# Isomerization of *all-trans*-9- and 13-Desmethylretinol by Retinal Pigment Epithelial Cells†

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*Received June 10, 1999; Revised Manuscript Received August 10, 1999*

ABSTRACT: Photoisomerization of 11-*cis*-retinal to *all-trans*-retinal triggers phototransduction in the retinal photoreceptor cells and causes ultimately the sensation of vision. 11-*cis*-Retinal is enzymatically regenerated through a complex set of reactions in adjacent retinal pigment epithelial cells (RPE). In this study using *all-trans*-9-desmethylretinol (lacking the $C_{19}$ methyl group) and *all-trans*-13-desmethylretinol (lacking the $C_{20}$ methyl group), we explored the effects of $C_{19}$ and $C_{20}$ methyl group removals on isomerization of these retinols in RPE microsomes. The $C_{19}$ methyl group may be involved in the substrate activation, whereas the $C_{20}$ methyl group causes steric hindrance with a proton in position $C_{10}$ of 11-*cis*-retinol; thus, removal of this group could accelerate isomerization. We found that *all-trans*-9-desmethylretinol and *all-trans*-13-desmethylretinol are isomerized to their corresponding 11-*cis*-alcohols, although with lower efficiencies than isomerization of *all-trans*-retinol to 11-*cis*-retinol. These findings make the mechanism of isomerization through the $C_{19}$ methyl group unlikely, because in the case of 9-desmethylretinol, the isomerization would have to progress by proton abstraction from electron-rich olefinic $C_9$. The differences between *all-trans*-retinol, *all-trans*-9-desmethylretinol, and *all-trans*-13-desmethylretinol appear to be a consequence of the enzymatic properties, and binding affinities of the isomerization system, rather than differences in the chemical or thermodynamic properties of these compounds. This observation is also supported by quantum chemical calculations. It appears that both methyl groups are not essential for the isomerization reaction and are not likely involved in formation of a transition stage during the isomerization process.

Our vision has evolved to operate in a broad range of illumination able to detect light intensities from a few photons to as many as $10^7$ photons per second. With each absorbed photon, photoisomerization of one 11-*cis*-retinal molecule to *all-trans*-retinal occurs with ∼0.6 quantum efficiency (*1*). 11-*cis*-Retinal is coupled via a Schiff base to a Lys residue ($^{296}$Lys in bovine rhodopsin) located within the transmembrane-spanning portion of the rod and cone photoreceptor opsins. Photoisomerization triggers conformational changes in these receptors that lead to activation of G-proteins and a subsequent cascade of reactions, which comprise phototransduction (*2*). During daytime light settings, photoisomerization occurs continuously with large amounts of *all-trans*-retinal generated. However, at a given moment, only a fraction of cone and rod pigments are bleached, because photoisomerization is countered by a set of enzymatic reactions (the visual cycle) in rods/cones where *all-trans*-retinal is reduced to *all-trans*-retinol, and in adjacent retinal pigment epithelial cells (RPE)[1] where *all-trans*-retinol is converted to 11-*cis*-retinal. The time constant for the entire visual cycle in humans is 400 s for rhodopsin in rods (*3*), occurs 4 times faster in cones (*4*), and is independent of bleach levels.

Understanding of the fundamental processes of vision requires that the visual cycle be delineated at the chemical level. Furthermore, mutations in any of the genes involved in retinoid transformations could result in retinal dystrophies and degeneration of photoreceptors. Thus, it is important to understand this metabolic pathway in order to provide molecular insights into human diseases, including genetic diseases and age-related macular degeneration.

The flow of retinoids in the visual cycle has been investigated with considerable detail for the rodent model in vivo (*5*). After an intense bleach, no free retinals accumulate; *all-trans*-retinal either is complexed with opsin in photoreceptor cells, or is reduced, and ultimately esterified with fatty acids in RPE. This reaction is followed by steady

†This research was supported by NIH Grants EY08061 (to K.P.) and EY04939 (to R.K.C.), by grants from the Ruth and Milton Steinbach Fund, and from the E. K. Bishop Foundation, and by an RPB award to the Departments of Ophthalmology at the University of Washington and the Medical University of South Carolina.

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[1] Abbreviations: apo-rCRALBP, recombinant apo-cellular retinaldehyde-binding protein; BSA, bovine serum albumin; BTP, 1,3-bis-[tris(hydroxymethyl)methylamino]propane; CTP, cytidine 5′-triphosphate; LRAT, lecithin:retinol acyltransferase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RPE, retinal pigment epithelium.

10.1021/bi9913294 CCC: $18.00 
Published on Web 09/21/1999

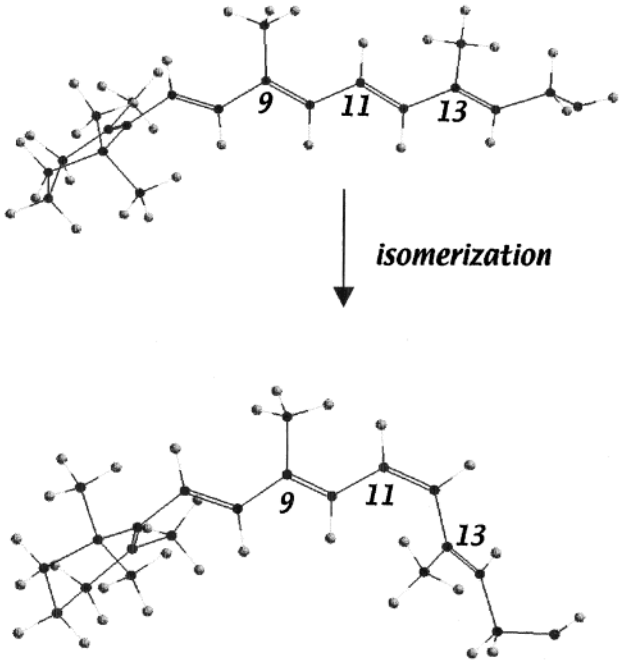


FIGURE 1: Isomerization of *all-trans*-retinol to 11-*cis*-retinol. The model of *all-trans*-retinol and 11-*cis*-retinol was built using SPARTAN PROGRAM from Wavefunction, Inc.

production of 11-*cis*-retinal which regenerates rhodopsin and cone pigments. These studies indicate that 11-*cis*-retinal is likely produced on demand, generating an 11-cis isomer once *all-trans*-retinol is cleared from photoreceptors.

The key reaction of the visual cycle is isomerization of *all-trans*-retinol to 11-*cis*-retinol (Figure 1). It has been proposed that isomerization occurs enzymatically without an exogenous source of energy (*6*, *7*), where hydrolysis of *all-trans*-retinyl carboxylic ester provides the 4 kcal/mol energy needed for isomerization (*8*, *9*). It was concluded that retinyl esters are essential intermediates in 11-*cis*-retinol formation (*10*). The proposed mechanism involves double bond migration and expulsion of the carboxylate during addition of a nucleophilic group to $C_{11}$. Rotation about the $C_{11}-C_{12}$ single bond is followed by attack of water at $C_{15}$ and concomitant reshuffling of the double bonds, which locks the retinol in the 11-cis configuration (*8*). Alternatively, the $C_{19}$ methyl group could be important during isomerization, for example, for donating the proton to allow double bond reshuffling (*11*). The possibility of a proton abstraction from the $C_{19}$ methyl group of *all-trans*-retinyl ester was further supported by lack of isomerization of *all-trans*-9-desmethylretinol, whereas *all-trans*-13-desmethylretinol was a good substrate for isomerization (*11*). Against this idea, however, was a lack of deuterium isotope effects during isomerization (*11*). More recently, Winston and Rando (*12*) proposed that BSA or apo-rCRALBP enhanced isomerization, due to removal of 11-*cis*-retinol (relieve from the product inhibition).

