase activity (752 pmol of 11-*cis*-retinol formed/mg of protein, SD = 50.4, n = 3). Decreasing the concentration of BSA to 0.2% (= 31 μM) (*35*) abolishes isomerohydrolase activity almost completely since only 114 pmol of 11-*cis*-retinol/mg of protein was formed (SD = 55.4, n = 3). Isomerohydrolase activity can be completely restored, however, in the presence of CRALBP. Upon the addition of 20 μM CRALBP, the membranes produced 1137.8 pmol of 11-*cis*-retinol/mg of protein (SD = 33.7, n = 3). Furthermore, the addition of a protein that does not bind retinoids, ovalbumin (20 μM), had no effect, as expected, with only 88.0 pmol of 11-*cis*-retinol being formed (SD = 4.3, n = 3). Hence, CRALBP stimulates the formation of 11-*cis*-retinol about 13-fold over the ovalbumin control. At the 20 μM concentration used in this experiment, CRALBP is about 10 times more effective than BSA at 0.2% (32 μM). This result clearly demonstrates that CRALBP can promote the formation of 11-*cis*-retinol more efficiently than BSA can and that this stimulating effect is restricted to proteins that can bind retinoids.

As in the case of BSA (Figure 2), the effect of CRALBP is concentration-dependent (Figure 4). The formation of 11-*cis*-retinol is roughly linear to the CRALBP concentration up to 1 μM CRALBP. The maximal stimulation of 11-*cis*-retinol generation occurred at 5 μM CRALBP (1231 pmol/mg of protein, SD = 64.1, n = 3). Increasing the concentration of CRALBP to 20 μM does not further increase 11-*cis*-retinol formation. It should be noted that under all conditions the binding protein is in excess over the 11-*cis*-retinol formed. The presence of 0.1 μM CRALBP (10 pmol/sample) leads to the formation of 292 pmol of 11-*cis*-retinol/mg of protein (SD = 63.4, n = 3), which equals 1.46 pmol/sample, and a ratio of binding protein:11-*cis*-retinol = 6.8. This value increases with increasing CRALBP concentration.

Activity of Isomerohydrolase Is Inhibited by Product Formation. The experiments described above show that in vitro 11-*cis*-retinoid synthesis is stimulated by the presence of a retinoid binding protein. One possible mechanism for this stimulation involves the retinol binding proteins binding to and removing an endogenous inhibitor of the isomerohydrolase. However, membranes washed three times (before or after UV illumination) by sedimentation in 2 mL of buffered 10% BSA solutions, 1 or 5 M NaCl, or 1 or 5 M

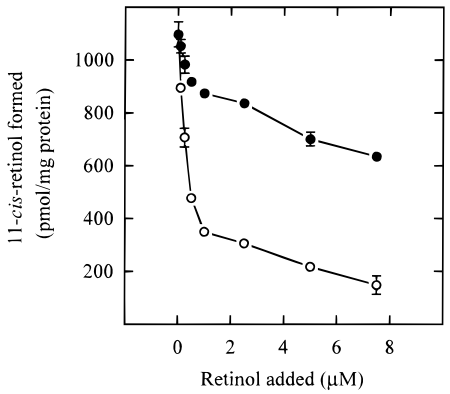

FIGURE 5: Inhibition of isomerohydrolase activity in the presence of 11-*cis*-retinol or 13-*cis*-retinol. Samples were prepared for measuring 11-*cis*-retinol formation as described in Materials and Methods. The samples were incubated at 37 °C for 2 h (100 mM Tris/HCl, pH 8.0, 100 μL total volume, 5% BSA) with nonradioactive 11-*cis*-retinol (○), or 13-*cis*-retinol (●), respectively, added to end concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, or 7.5 μM. Each measured point represents the results of duplicate determinations. Where no error bars are given, the error bars were smaller than the symbol used. The membranes contained 6.0 nmol of total retinoids/mg of protein (SD = 0.49, n = 35).

urea, respectively) show the same rate of 11-*cis*-retinol formation as untreated controls (Winston and Rando, unpublished results). Therefore, the removal of a potential endogenous inhibitor does not seem to play a role in 11-*cis*-retinol biosynthesis. A likely mechanism for the stimulatory role of BSA involves the partitioning of 11-*cis*-retinol from the membranes, where it could potentially inhibit the membrane-bound isomerohydrolase, into the soluble fraction.

