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Multienzyme Analysis of Visual Cycle

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Introduction

The visual cycle is a fundamental chemical transformation that generates 11-*cis*-retinal from all-*trans*-retinal. The chromophore 11-*cis*-retinal recombines with apoprotein opsin to form rhodopsin, the light-sensitive receptor of the visual transduction cascade.¹ Several reactions are required for this transformation. First, all-*trans*-retinal, the bleaching product, is reduced by all-*trans*-retinol dehydrogenase (RDH) in the rod outer segment (ROS). Next, all-*trans*-retinol diffuses to the retinal pigment epithelium cells (RPE). Within RPE further reactions take place: (1) all-*trans*-retinol is isomerized to 11-*cis*-retinol, and (2) 11-*cis*-retinol is oxidized to 11-*cis*-retinal by 11-*cis*-retinol dehydrogenase (11-*cis*-RDH²). Last, 11-*cis*-retinal diffuses back to the ROS, where rhodopsin is regenerated. Both diffusion of all-*trans*-retinol to the RPE and uptake of 11-*cis*-retinal by photoreceptors remain poorly understood.

11-*cis*-Retinol is produced enzymatically by an isomerase, which still has defied purification and molecular characterization. The proposed substrates for this reaction are all-*trans*-retinyl carboxylic esters (predominantly palmitoyl), which undergo all-*trans* to 11-*cis* isomerization coupled to ester hydrolysis.³ Retinoid-binding proteins, such as cellular retinaldehyde-binding protein (CRALBP) or albumin (BSA), enhance the formation of 11-*cis*-retinol.⁴ Several puzzling features, however, are inconsistent with this model, in which all-*trans*-retinyl esters are substrates for a putative isomero-hydrolase.⁵

Phosphatidylcholine is an acyl donor for all-*trans*-retinyl ester synthesis. This reaction is catalyzed by lecithin: retinol acyltransferase (LRAT, EC 2.3.1.135) in RPE.⁶ LRAT is also responsible for the formation of 11-*cis*-retinyl esters.⁷ 11-*cis*-Retinyl esters are substrates for an 11-*cis*-retinyl ester-specific hydrolase, and are another source for 11-*cis*-retinol.⁸ Storage and time-dependent release of 11-*cis*-retinol from the ester pool could be important for the regulation of the visual cycle. To understand the production of 11-*cis*-retinal in RPE, an assay system is required for the simultaneous detection of LRAT, retinol isomerase, and retinyl ester hydrolase activities. Techniques designed to study these enzymatic reactions in RPE are summarized below.

Materials and Methods

General

All procedures involving retinoids are performed under dim red illumination to prevent photoisomerization and photodecomposition. Retinoids are stored under argon at -80° . Bovine RPE microsomes are the source for LRAT, isomerase, and retinyl ester hydrolase. Native RPE microsomes contain endogenous retinoids (mostly retinyl esters). In some experiments, these retinoids are destroyed by UV light treatment.³

Source of Bovine Eyes and all-*trans*-Retinol

Fresh bovine eyes are obtained from a local slaughterhouse (Schenk Packing Company, Stanwood, WA). all-*trans*-Retinol is purchased from Sigma (St. Louis, Mo). all-*trans*-[11,12-³H(N)]retinol is purchased from NEN Life Science Products (Boston, MA).

Preparation of Retinal Pigment Epithelium Microsomes

A microsomal membrane fraction is obtained from fresh bovine RPE as described previously.⁹ The microsomal fraction is resuspended in 10 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 7.0, containing 1 μ M leupeptin and 1 mM dithiothreitol (DTT) to a final protein concentration of 2.3 mg/ml according to the Bradford method,¹⁰ and stored in small aliquots at -80° .

Ultraviolet Treatment

To destroy endogenous retinoids, RPE microsomes (200- μ l aliquots) are irradiated in a quartz cuvette for 5 min at 0° , using a Chromato UVE-transilluminator (UVP, Upland, CA). UV treatment produces RPE microsomes without detectable amounts of all retinoids (Fig. 2B, top, inset).

Aporecombinant Cellular Retinaldehyde-Binding Protein

Aporecombinant CRALBP (apo-rCRALBP) is expressed in *Escherichia coli* and purified to apparent homogeneity by Ni^{2+} -NTA affinity chromatography.¹¹ Two fractions with the highest protein content [checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)] are dialyzed overnight against 10 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP), 250 mM NaCl. Fractions of freshly purified and dialyzed apo-rCRALBP are stored at 4° in the presence of 0.02% (w/v) NaN_3 and used within 1 week.