Recently, we proposed that another mechanism should be considered as well (*13*). Isomerization might proceed through an unidentified retinoid intermediate (or a specific subpopulation of retinyl esters), and apo-retinoid-binding proteins appear to play a key role in the isomerization processes. The retinoid-binding proteins may overcome the thermodynamically unfavorable isomerization and drive the reaction by removing the 11-cis product. The consequence of isomerization proceeding only in the presence of the acceptor protein would be that an 11-*cis*-retinol/11-*cis*-retinal pool does not accumulate in RPE because they are formed on demand.

Here, we showed that the optimal isomerization condition requires the presence of not only apo-rCRALBP but also BSA in vitro. Isomerization of *all-trans*-retinol to the 11-cis isomer would cause the methyl group in the $C_{13}$ position ($C_{20}$ methyl) to be pushed toward a proton at $C_{10}$ (Figure 1). Because it is a prohibited interaction, the polyene chain becomes twisted (*14*). Thus, the removal of the $C_{20}$ methyl group could produce a condition for more rapid isomerization. Moreover, it was unclear why the removal of $C_{19}$ ceased isomerization (*11*). With this improved assay, we asked whether *all-trans*-9-desmethyl- and *all-trans*-13-desmethylretinol are substrates for isomerization and how they compare with physiologically relevant *all-trans*-retinol. We found that both *all-trans*-9-desmethyl- and *all-trans*-13-desmethylretinol are substrates for isomerization, but they are less efficiently converted to corresponding 11-*cis*-alcohols than *all-trans*-retinol. These results eliminate the possibility that the $C_{19}$ methyl group is essential for isomerization.

## MATERIALS AND METHODS

### *Materials*

Fresh bovine eyes were obtained from a local slaughterhouse (Schenk Packing Co., Inc., Stanwood, WA). Apo-recombinant CRALBP (apo-rCRALBP) was expressed in *E. coli* and purified by $Ni^{2+}$-affinity chromatography (*15*). Delipidated BSA was purchased from Sigma.

### *Methods*

*RPE Microsomes.* The microsomal fraction was isolated from fresh bovine RPE (*16*) and resuspended in 10 mM MOPS, pH 7.0, containing 1 $\mu$M leupeptin and 1 mM dithiothreitol (DTT). The total protein concentration was 3.2 mg/mL as determined using the Bradford method (*17*). Samples were stored for up to 3 months in small aliquots at $-80$ °C.

*UV Treatment.* To destroy endogenous retinoids, RPE microsomes (200 $\mu$L aliquots) were irradiated in a quartz cuvette for 5 min at 0 °C using a ChromatoUVE-transilluminator (model TM-15 from UVP, Inc.) (*8*, *13*).

*Synthesis of 9- and 13-Desmethylretinols.* All experiments with the retinol analogues were performed under dim red light. The desmethylretinols (all-trans isomers) were synthesized using the general methods previously reported (*18*). Diethyl(3-cyano-2-methylprop-2-enyl)phosphonate was prepared from 1-chloro-3-cyano-2-methylpropene by heating with triethyl phosphite. Sodium bis(dimethylsilyl)amide (Aldrich) in dry tetrahydrofuran was used as the base, and diisobutylaluminum hydride (Aldrich) was used as the reducing agent. The products were purified by TLC (5% ethyl acetate/hexane) and HPLC (Varian 5000 gradient system, Altech Econosphere Silica SU column, 6 $\times$ 250 mm, 2.0% ethyl acetate/0.1% methanol/97.9% hexane). *all-trans*-9-Desmethylretinal and *all-trans*-13-desmethylretinal were characterized by NMR employing a Varian VXR400 spectrometer and deuterated chloroform as solvent. The analogues were stored at $-70$ °C under argon. Due to instability of

products, experiments were performed within 2 weeks of synthesis.

11-*cis*-Retinal and its desmethyl analogues were isolated from the photolyzed sample of corresponding *all-trans*-retinals (*18*). The 11-*cis*-alcohols were obtained by reducing the 11-*cis*-retinals with $NaBH_4$ (*19*).

*Retinoid Preparations.* To prevent isomerization and oxidation, all procedures involving retinoids were performed under dim red light, and the retinoids were stored under argon at −80 °C. *all-trans*-Retinol, *all-trans*-9-desmethylretinol, and *all-trans*-13-desmethylretinol were purified on a normal phase HPLC column (Altex, Ultrasphere-Si 5u, 4.6 × 250 mm, flow rate 1.4 mL/min, 10% ethyl acetate in hexane) (*20*). Purified material was dried under argon and stored in vials (0.5 nmol aliquots at −80 °C) for up to 3 months. Retinoid concentrations in 10% ethyl acetate/90% hexane were determined spectrophotometrically: 11-*cis*-retinol, 318 nm, $\epsilon = 34\,320\ M^{-1}\ cm^{-1}$; 11-*cis*-9-desmethylretinol, 320 nm, $\epsilon = 34\,320\ M^{-1}\ cm^{-1}$; 11-*cis*-13-desmethylretinol, 324 nm, $\epsilon = 34\,320\ M^{-1}\ cm^{-1}$; *all-trans*-retinol, 325 nm, $\epsilon = 51\,770\ M^{-1}\ cm^{-1}$; *all-trans*-9-desmethylretinol, 320 nm, $\epsilon = 51\,770\ M^{-1}\ cm^{-1}$; *all-trans*-13-desmethylretinol, 325 nm, $\epsilon = 51\,770\ M^{-1}\ cm^{-1}$.

*Reaction Conditions for Isomerase and LRAT.* Substrates [*all-trans*-retinol, *all-trans*-9-desmethylretinol, or *all-trans*-13-desmethylretinol (0.5 nmol)] were dried from ethyl acetate/hexane under argon in a 1.5 mL polypropylene tube. To the dried substrate were added 20 μL of BSA (in 10 mM BTP, pH 7.0, to give a final concentration of 1%) and 20−30 μL of apo-rCRALBP (in 10 mM BTP, pH 7.5, containing 250 mM NaCl, to give a final concentration of 25 μM). Next, 10 mM BTP, pH 7.0, containing 1 mM CTP was added to bring the total volume to 175 μL. For some experiments, additional compounds in the same buffer were added. Finally, 25 μL of RPE microsomes (80 μg of protein) was added to this mixture, and the reactions were incubated at 37 °C for the indicated times.