To further explore the potential inhibition of isomerohydrolase by 11-*cis*-retinol, the formation of 11-*cis*-retinol was measured in the presence of either nonradioactive 11-*cis*-retinol or nonradioactive 13-*cis*-retinol. Isomerohydrolase is specific for *all-trans*-retinyl ester and 11-*cis*-retinol as substrates (*36*) and is, therefore, not expected to have high affinity for 13-*cis*-retinol. As depicted in Figure 5, 11-*cis*-retinol is a significantly better inhibitor of isomerohydrolase than its 13-*cis* congener. The formation of [11,12-$^3H_2$]-11-*cis*-retinol in the presence of cold 11-*cis*-retinol is significantly ($p < 0.05$) lower than in the presence of 13-*cis*-retinol. 13-*cis*-Retinol is therefore not an effective inhibitor of isomerohydrolase. With 11-*cis*-retinol, isomerohydrolase activity reaches half of its maximum value (1096 pmol/mg of protein, SD = 46.9, n = 2) at about 400 nM added cold 11-*cis*-retinol. This low IC_{50} value indicates that 11-*cis*-retinol may indeed act as a specific inhibitor of isomerohydrolase.

DISCUSSION

In vertebrate eyes, the rate of dark adaptation after a strong bleach (photochemical adaptation) is limited by the relatively slow regeneration of opsin molecules with 11-*cis*-retinal to form rhodopsin (*22*). Full dark adaptation requires about 1 h in human and bovine eyes (*37*). The rate-limiting step(s) in the visual cycle has not yet been identified, but the sluggish rate of 11-*cis*-retinoid biosynthesis in vitro suggests that the isomerohydrolase should be considered (*8, 19*). 11-*cis*-Retinoid formation had previously been shown to proceed in vitro with a maximum rate of 1.3 pmol of 11-*cis*-retinol formed min^{-1} (mg of protein)$^{-1}$ (*29*) or 0.4 nmol h^{-1} (mg of protein)$^{-1}$ (= 6.7 pmol/min) (*30*) for bovine eyes. At the end of the in vitro reaction, 11-*cis*-retinol accounted for about 10−15% of total retinoids (*29*). These rates would imply a rate-limiting role for the isomerohydrolase in the visual cycle. However, here we show that retinoid binding proteins dramatically stimulate the rate of 11-*cis*-retinol formation. The reaction proceeds with maximum rate of about 44.3 pmol min^{-1} (mg of protein)$^{-1}$, which is 6.7−34-fold higher than previously measured rates (*29, 30*). After 2 h, the relative amount of 11-*cis*-retinol is around 32% of total retinoids. The finding that isomerohydrolase is not particularly sluggish in vitro shows that the isomerohydrolase reaction may not be a rate-limiting step in the visual cycle. This would be consistent with other reports suggesting that the slowest step in the overall visual cycle may be in the processing of *all-trans*-retinol in the photoreceptors (*38, 39*). As discussed in greater detail below, the reason for an accumulation of *trans*-retinyl ester in the RPE in the dark-adapted eye is most likely not a slow isomerohydrolase reaction per se but rather due to inhibition of isomerohydrolase under conditions where the reaction product is not being removed.

It should be mentioned that measurements of the activity of isomerohydrolase were performed at retinoid concentrations that approximate those found under physiological conditions. In bovine eyes, a net amount of 3−8 nmol of retinoids was found to be shifted between RPE and photoreceptors under varying light conditions (*25*). The net formation of 11-*cis*-retinal is reported to be 4.2 nmol/eye (*40*). In the two sets of experiments conducted for determining the time course of 11-*cis*-retinol formation (see Figure 1), we measured retinoid amounts of 3.1 and 6.8 nmol/mg of protein, respectively. This equals amounts per eye of 0.70 and 1.53 nmol. The physiologically relevant values are expected, however, to be substantially higher, since a substantial amount of RPE microsomal membranes are lost during the preparation procedure.

In light of the results presented here, it is interesting to consider the role of soluble retinoid binding proteins in the operation of the visual cycle. It has been suggested that soluble retinoid binding proteins might play an important role in the regulation of the visual cycle (*41, 35*). These proteins are generally responsible for the binding, stabilization, and transport of retinols and retinals, as these small organic molecules dissolve only poorly in aqueous solutions, and are susceptible to oxidative degradation (*42, 43*). Carlson and Bok (*41*) demonstrated that interphotoreceptor retinoid binding protein (IRPB) promotes the formation of 11-*cis*-retinal from cultured retinal pigment epithelium cells. In the eye, IRBP mediates the transport of *all-trans*-retinol and 11-*cis*-retinal between photoreceptors and RPE (*44, 45*). In the cytosol of RPE cells, cellular retinoid binding protein (CRALBP), which is a binding protein with high affinity for 11-*cis*-retinal and 11-*cis*-retinol, has been suggested to have a substrate-directing role in the visual cycle (*35*). The presence of CRALBP led to a 10-fold increase in 11-*cis*-retinol oxidation to 11-*cis*-retinal. In the absence of CRALBP, 11-*cis*-retinol was readily esterified to 11-*cis*-retinyl ester. On the basis of these findings, Saari et al. have produced a model for the regulation of the visual cycle in which 11-*cis*-retinol is found at a critical branch in the visual cycle. In this model, the amount of CRALBP in the RPE is critical