Preparation of all-trans-Retinol as Substrate for Enzyme Reaction

all-*trans*-5-[11,12- ^3H (N)] Retinol is diluted with all-*trans*-retinol to give the desired specific radioactivity (550,000 dpm/nmol), and purified on a normal-phase high-performance liquid chromatography (HPLC) column [Ultrasphere-Si 5 μ , 4.6×250 mm (Altex), flow rate 1.4 ml/min, 10% (v/v) ethyl acetate in hexane] according to modified procedure published previously.¹² Aliquots of the freshly purified all-*trans*-retinol in 10% (v/v) ethyl acetate-hexane are transferred to 1.5-ml polypropylene tubes (containing between 0.5 and 10.0 nmol of all-*trans*-retinol) and dried under argon. For this procedure, the tubes are placed in a water bath at 37° . A gentle flow of argon is directed to the surface of the liquid with a Pasteur pipette. This evaporation procedure does not last longer than 2 min. Purified and dried all-*trans*-retinol is stored (0.5–10 nmol per vial at -80°) for up to 3 months.

Assay Conditions for Lecithin: Retinol Acyltransferase, Isomerase, and Hydrolase

The 1.5-ml polypropylene tube with purified and dried substrate, all-*trans*-[^3H] retinol (0.5–10.0 nmol, 550,000 dpm/nmol) is used as the reaction vial. Twenty microliters of 5% BSA in 10 mM BTP, pH 7.0, is added, followed by 30–40 μ l of apo-rCRALBP in 10 mM BTP, pH 7.5, containing 250 mM NaCl to give a final concentration of 25 μ M. Next, 10 mM BTP, pH 7.0, is added to bring the final volume to 165 μ l. For some experiments, additional compounds (i.e., phosphate-containing compounds, alcohols), solubilized in 10 mM BTP, pH 7.0, are added. Finally, 35 μ l of RPE microsomes (~ 80 μ g of protein) is added to this mixture, and the reactions are incubated at 37° for the indicated times.

Extraction of Retinoids from Reaction Mixtures

The reaction mixture (180 μ l of 200 μ l) is transferred to a new vial containing 300 μ l of ice-cold methanol, and 300 μ l of hexane is added. The sample is vortexed for 2 min and centrifuged for 4 min at 14,000g to separate organic and aqueous phases. all-*trans*-Retinol and 11-*cis*-retinol are extracted with hexane in 75–95% yield. These yields are determined with all-*trans*-[^3H]retinol and 11-*cis*-[^3H]retinol as tracers. To estimate the yield of retinyl ester

extraction, all-*trans*-[³H]retinol is incubated for 1 hr with RPE microsomes as a source of LRAT. Most of the all-*trans*-[³H]retinol is converted to more hydrophobic all-*trans*-[³H]retinyl esters, which can be extracted in ~60% yield.

High-Performance Liquid Chromatography Separation of Retinoids

Retinoids are separated using a Hewlett Packard, (HP; Palo Alto, CA) HP1100 HPLC with a diode-array detector and HP Chemstation A.04.05 software. The latter feature allows the online recording of UV spectra and identification of retinoid isomers according to their specific absorption maxima between 280 and 400 nm. A normal-phase, narrow-bore column [Alltech (Deerfield, IL) Silica 5 μ Solvent Miser, 2.1 $\times$ 250 mm] and an isocratic solvent composed of 4% (v/v) ethyl acetate in hexane at a flow rate of 0.7 ml/min are used to separate retinyl esters from 11-*cis*-retinal (as oximes⁵), all-*trans*-retinal (as oximes⁵), and 11-*cis*-retinol and all-*trans*-retinol (Fig. 1B). Thirty microliters of the hexane extract is injected onto the HPLC column. Because retinals are not present, except for trace amounts in the case of native RPE microsomes (<0.1 nmol/mg of RPE protein), a similar isocratic solvent composed of 10% (v/v) ethyl acetate in hexane at a flow rate of 0.3 ml/min is used. The separation with this isocratic system can be done faster and is sufficient for the separation of retinyl esters from 11-*cis*-retinol and all-*trans*-retinol (traces in Fig. 2).