*HPLC Analysis of Retinoids.* The reaction mixture (180 μL out of 200 μL) was transferred to a new vial containing 300 μL of ice-cold methanol, and 300 μL of hexane was added. In some cases, to isolate 11-*cis*-retinols bound to rCRALBP, the reaction mixture was spun down at 175000*g* for 30 min at 4 °C. The supernatant (180 μL of 200 μL) was transferred to a new vial containing 300 μL of ice-cold methanol, and 300 μL of hexane was added as described previously. In both cases, the sample was vortexed for 2 min and centrifuged for 4 min at 14000*g* at 4 °C to separate organic and aqueous phases. Aliquots of hexane extract (typically 50 μL) were injected into the HPLC column. Retinoids were separated using an HP1100 HPLC (with a diode-array detector 280−400 nm) and a normal phase, narrow-bore column (Alltech, Silica 5 μm Solvent Miser, 2.1 × 250 mm). An isocratic solvent composed of 10% ethyl acetate in hexane at a flow rate of 0.3 mL/min was used.

*Hydrolysis of Retinyl Esters.* The HPLC-purified retinyl ester fractions (typically 200 μL) were dried under argon, and hydrolyzed in 230 μL of ethanol and 20 μL of 6 M KOH. The sample was incubated for 30 min at 55 °C, diluted with 100 μL of water, chilled on ice for 2 min, and extracted with 300 μL of hexane. The retinoids in hexane were analyzed directly by HPLC. The data are presented with standard deviation (SD).

*Binding Assay with Apo-rCRALBP.* Freshly purified 11-*cis*-retinol, 11-*cis*-9-desmethylretinol, and 11-*cis*-13-desmethylretinol (16 nmol of each) were dried under argon in 1.5 mL polypropylene tubes. The dry retinols were each solubilized with 2 μL of ethanol and 50 μL of 10 mM BTP, pH 7.0, containing 250 mM NaCl, and 16 nmol of apo-rCRALBP. The sample was incubated for 12 h at 4 °C, and unbound retinols were separated from rCRALBP on DEAE-cellulose columns according to the following procedure: one-third of the volume of a Pasteur pipet was filled with DEAE-cellulose and equilibrated with 10 mM BTP, pH 7.0. Next, the CRALBP/retinoid mixtures were loaded and washed with 5 mL of 10 mM BTP, pH 7.5, to purge unbound retinols. The rCRALBP/retinoid complex was eluted with 0.5 M NaCl in 10 mM BTP, pH 7.5, and the UV spectra were recorded using an HP 8452A Diode Array Spectrophotometer.

*Quantum-Chemical Calculations.* Quantum-chemical calculations were performed using the Gaussian-94 suite of programs (Gaussian, Inc., Pittsburgh, PA). Three methods were used: (i) the semiempirical AM1 method for a general purpose quantum-mechanical molecular model; this method is well tested and is currently widely used to study conformational changes in hydrocarbons and their derivatives (*21*); (ii) ab initio calculations carried out within the Hartree−Fock approach; and (iii) a recently developed hybrid Hartree−Fock/Density−Functional method, B3LYP (*22*). The 6−31 g* basis set was used with both ab initio methods.

Energies of three conformations of each retinol molecule were obtained using full geometry optimization. The only fixed geometric parameter was the torsion angle formed by $C_{10}$, $C_{11}$, $C_{12}$, and $C_{13}$. The angle was set equal to 0° for the *cis*-retinols, 180° for the *trans*-retinols, and 90° for the transition states. The reference zero energy was taken to be the energy of the trans isomer in all cases.

## RESULTS

*Influence of BSA and Apo-rCRALBP on Isomerization.* RPE microsomes used in these studies were isolated from bovine eyes using a standard sucrose differential centrifugation procedure (*16*) and characterized for their ability to isomerize *all-trans*-retinol, *all-trans*-9-desmethylretinol, and *all-trans*-13-desmethylretinol. Recombinant apo-CRALBP, expressed in *E. coli* and purified to homogeneity (*15*), and delipidated BSA were used in the isomerization assay. The assay was previously described (*13*, *23*). Briefly, retinoids were extracted with hexane, polar retinoids were extracted at 75−95% yield, as determined using [$^3$H]retinol tracers, and nonpolar retinyl esters were extracted in ∼60% yield (*13*). Extracted retinoids were separated on a normal phase silica column under isocratic conditions (for example, see Figure 2). *all-trans*-Retinyl esters and 11-*cis*-retinyl esters (peak 1) were eluted 0.5 min after the solvent front, followed by 11-*cis*-retinol (peak 2) and *all-trans*-retinol (peak 3), all with a chromatographic yield of >95%. 9-*cis*-Retinol eluted ∼1 min earlier than *all-trans*-retinol, and 13-*cis*-retinol eluted ∼1 min after the 11-*cis*-retinol peak (not shown). Because the analysis of retinoid conversions in RPE microsomes is complicated by the presence of endogenous retinoids, RPE microsomes were exposed to UV irradiation for 5 min to destroy all of the endogenous retinoids (*8*, *12*, *13*). In the presence of 1% BSA and exogenous 0.5 nmol *all-trans*-

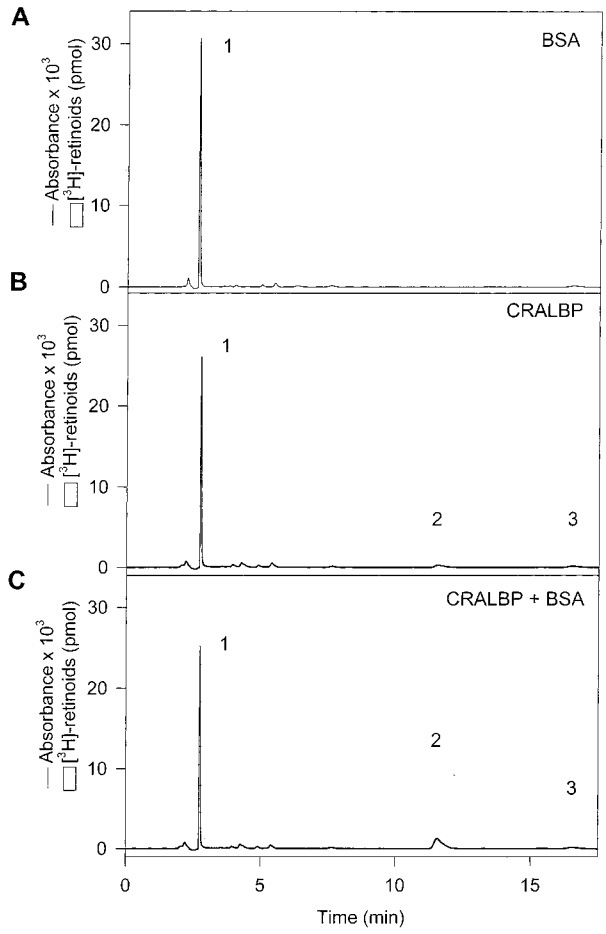


FIGURE 2: Influence of BSA and apo-rCRALBP on the formation of 11-*cis*-retinol. (A) UV-treated RPE microsomes (80 $\mu$g of protein in 200 $\mu$L) were incubated with 2.5 $\mu$M [$^3$H]-*all-trans*-retinol (550 000 dpm/nmol) for 90 min at 37 °C in 10 mM BTP, pH 7.0, containing 1 mM CTP and 1% BSA. After the reaction was quenched with methanol, retinoids were extracted into hexane, and one-sixth of the extract was analyzed by HPLC. (B) RPE microsomes were incubated with 25 $\mu$M apo-rCRALBP and without BSA. (C) RPE microsomes were incubated with 25 $\mu$M apo-rCRALBP and 1% BSA. HPLC fractions were collected, and radioactivity was counted using a Beckman LS 3801. Peak 1, *all-trans*-retinyl ester; peak 2, 11-*cis*-retinol; and peak 3, *all-trans*-retinol. Retinoids were detected at 325 nm.

retinol, but without apo-rCRALBP, formation of 11-*cis*-retinol was low (at the detection limit level) (Figure 2A), even though formation of retinyl esters was unaffected as compared to a sample without BSA (data not shown). In the presence of 25 $\mu$M apo-rCRALBP, however, 11-*cis*-retinol was readily formed (1.8 pmol, peak 2), while addition of 1% BSA and 25 $\mu$M apo-rCRALBP to the reaction mixture synergistically promoted isomerization (6.6 pmol) (Figure 2C). In the reconstitution assays, it appears that these two proteins are necessary to monitor efficient conversion of *all-trans*-retinol to 11-*cis*-retinol. A combination of BSA and apo-rCRALBP was used in further described assays.