for determining whether 11-*cis*-retinol is esterified (in the absence of CRALBP) or oxidized (in the presence of CRALBP).

Our results suggest, however, that CRALBP also plays a crucial role one step upstream in the visual cycle by regulating isomerohydrolase activity either directly or indirectly. In the studies reported here, the fate of retinoids is followed in two subsequent steps in the visual cycle; namely, the esterification of *all-trans*-retinol and the subsequent cleavage of the ester bond and isomerization of the *trans*-retinoid into the 11-*cis* form. In the RPE membrane system reported here, the potential influence of soluble retinoid binding proteins on the *de novo* formation of 11-*cis*-retinoids can be investigated. In the absence of soluble retinoid binding proteins only small amounts of 11-*cis*-retinol and 11-*cis*-retinyl esters are formed. Upon addition of CRALBP (or BSA), the amount of 11-*cis*-retinol increases drastically, while almost no 11-*cis*-retinyl esters are produced. Thus, the addition of retinol binding proteins to the medium appears to stimulate the *de novo* synthesis of 11-*cis*-retinoids rather than to shift the equilibrium between esterification to 11-*cis*-retinyl ester and solubilization as 11-*cis*-retinol.

A number of possible mechanisms could account for this phenomenon. Retinoid binding proteins might stimulate 11-*cis*-retinoid biosynthesis by activating visual cycle enzymes. This is unlikely given the lack of specificity with respect to binding protein type. Another potential mechanism for the stimulation of 11-*cis* formation is the release of isomerohydrolase inhibition by inhibitor removal. As thorough prior washing of membranes with BSA did not stimulate 11-*cis*-retinol production, it is unlikely that endogenous isomerohydrolase inhibitors are important. Rather, the activity of isomerohydrolase appears to depend more on the concentration of membrane-bound 11-*cis*-retinoids. The fact that 11-*cis*-retinol is apparently removed from membrane by binding to a soluble retinoid binding protein (CRALBP or BSA) suggests that it might possibly be a product inhibitor of the isomerohydrolase, leading to inhibition of the visual cycle. Studies reported here show that added 11-*cis*-retinol has a strong inhibitory effect on isomerohydrolase at nanomolar concentrations, while the effect of added 13-*cis*-retinol is significantly weaker.

These findings suggest a model for the regulation of the visual cycle as depicted in Figure 6. Here, the activity of isomerohydrolase depends on the concentration of 11-*cis*-retinoids in the membrane. When 11-*cis*-retinol is not removed by binding to a soluble retinoid binding protein (CRALBP), it accumulates in the membrane, leading to product inhibition of isomerohydrolase and cessation of visual cycle activity. Under conditions that require constant regeneration of 11-*cis*-retinoids following photoreceptor bleaching, 11-*cis*-retinal is transported from the RPE to photoreceptor, making apo-CRALBP molecules available for the binding of 11-*cis*-retinol. The removal of 11-*cis*-retinol from membranes by binding to CRALBP releases the membrane-bound isomerohydrolase from inhibition. 11-*cis*-Retinol bound to CRALBP is readily oxidized and exported, leaving apo-CRALBP molecules behind that can bind 11-*cis*-retinol. Alternatively, 11-*cis*-retinyl ester may be an inhibitor of isomerohydrolase. Since LRAT is a freely reversible enzyme (*46*), the binding of 11-*cis*-retinol to a binding protein will have the effect of decreasing the levels