Hydrolysis of Retinyl Esters

Hexane from the extracts containing retinyl esters or from the HPLC-purified retinyl ester fractions (typically 200 μ l) is evaporated under argon as described above. The retinyl esters are dissolved in 230 μ l of absolute ethanol and hydrolyzed with 20 μ l of 6 M KOH for 30 min at 55°. To extract the products, the sample is diluted with 100 μ l of water, chilled on ice for 2 min, and extracted with 300 μ l of hexane. The retinoids in hexane are analyzed directly by HPLC.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SDS–PAGE is performed according to Laemmli¹³ using 12% SDS–acrylamide gels in a Hoefer (San Francisco, CA) minigel apparatus and low molecular weight markers from Pharmacia Biotech (Piscataway, NJ). The gels are stained with Coomassie Brilliant Blue R-250 and destained with 50% (v/v) methanol and 7% (v/v) acetic acid.

Calculation of Results

The HPLC separation system is calibrated by injection of various amounts of retinoids. Graphs relating HPLC areas to different injected amounts of retinoids are constructed for all-*trans*-retinyl palmitate, 11-*cis*-retinol, and all-*trans*-retinol at 325 nm. Values (picomoles per unit area) are calculated over the linear portion of this relationship. all-*trans*-Retinyl palmitate and all-*trans*-retinol concentrations in hexane are determined spectrophotometrically at 325 nm with ϵ values of 51,770 and 49,260 M⁻¹ cm⁻¹, respectively.^{14,15} Radioactive HPLC fractions are collected and picomole values are calculated according to the specific radioactivity (550,000 dpm/nmol). The apo-rCRALBP concentration in buffer (10 mM BTP, 250 mM NaCl) is determined spectrophotometrically at 280 nm with ϵ of 9.7 ml mg⁻¹ cm⁻¹.¹⁶

Comments

Stability of Proteins

LRAT activity was stable for 6 months, but significant variations in ester hydrolase and isomerase activities of RPE microsome preparations were observed depending on length of storage at –80°. The activities of isomerase and hydrolase declined by ~50% over 3 months. Therefore, less than 2-month-old preparations were used for all studies. SDS–PAGE analysis

showed a typical composition of proteins (Fig. 1A, lane II) as observed by others.^{2,17} The protein pattern did not vary significantly between preparations, for RPE microsomal protein and apo-rCRALBP (Fig. 1A, lane III). The latter protein was used within 1 week of purification.

High-Performance Liquid Chromatography Separation

all-*trans*-Retinyl esters and 11-*cis*-retinyl esters were eluted 0.5 min after the solvent front (peak 1 in Figs. 1B and 2), followed by 11-*cis*-retinol (peak 4) and all-*trans*-retinol (peak 6), all with a chromatographic yield of >95%. 9-*cis*-Retinol eluted ~1 min earlier than all-*trans*-retinol, while 13-*cis*-retinol eluted on the descending side of the 11-*cis*-retinol peak (data not shown).

Enzyme Assay

Retinoid analysis of native RPE microsomes, which were incubated without additional compounds, extracted with hexane, and separated by HPLC, showed only the presence of retinyl esters (Fig. 2A, top). Native RPE microsomes contained 0.0–0.9 nmol of all-*trans*-retinol per milligram of protein, 0.0–0.3 nmol of 11-*cis*-retinol, 8.6 ± 0.9 nmol of all-*trans*-retinyl esters, and 9.6 ± 1.4 nmol of 11-*cis*-retinyl esters. Retinyl esters were in 11-*cis* and all-*trans* configurations (Fig. 2A, top, inset). These isomers could be demonstrated via the following procedures: (1) HPLC separation and collection of the ester fraction, (2) hydrolysis of the esters, and (3) another round of HPLC separation to quantify the two retinol isomers. Different preparations of RPE microsomes had similar trace amounts of retinols and retinals; however, they differed in amounts of endogenous esters (with similar ratios between 11-*cis*- and all-*trans*-retinyl esters). In some cases, the ester pool was as high as ~60 nmol/mg of protein. 11-*cis*-Retinol was released from 11-*cis*-retinyl esters by a hydrolase and accumulated when native RPE microsomes (with a high endogenous amount of 11-*cis*-retinyl esters) were incubated in the presence of apo-rCRALBP (Fig. 2A, bottom). Treatment of RPE microsomes with UV light destroyed all the endogenous retinoids (Fig. 2B, top, inset). Incubation of UV-treated RPE microsomes with all-*trans*-[³H]retinol in the absence of apo-rCRALBP led to esterification of the substrate by LRAT (Fig. 2B, top). When apo-rCRALBP was present during the incubation period, all-*trans*-retinol was both esterified by LRAT and converted to 11-*cis*-retinol by the isomerase (Fig. 2B, bottom). In our studies, apo-rCRALBP could not be substituted by albumin, as observed by others.⁴