*Isomerization of all-trans-9-Desmethylretinol and all-trans-13-Desmethylretinol.* 11-*cis*-Alcohols of 9-desmethyl- or 13-desmethylretinol were formed when the corresponding *all-trans*-alcohols were added to RPE microsomes in the presence of BSA and apo-rCRALBP (Figure 3, peaks 2). Formation of the corresponding retinyl esters did not differ significantly between these substrates. However, it appears that higher amounts of free *all-trans*-9-desmethylretinol and *all-trans*-13-desmethylretinol remained (or is hydrolyzed from corresponding esters) in the reaction mixture than in the control experiment when the substrate was *all-trans*-retinol. This effect likely resulted from more favorable competition of apo-rCRALBP for binding of *all-trans*-desmethyl alcohols and hydrolysis of desmethylretinyl esters as opposed to esterification by LRAT (Figure 3).

To examine the possibility that 11-*cis*-alcohols were formed and then converted to the corresponding 11-*cis*-retinyl esters, the HPLC ester fraction was collected and saponified. Analysis of retinols revealed the presence of only *all-trans*-retinol (Figure 3, insets), suggesting that 11-*cis*-alcohols were not re-esterified.

*Partition of Retinoids in the Isomerization Mixture.* To identify whether retinoids were bound to membranes or to soluble BSA and CRALBP, the reaction mixture was ultracentrifuged and separated to membrane and soluble fractions. 11-*cis*- and *all-trans*-alcohols were bound to soluble apo-rCRALBP/BSA as determined after pelleting the RPE microsomes and analyzing the retinol content of the membrane and supernatant phases. The 11-*cis*-alcohols were identified by their coelution with authentic synthetic standards, and by the UV spectra of the product eluted from the column (data not shown). These spectra were identical to the spectra of synthetic standards. These data, and the analysis of the ester pools (Figure 3), suggest that after isomerization, the product was transferred to solution by the binding proteins.

*all-trans-Retinol Is Optimal for Isomerization.* To follow the fate of exogenous *all-trans*-alcohols, washed RPE microsomes (80 $\mu$g of protein) were mixed with 0.5 nmol of *all-trans*-retinol, *all-trans*-9-desmethylretinol, or *all-trans*-13-desmethylretinol (Figure 3 and Figure 4). These retinoids were efficiently converted to *all-trans*-retinyl (or desmethyl) esters by endogenous LRAT (Figures 3 and 4). No detectable amounts of 11-*cis*-retinyl esters were formed during the reaction (Figure 3, insets). In the presence of apo-rCRALBP and 1% BSA, the average of three experiments showed formation of 9.5 pmol mg$^{-1}$ min$^{-1}$ of 11-*cis*-retinol, 1.3 pmol mg$^{-1}$ min$^{-1}$ of 11-*cis*-9-desmethylretinol, and 2.3 pmol mg$^{-1}$ min$^{-1}$ 11-*cis*-13-desmethylretinol (Figure 4). The isomerization reaction reached equilibrium at ~40 min (Figure 5). The relative initial rates of isomerization appear to be similar for all three substrates. As before for *all-trans*-retinol, no significant amounts of 11-cis isomers of *all-trans*-9-desmethylretinol or *all-trans*-13-desmethylretinol were formed without apo-rCRALBP (Figure 4).

The differences in isomerization levels between *all-trans*-retinol and its analogues could be due to lower affinity of CRALBP for 11-*cis*-desmethylretinols. To test this hypothesis, two sets of experiments were performed. First, the apo-rCRALBP titration showed that maximum production is achieved at a concentration <10 $\mu$M. This concentration is much lower than the 25 $\mu$M typically used in the assays (Figure 6A). Interestingly, the amounts of free *all-trans*-alcohols went up continuously with increased amounts of added binding protein (Figure 6B), suggesting that CRALBP, albeit with lower affinity, binds all-trans isomers. The esters were unaffected (data not shown).

Second, apo-rCRALBP was reconstituted with synthetic 11-*cis*-retinol, 11-*cis*-9-desmethylretinol, and 11-*cis*-13-desmethylretinol. The excess of free retinols was separated from