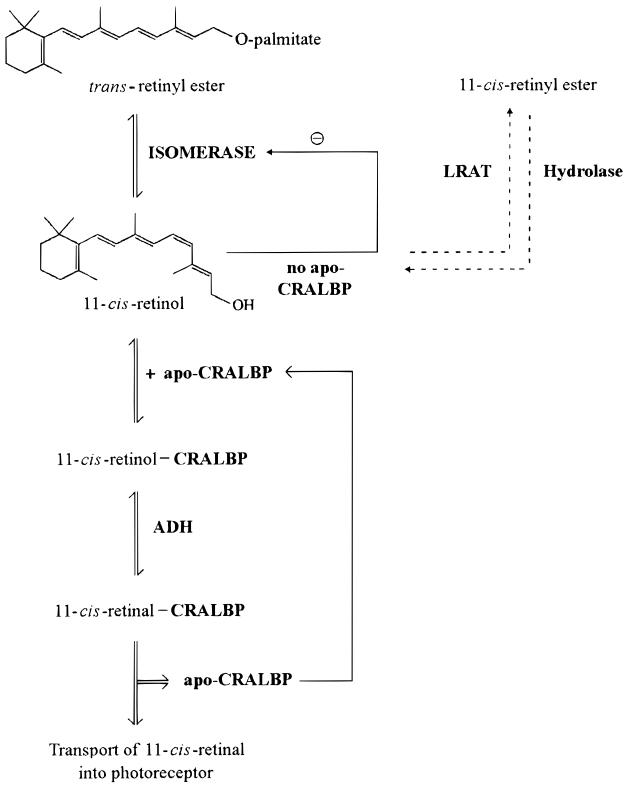

FIGURE 6: Model for the regulation of the visual cycle byproduct inhibition of isomerohydrolase. In retinal pigment epithelium, *trans*-retinyl ester located in the microsomal membranes is the substrate for the formation of 11-*cis*-retinol. Under bleaching conditions that require the constant biosynthesis of 11-*cis*-retinal, the 11-*cis*-retinol formed is sequestered into the cytosol by binding to apo-CRALBP. CRALBP-bound 11-*cis*-retinol is substrate for an alcohol dehydrogenase (ADH), which catalyzes oxidation to 11-*cis*-retinal, and the latter is then transported back into the photoreceptor layer. After longer periods of dark adaptation, when rhodopsin is fully regenerated, 11-*cis*-retinal bound to CRALBP builds up in the RPE. There is no more apo-CRALBP available for binding of 11-*cis*-retinol. Under these conditions, 11-*cis*-retinol formation leads to product inhibition of the isomerohydrolase. The slow buildup of 11-*cis*-retinyl ester can be explained as a slow constitutive component of 11-*cis*-retinol formation. As retinols are toxic to membranes, the 11-*cis*-retinol formed under these conditions is readily esterified by LRAT.

of 11-*cis*-retinyl ester. At this point, it is not possible to immediately decide whether 11-*cis*-retinol and/or its ester are inhibitors of isomerohydrolase. It is difficult to test the possibility that long-chain 11-*cis*-retinyl esters inhibit the isomerohydrolase, since they cannot simply be added to a membrane solution because of their insolubility in water.

Interestingly, the stimulating effect of CRALBP on isomerohydrolase activity can be mimicked in vitro by BSA, suggesting that specific protein−protein interactions are unlikely to be involved in the stimulation of 11-*cis*-retinol formation. BSA binds retinol and retinal with low affinity (*47*) and is usually used to introduce these organic compounds into buffer-based assays (*13*, *28*). As BSA is not the natural binding protein for 11-*cis*-retinoids in the RPE, it is not likely to possess a specific binding site for any of the enzymes involved in the visual cycle. A similar lack of specific protein−protein interaction has been reported by Saari et al. (*35*) for LRAT and CRALBP.

The studies presented here suggest that the dark buildup of 11-*cis*-retinoids in the RPE self-inhibits further isomer-

ization. It has been shown, however, that 11-*cis*-retinyl esters build up in amphibian RPE membranes in vivo and in vitro in the dark, but they never reach more than approximately 50% of the retinyl ester pool (*24*, *48*, *20*). Also, the formation of 11-*cis*-retinyl esters has been found to be very sluggish. In frog eyes for example, it takes 24 h for the 11-*cis* form to reach 50% of the total ester pool (*24*). This would be expected if product inhibition occurs. Upon photoreceptor bleaching, the 11-*cis*-retinyl esters formed in the dark are processed into 11-*cis*-retinol(al); 11-*cis*-retinyl esters are, however, not used preferentially over *trans*-retinyl esters for rhodopsin regeneration (*24*).

It will be of further interest to determine whether other regulatory elements exist in the visual cycle. Potential candidates to be tested involve posttranslational modifications such as phosphorylation or second messengers such as, for example, cAMP, cGMP, Ca^{2+}, or inositol phosphates, that are known to play regulatory roles in a variety of physiological processes. Using the assay system developed here, it should be possible to test whether elements of the visual cycle are sensitive to various physiological stimuli.

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