Kinetics and Further Tests

This reliable enzyme assay allows us to measure the kinetic parameters of these reactions, as well as the influence of different compounds on the activities of LRAT, retinyl ester hydrolase, and retinol isomerase. The enzymatic activities of all three enzymes were a function of the concentration of native RPE microsomes (Fig. 3A) or UV-treated RPE microsomes (Fig. 3B). The influence of different substrate concentrations on LRAT and isomerase in UV-treated RPE microsomes is shown in Fig. 4. Using this assay, we tested how different compounds, such as phosphate-containing compounds or alcohols, influenced the activities of visual cycle enzymes. ATP, for example, strongly influenced the activities of isomerase and hydrolase. Their activities are increased by a factor of ~3 when ATP is present. ATP had initially no influence on LRAT activity. The apparent decrease in the ester levels after longer incubation resulted from high production and removal of 11-*cis*-retinol (Fig. 5). The ATP effect was not specific only for high-energy compounds, because a variety of phosphate-containing compounds also stimulated isomerase and hydrolase (Tables I–III). Alcohols, on the other hand, are strong inhibitors of enzymes from the visual cycle. Branched alcohols, in particular, such as 2-propanol, isobutanol, or isopentanol, profoundly inhibited isomerase activity but had little effect on LRAT activity (Table IV). The mechanistic explanation of these observations awaits further investigation.

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Fig. 1. Separation of retinoids and characterization of RPE microsomes and apo-rCRALBP. (A) SDS-PAGE analysis of RPE microsomes (lane II) and apo-rCRALBP (lane III). Molecular weight markers are shown in lane I. (B) HPLC separation of retinyl esters (1), *syn* 11-*cis*-retinal oxime (2), *syn* all-*trans*-retinal oxime (3), 11-*cis*-retinol (4), *anti* 11-*cis*-retinal oxime (5), all-*trans*-retinol (6), and *anti* all-*trans*-retinal oxime (7).

Fig. 2.

Influence of apo-rCRALBP on the formation of 11-*cis*-retinol in native (A) and UV-treated (B) RPE microsomes. RPE microsomes (35 μ l; 2.3 mg/ml) were incubated for 60 min at 37° in 10 mM BTP, pH 7.0, containing 1% BSA. After quenching the reaction with methanol, retinoids were extracted with hexane and one-tenth of the extract was analyzed by HPLC. (A) *Top*: HPLC traces before and after saponification (inset). *Bottom*: RPE microsomes were incubated in the presence of 25 μ M apo-rCRALBP. (B) *Top*: UV-treated RPE microsomes were incubated with 2.5 μ M all-*trans*-[³H]retinol (550,000 dpm/nmol). UV-treated RPE microsomes did not contain endogenous retinoids (inset). *Bottom*: UV-treated RPE microsomes were incubated with 2.5 μ M all-*trans*-[³H]retinol in the presence of 25 μ M apo-rCRALBP. HPLC fractions were collected and radioactivity was counted with a Beckman LS 3801. For peak identification, see Fig. 1.

Fig. 3.

Formation of all-*trans*-[³H]retinyl esters and 11-*cis*-retinol (total and ³H-labeled) as a function of RPE microsome concentrations. Native RPE microsomes (A) and UV-treated RPE microsomes (B) (in both cases 35 μl; 2.3 mg/ml) were incubated with 2.5 μM all-*trans*-[³H]retinol (550,000 dpm/nmol) in the presence of 25 μM apo-rCRAALBP for 60 min at 37° in 10 mM BTP, pH 7.0, containing 1% BSA. After quenching the reaction with methanol, retinoids were extracted with hexane and one-tenth of the extract was analyzed by HPLC. HPLC fractions were collected and radioactivity was counted with a Beckman LS 3801.

Fig. 4.

Formation of all-*trans*-[³H]retinyl esters and 11-*cis*-[³H]retinol as a function of all-*trans*-[³H]retinol concentrations. RPE microsomes (35 μl; 2.3 mg/ml) were incubated with different concentrations of all-*trans*-[³H]retinol in the presence of 25 μM apo-rCRALBP for 60 min at 37° in 10 mM BTP, pH 7.0, containing 1% BSA. After quenching the reaction with methanol, retinoids were extracted with hexane and one-tenth of the extract was analyzed by HPLC. HPLC fractions were collected and radioactivity was counted with a Beckman LS 3801.

Inset: A high range of the substrate concentration.