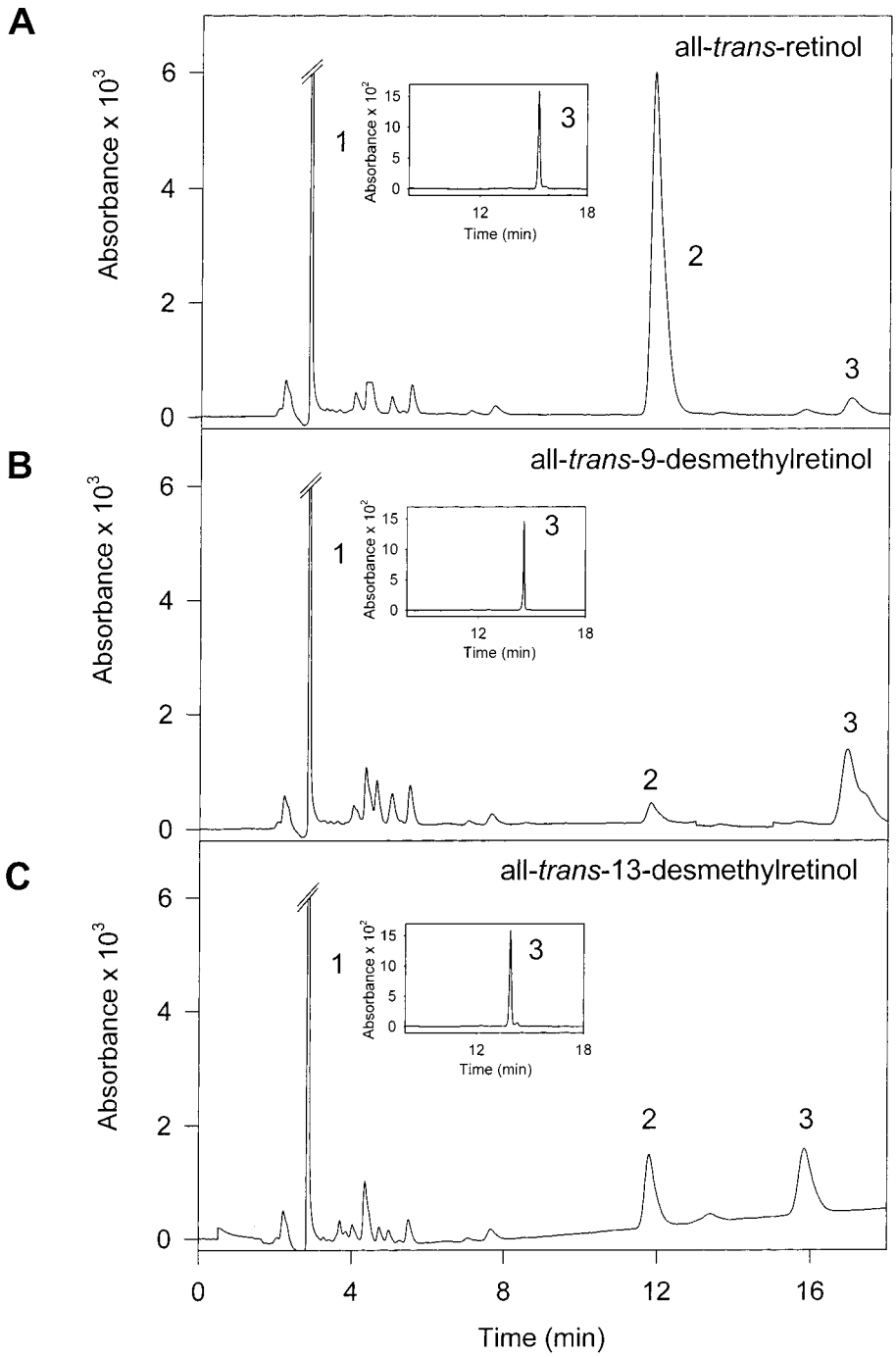


FIGURE 3: Conversion of *all-trans*-retinol and its 9-desmethyl- and 13-desmethyl analogues in UV-treated RPE microsomes. UV-treated RPE microsomes (80 $\mu$g of protein in 200 $\mu$L) were incubated with 2.5 $\mu$M *all-trans*-retinol (A), 2.5 $\mu$M *all-trans*-9-desmethylretinol (B), and 2.5 $\mu$M *all-trans*-13-desmethylretinol (C) for 90 min at 37 °C in 10 mM BTP, pH 7.0, containing 1 mM CTP, 25 $\mu$M apo-rCRALBP, and 1% BSA. After the reaction was quenched with methanol, retinoids were extracted into hexane, and one-sixth of the extract was analyzed by HPLC. The ester HPLC fractions of 3 different experiments were collected and saponified as described under Materials and Methods. The results of the ester saponification are shown in the insets. Peak 1, *all-trans*-retinyl ester; peak 2, 11-*cis*-retinol; and peak 3, *all-trans*-retinol and their desmethyl analogues. The unidentified peaks eluted between 3 and 6 min are products of decomposition of retinols. Retinoids were detected at 325 nm.

CRALBP on a DEAE-cellulose column (modified from ref *24*). 11-*cis*-13-Desmethylretinol bound readily to rCRALBP, as did 11-*cis*-retinol, and the ratios of protein (280 nm) and retinol absorption peaks (344 nm) were 1.1 and 1.3, respectively. However, 11-*cis*-9-desmethylretinol bound only ~60% as efficiently, with a 280 nm/344 nm ratio of 1.8 (Figure 7).

*11-cis-Retinol Dehydrogenase Does Not Enhance Isomerization.* To test if oxidation of 11-*cis*-alcohols to 11-*cis*-aldehydes affects conversion of *all-trans*-alcohols to 11-*cis*-alcohols, NAD/NADP, a cofactor of 11-*cis*-retinol dehydrogenase, was added [note that RPE microsomes contain endogenous 11-*cis*-retinol dehydrogenase (*25*)]. In the presence of apo-rCRALBP and NAD/NADP, efficient production of 11-*cis*-retinal was observed (as its oxime derivative, mixture of syn and anti isomers), while NAD/NADP alone had only a small effect (Table 1). It should be noted that CRALBP has a higher affinity for 11-*cis*-retinal than for 11-

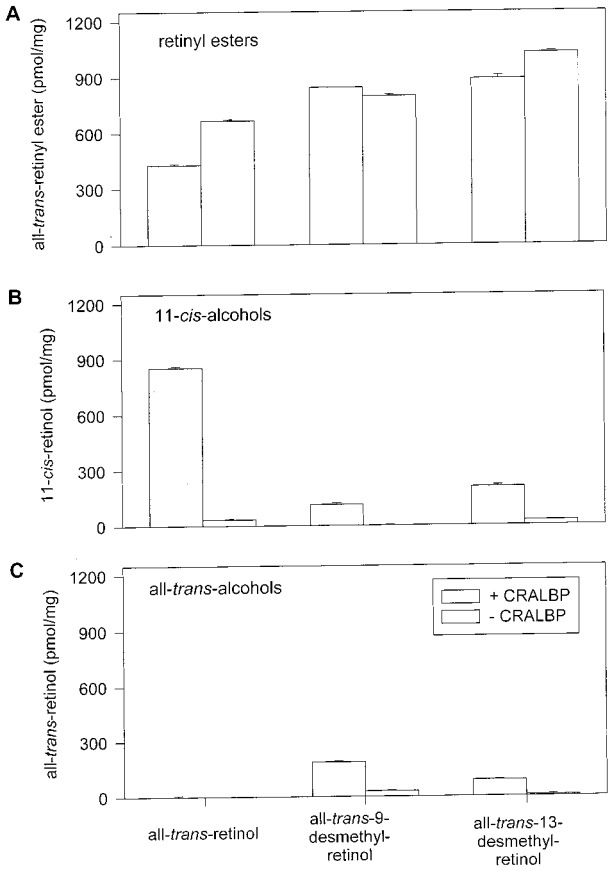


FIGURE 4: Enhanced isomerization of *all-trans*-retinol and its *all-trans*-9-desmethylretinol and *all-trans*-13-desmethylretinol analogues in RPE microsomes in the presence of apo-CRALBP. UV-treated RPE microsomes (80 $\mu$g of protein in 200 $\mu$L) were incubated with either 2.5 $\mu$M *all-trans*-retinol, 2.5 $\mu$M *all-trans*-9-desmethylretinol, or 2.5 $\mu$M *all-trans*-13-desmethylretinol for 90 min at 37 °C in 10 mM BTP, pH 7.0, containing 1 mM CTP, 1% BSA, and 25 $\mu$M apo-rCRALBP (shaded bars) or 1% BSA without CRALBP (white bars). After the reaction was quenched with methanol, retinoids were extracted into hexane, and one-sixth of the extract was analyzed by HPLC. (A) Conversion of the three different substrates to retinyl esters. (B) Conversion of the three different substrates to 11-*cis*-alcohols. (C) Unconverted *all-trans*-alcohols. The results are an average of three different experiments.

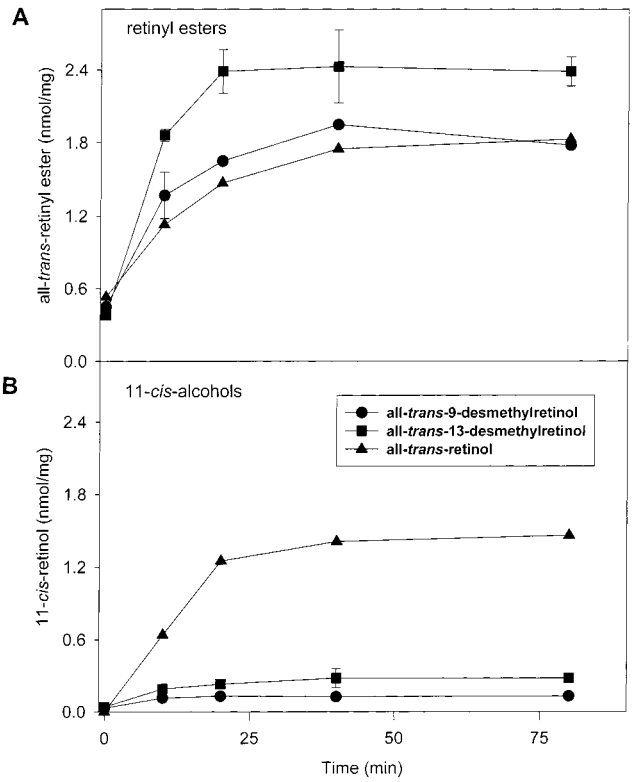


FIGURE 5: Time course of the formation of retinyl esters and 11-*cis*-retinols from different *all-trans*-retinol substrates. UV-treated RPE microsomes (80 $\mu$g of protein in 200 $\mu$L) were incubated with 2.5 $\mu$M *all-trans*-retinol (triangles), 2.5 $\mu$M *all-trans*-9-desmethylretinol (circles), and 2.5 $\mu$M *all-trans*-13-desmethylretinol (squares) at 37 °C in 10 mM BTP, pH 7.0, containing 1 mM CTP, 25 $\mu$M apo-rCRALBP, and 1% BSA. After the reaction was quenched at the indicated times with methanol, retinoids were extracted into hexane, and one-sixth of the extract was analyzed by HPLC. (A) Conversion of the three different substrates to retinyl esters. (B) Conversion of the three different substrates to 11-*cis*-alcohols. The results are an average of three different experiments.

*cis*-retinol, and that 11-*cis*-retinol bound to CRALBP is a good substrate of 11-*cis*-retinol dehydrogenase (*26*). These results suggest that conversion of 11-*cis*-alcohols to 11-*cis*-aldehydes did not enhance the isomerization.

*Energetic Considerations of all-trans-9-Desmethylretinol and all-trans-13-Desmethylretinol Isomerization.* To assess the role of substituents at positions $C_9$ and $C_{13}$ on cis-trans isomerization of *all-trans*-retinol, semiempirical and ab initio quantum-chemical calculations were performed (Materials and Methods). The quantum-mechanical energies of the three conformations were computed for the ground electronic state of *all-trans*-retinol, *all-trans*-9-desmethylretinol, *all-trans*-13-desmethylretinol, and *all-trans*-9,13-desmethylretinol, and conformations corresponding to the 11-*cis*-alcohols and the cis-trans isomerization transition state. The data presented in Table 2 showed that the relative energies of the three conformations change insignificantly in the presence or absence of $C_{19}$ and $C_{20}$ methyl groups. Thus, according to the AM1 calculations, the transition state for 13-desmethylretinol is only 1 kcal/mol lower than the transition state for the original retinol. The transition states of 9-desmethylretinol and 9,13-desmethylretinol are even higher in energy than the transition state of *all-trans*-retinol. The cis conformation is only 1−2 kcal/mol higher than trans, according to the AM1 method. Ab initio results produced somewhat larger differences for the energies of cis and trans conformations. Similarly to AM1, however, ab initio methods predict that the energies of the transition states remain insensitive to the presence of the methyl substituents. The role of methyl groups is 2-fold: first, they introduce additional steric repulsion that hinders the isomerization. Second, the methyl groups perturb the electronic structure of the retinol backbone. Based on the steric effects alone, one would expect a decrease in the transition state energy when the methyl substituents are removed. This prediction is substantiated in the case with 13-desmethylretinol. It is the second factor that is responsible for the increase in the transition state energy in 9-desmethylretinol and 9,13-desmethylretinol. Overall, the results of the quantum-chemical calculations allow us to conclude that the cis-trans conformational flexibility of the retinol molecule is essentially independent of the substituents at $C_9$ and $C_{13}$.

## DISCUSSION

*Apo-CRALBP and BSA Are Necessary in Vitro for Efficient Isomerization.* Our results show that the isomerase activity

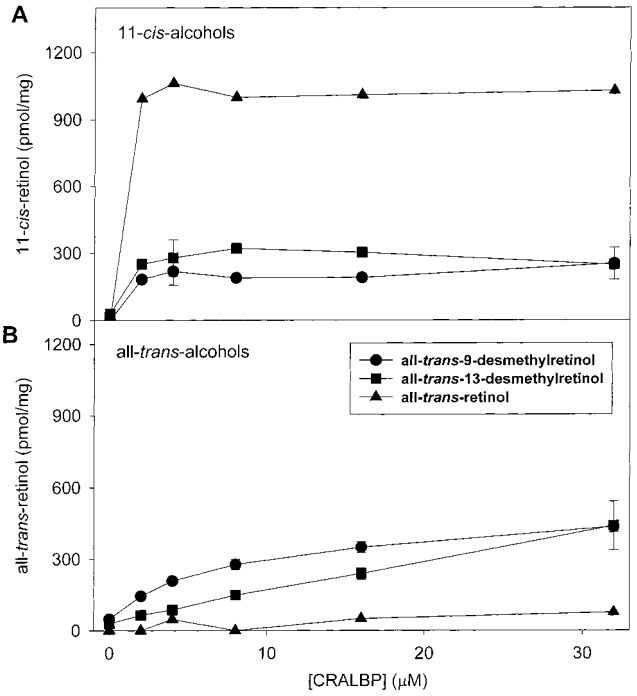


FIGURE 6: Effect of increasing apo-rCRALBP concentrations on isomerization. UV-treated RPE microsomes (80 $\mu$g of protein in 200 $\mu$L) were incubated with 2.5 $\mu$M *all-trans*-retinol (triangles), 2.5 $\mu$M *all-trans*-9-desmethylretinol (circles), and 2.5 $\mu$M *all-trans*-13-desmethylretinol (squares) for 90 min at 37 °C in 10 mM BTP, pH 7.0, containing 1 mM CTP, the indicated concentrations of apo-rCRALBP, and 1% BSA. After the reaction was quenched with methanol, retinoids were extracted into hexane, and one-sixth of the extract was analyzed by HPLC. (A) Conversion of the three different substrates to 11-*cis*-alcohols. (B) Amounts of *all-trans*-alcohol substrates. The results are an average of three different experiments.

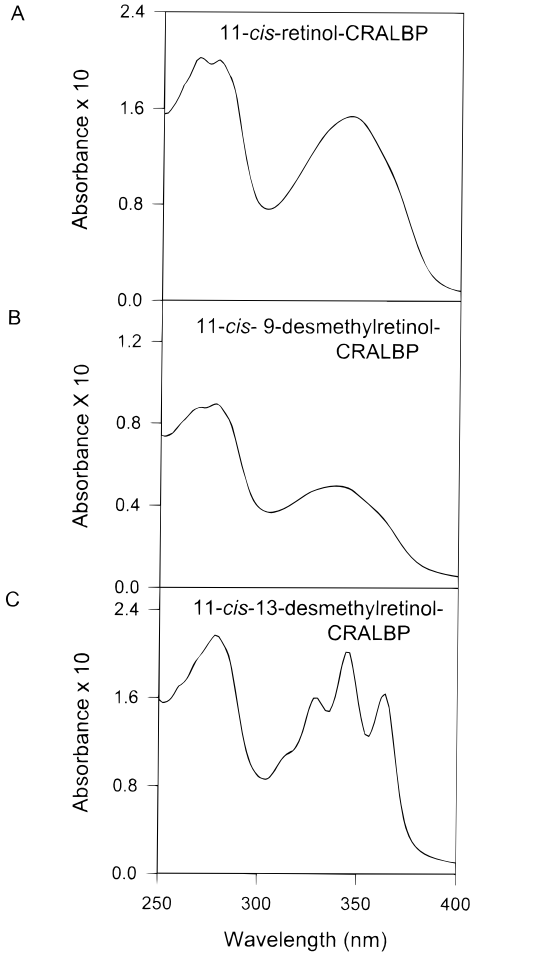


FIGURE 7: Binding of different 11-*cis*-retinol, 11-*cis*-9-desmethylretinol, and 11-*cis*-13-desmethylretinol to apo-rCRALBP. 11-*cis*-Retinol (A), 11-*cis*-9-desmethylretinol (B), and 11-*cis*-13-desmethylretinol (C) in 2 $\mu$L of ethanol (16 nmol each) were incubated with 48 $\mu$L of apo-rCRALBP (16 nmol in 10 mM BTP, pH 7.5). rCRALBP loaded with retinols was separated from free retinols on DEAE-cellulose (Materials and Methods), and the UV spectra were recorded.

is reliably measured in RPE microsomes only in the presence of apo-rCRALBP, and BSA cannot substitute for apo-CRALBP (*13*). However, the most efficient isomerization requires both BSA and apo-CRALBP (Figure 2). One plausible explanation of this observation is that both proteins are exerting their effects by a different mechanism: apo-CRALBP by binding the product of isomerization, and BSA by providing additional cofactor necessary for isomerization, such as phospholipids. These results are in odds with earlier reports that isomerization proceeded without binding proteins (*6*−*8*, *10*, *11*) and only partially in agreement with more recent studies (*12*), which first reported isomerase activity not only in the presence of apo-CRALBP but also in the presence of BSA alone. Apo-rCRALBP/BSA had no effect on the LRAT activity in our assay using *all-trans*-retinol as substrate, probably because these proteins do not bind avidly *all-trans*-retinol.

*all-trans-9-Desmethylretinol and all-trans-13-Desmethylretinol as a Substrate for Isomerization.* One valuable approach to study isomerization is application of *all-trans*-retinol analogues as substrates. Specific modification of the structure of the natural substrate may provide additional insights into the importance of a particular group(s) in the parental molecule. *all-trans*-9-Desmethylretinol and *all-trans*-13-desmethylretinol (Figure 1) are particularly interesting. Abstraction of a proton from the $C_{19}$ methyl group could be one of the mechanisms of the isomerization reaction (*11*), and lack of this methyl group should prevent isomerization. Indeed, it was reported that *all-trans*-9-desmethylretinol is not a substrate for the isomerization (*11*). The methyl group in the $C_{13}$ position in 11-cis configuration ($C_{20}$) impinges on the $C_{10}$, proton causing the polyene chain of retinol to twist (*14*). The lack of the $C_{20}$ methyl group should release this tension and speed up isomerization. Due to the low isomerization rate in the previously published assays (*11*), reevaluation of this analogue was in order.

all-*trans*-9-Desmethylretinol and *all-trans*-13-desmethylretinol are comparable substrates for LRAT (Figure 5), which has relaxed specificity for its alcohol substrate (*11*), and they are efficiently isomerized to their corresponding 11-*cis*-alcohols (Figures 3−5). Their characteristic spectra and the retention times, identical with authentic synthetic standards, identified the isomerization products as 11-cis isomers.

These findings make the mechanism of isomerization through abstraction of a proton from the $C_{19}$ methyl group very unlikely, because in the case of 9-desmethylretinol, the isomerization would have to progress by abstraction of a proton from the olefinic $C_9$ located in a different position and with much lower reactivity due to the electron-rich environment.

Table 1: Oxidation of 11-*cis*-Retinols in RPE Microsomes in the Presence of Apo-rCRALBP[a]

| substrate | condition 1 (−NAD/NADP) 11-*cis*-alcohols (pmol/mg) | condition 2 (+NAD/NADP) 11-*cis*-alcohols (pmol/mg) | 11-*cis*-aldehyde oximes (pmol/mg) | total 11-*cis*-retinoids (pmol/mg) |
|---|---|---|---|---|
| *all-trans*-retinol | 853 ± 1 | 78 ± 2 | 586 ± 1 | 664 |
| *all-trans*-9-desmethylretinol | 114 ± 1 | 8 ± 1 | 75 ± 5 | 83 |
| *all-trans*-13-desmethylretinol | 208 ± 1 | 37 ± 2 | 79 ± 1 | 116 |

[a] RPE microsomes (80 $\mu$g of protein in 200 $\mu$L), which served as the source of endogenous retinyl esters, retinyl ester hydrolase, isomerase, and 11-*cis*-retinol dehydrogenase, were incubated at 37 °C in 10 mM BTP, pH 7.0, 25 $\mu$M apo-rCRALBP, 1 mM CTP, 1% BSA, and with or without 1.0 mM NAD/1.0 mM NADP. After 90 min, the reaction was quenched with methanol and $NH_2OH$ (5 mM final concentration) to form retinal oximes. Retinoids were extracted into hexane and were analyzed by HPLC as described under Materials and Methods.

Table 2: Quantum-Chemical Calculations of Energies for the *all-trans*-Alcohols, 11-*cis*-Alcohols, and Transition State (TS)[a]

| retinoids | AM1 (kcal/mol) | HF (kcal/mol) | B3LYP (kcal/mol) |
|---|---|---|---|
| retinol | | | |
| *cis*- | 2.1 | 6.0 | 4.9 |
| *trans*- | 0 | 0 | 0 |
| TS | 36.6 | 45.7 | 36.4 |
| 9,13-desmethylretinol | | | |
| *cis*- | 1.1 | 2.0 | 1.9 |
| *trans*- | 0 | 0 | 0 |
| TS | 37.3 | 46.1 | 36.8 |
| 9-desmethylretinol | | | |
| *cis*- | 2.1 | − | − |
| *trans*- | 0 | − | − |
| TS | 37.1 | − | − |
| 13-desmethylretinol | | | |
| *cis*- | 1.2 | − | − |
| *trans*- | 0 | − | − |
| TS | 35.6 | − | − |

[a] Quantum-chemical calculations were performed using the Gaussian-94 suite of programs (Materials and Methods). AM1 was a general purpose semiempirical method (*21*); ab initio calculations used the Hartree−Fock (HF) method and a combined Hartree−Fock/Density-Functional approach (B3LYP) (*22*).

An unanswered question is why the isomerizations of *all-trans*-retinol, *all-trans*-9-desmethylretinol, and *all-trans*-13-desmethylretinol do not progress to completion, but rather plateaued with different levels of 11-*cis*-alcohol formed for all three substrates. One possibility is that the isomerization system reaches equilibrium between different components of retinoid metabolism in RPE microsomes and retinoid-binding capacities of proteins used in the assay. However, the results from titration experiments are contrary to this idea. When the concentrations of apo-rCRALBP were increased, there was no steady increase in 11-*cis*-alcohol production. In contrast, increased concentration of apo-rCRALBP caused more free *all-trans*-retinol to be withdrawn (Figure 6). Because apo-rCRALBP has a higher affinity for 11-*cis*-isomers, it appears that the amount of rCRALBP was sufficient to bind more 11-*cis*-alcohols than was produced. Another more provocative explanation is, however, that a second substrate is needed for isomerization that is exhausted during this reaction. In this model, the isomerase would establish a thermodynamic equilibrium between the unidentified *all-trans*-retinol derivative and 11-*cis*-retinol. CRALBP, by binding and withdrawing 11-*cis*-retinol, would promote production of 11-*cis*-retinol more efficiently. The formation of the unidentified *all-trans*-retinol derivative may require the second substrate, such as, for example, a specific phospholipid present in the vicinity of the isomerase or extracted by BSA. This derivative may be formed with different efficiencies for *all-trans*-retinol and its desmethyl analogues. In support of this idea are recent findings that slight perturbation of the RPE phospholipid composition abolished isomerization, even though retinyl esters are readily formed, and that isomerization can be restored by addition of the RPE lipid extract (H. Stecher and K. Palczewski, unpublished).

According to the isomerohydrolase model, there is no need for native lipids to promote isomerization, once the retinyl esters are made, because in this model the isomerization reaction would be thermodynamically favorable. Winston and Rando proposed that 11-*cis*-retinol inhibits isomerohydrolase with $IC_{50}$ = 400 nM (*12*). In our reaction conditions, the total concentration of 11-*cis*-retinol is ~1.5 $\mu$M, from which the vast majority partitions to soluble CRALBP. There is also another important reason significant amounts of 11-*cis*-retinol cannot accumulate in the membranes. It is known that this alcohol would be efficiently esterified due to LRAT-permissive specificity for *all-trans*- and 11-*cis*-alcohols (*27*). Our analysis of the ester pool showed, however, lack of any 11-*cis*-alcohol in the ester pools (Figure 3).

The differences in the levels of conversion to 11-*cis*-alcohols between substrates could be due to 10−20 times higher concentrations of free *all-trans*-alcohols for desmethyl analogues as compared with *all-trans*-retinol. These *all-trans*-alcohols likely partition to an insoluble membrane fraction in the reaction mixture due to their lower affinity for CRALBP than 11-*cis*-alcohols (Figure 7). The lower affinity between *all-trans*-9-desmethylretinol and CRALBP, the unknown LRAT efficiency, and the rate of opposing retinyl hydrolase activity for desmethyl analogues could account for a lower conversion rate as compared to *all-trans*-retinol. Therefore, the differences between *all-trans*-retinol and *all-trans*-9-desmethylretinol and *all-trans*-13-desmethylretinol appear to be a consequence of the enzymatic properties of enzymes involved in isomerization, and binding affinities to proteins involved in the isomerization assay, rather than the differences in the chemical or thermodynamic properties of these compounds (Table 2). It is unclear why in the previous studies (*11*) no isomerization activity was observed with *all-trans*-9-desmethylretinol. One possible explanation is that low levels of 11-cis isomerization product, observed previously without apo-CRALBP, were difficult to separate from 13-*cis*-alcohol generated spontaneously from *all-trans*-alcohol.

In summary, this study provides further improvements in the isomerization assay of *all-trans*-retinol to 11-*cis*-retinol. We also have tested the importance of $C_{19}$ and $C_{20}$ methyl

groups in the structure of *all-trans*-retinol. It appears that neither $C_{19}$ nor $C_{20}$ methyl groups are essential for the isomerization reaction, and that these methyls are unlikely to be involved in formation of a transition stage during the isomerization process.

## ACKNOWLEDGMENT

We thank Dr. John W. Crabb for the CRALBP construct, J. Preston Van Hooser for help during the course of this study, and Joshua McBee for critical reading of the manuscript and for preparation of Figure 1.

## REFERENCES

1. Wald, G. (1935) *J. Gen. Physiol. 19*, 351−371.
2. Polans, A., Baehr, W., and Palczewski, K. (1996) *Trends Neurosci. 19*, 547−554.
3. Alpern, M. (1971) *J. Physiol. 217*, 447−471.
4. Alpern, M., Maaseidvaag, F., and Ohba, N. (1971) *Vision Res. 11*, 539−549.
5. Dowling, J. E. (1960) *Nature 188*, 114−118.
6. Bernstein, P. S., Law, W. C., and Rando, R. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1849−1853.
7. Fulton, B. S., and Rando, R. R. (1987) *Biochemistry 26*, 7938−7945.
8. Deigner, P. S., Law, W. C., Canada, F. J., and Rando, R. R. (1989) *Science 244*, 968−971.
9. Rando, R. R., and Chang, A. (1983) *J. Am. Chem. Soc. 105*, 2879−2882.
10. Trehan, A., Canada, F. J., and Rando, R. R. (1990) *Biochemistry 29*, 309−312.
11. Canada, F. J., Law, W. C., Rando, R. R., Yamamoto, T., Derugini, F., and Nakanishi, K. (1990) *Biochemistry 29*, 9690−9697.
12. Winston, A., and Rando, R. R. (1998) *Biochemistry 37*, 2044−2050.
13. Stecher, H., Gelb, M. H., Saari, J. C., and Palczewski, K. (1999) *J. Biol. Chem. 274*, 8577−8585.
14. Pauling, L. (1949) *Helv. Chim. Acta 32*, 2241−2246.
15. Crabb, J. W., Chen, Y., Goldflam, S., West, K., and Kapron, J. (1998) in *Methods in Molecular Biology*: *Retinoid protocols* (Redfern, C. P. F., Ed.) Vol. 89, pp 91−104, Humana Press, Totowa, NJ.
16. Saari, J. C., and Bredberg, D. L. (1990) *Methods Enzymol. 190*, 156−163.
17. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
18. von Isler, O., Montavon, M., Ruegg, R., and Zeiler, P. (1956) *Helv. Chim. Acta 39*, 259−273.
19. Bridges, C. D. B., Fong, S.-L., and Alvarez, R. A. (1980) *Vision Res. 20*, 355−360.
20. Landers, G. M., and Olson, J. A. (1988) *J. Chromatogr. 438*, 383−392.
21. Dewar, M. J. S., Zoebisch, E. G., and Healy E. F. (1985) *J. Am. Chem. Soc. 107*, 3902−3909.
22. Becke A. D. (1993) *J. Chem. Phys. 98*, 5648−5652.
23. Stecher, H., and Palczewski, K. (1999) *Methods Enzymol. 316*, 213−230.
24. Saari, J. C., and Bredberg, D. L. (1987) *J. Biol. Chem. 262*, 7618−7622.
25. Zimmerman, W. F., Lion, F., Deamen, F. J. M., and Bonting, S. L. (1975) *Exp. Eye Res. 21*, 325−332.
26. Saari, J. C., Bredberg, D. L., and Noy, N. (1994) *Biochemistry 33*, 3106−3112.
27. Saari, J. C., and Bredberg, D. L. (1988) *J. Biol. Chem. 263*, 8084−8090.

BI9913294