Fig. 5.

The effect of ATP on the formation of all-*trans*-[³H]retinyl esters and 11-*cis*-retinol (total and ³H-labeled). Native RPE microsomes (35 μ l; 2.3 mg/ml) were incubated with all-*trans*-[³H]retinol in the presence of 25 μ M apo-rCRALBP for 60 min at 37° in 10 mM BPT, pH 7.0, containing 1% BSA. After quenching the reaction with methanol, retinoids were extracted with hexane and one-tenth of the extract was analyzed by HPLC. HPLC fractions were collected and radioactivity was counted with a Beckman LS 3801. *Top*: Formation of all-*trans*-[³H]retinyl ester (A), 11-*cis*-[³H]retinol (B), and total 11-*cis*-retinol (C) as a function of ATP concentration. *Bottom*: Time-dependent formation with and without 5 mM ATP of all-*trans*-[³H]retinyl ester (D) 11-*cis*-[³H]retinol (E), and total 11-*cis*-retinol (F).

TABLE I
Effect of Phosphate-Containing Compounds on Isomerase and Hydrolase Activity

Compounds	Concentration (mM)	11- <i>cis</i> -[³ H]Retinol (pmol/mg/min) (a)	11- <i>cis</i> -Retinol (pmol/mg/min) (b)	Ratio (b/a)
—	—	3.24	51.84	16.0
AMP	5	5.41	77.92	14.4
ADP	5	9.96	155.84	15.6
ATP	5	14.28	207.79	14.5
cAMP	5	4.11	60.61	14.7
Adenosine (2',5'- and 3',5'- diphosphate	5	8.87	160.17	18.0
ATP _γ S	5	11.25	194.80	17.3
CTP	5	14.50	203.46	14.0
GMP	5	6.06	95.24	15.7
GTP	5	15.58	168.83	10.8
cGMP	5	3.46	51.95	15.0
GTP _γ S	5	9.09	168.83	18.6
Sodium phosphate (P _i)	10	7.78	133.92	17.2
	100	9.07	151.2	16.6
Tetrasodium pyrophosphate (PP _i)	10	15.34	211.68	13.8
	20	15.80	216.45	13.7
	100	9.72	194.4	20.0
Tripolyphosphate (PPP _i)	25	14.07	199.13	14.1
Imidodiphosphate (PNP _i)	10	6.26	129.6	20.7
	100	10.37	168.48	16.2

TABLE II

Effect of ATP on Isomerase Activity

Time (min)	Preincubation with all- <i>trans</i> -retinol (2.5 μ M)					
	all- <i>trans</i> -Retinyl ester (nmol/mg)		11- <i>cis</i> -Retinol (nmol/mg)		all- <i>trans</i> -Retinol (nmol/mg)	
	+ATP	-ATP	+ATP	-ATP	+ATP	-ATP
0	2.40 $\pm$ 0.01	2.30 $\pm$ 0.01	0.00	0.00	0.00	0.00
15	1.44 $\pm$ 0.20	1.64 $\pm$ 0.06	0.60 $\pm$ 0.21	0.42 $\pm$ 0.15	0.15 $\pm$ 0.02	0.00
30	1.74 $\pm$ 0.18	1.70 $\pm$ 0.03	0.90 $\pm$ 0.18	0.52 $\pm$ 0.16	0.13 $\pm$ 0.01	0.00

TABLE III

Effect of ATP on Hydrolase Activity

Time (min)	Preincubation with 11- <i>cis</i> -retinol (2.5 μ M)					
	all- <i>trans</i> -Retinyl ester (nmol/mg)		11- <i>cis</i> -Retinol(nmol/mg)		all- <i>trans</i> -Retinol (nmol/mg)	
	+ATP	-ATP	+ATP	-ATP	+ATP	-ATP
0	1.84 $\pm$ 0.01	1.80 $\pm$ 0.01	0.00	0.00	0.00	0.00
5	1.53 $\pm$ 0.09	1.56 $\pm$ 0.10	0.33 $\pm$ 0.03	0.31 $\pm$ 0.01	0.00	0.00
10	1.73 $\pm$ 0.01	1.44 $\pm$ 0.06	0.63 $\pm$ 0.04	0.54 $\pm$ 0.03	0.00	0.00

TABLE IV

Effect of Alcohols on LRAT and Isomerase Activity

Alcohol	Structure	all- <i>trans</i> [³ H] Retinyl ester (pmol/mg/min) (<i>a</i>)	11- <i>cis</i> -[³ H] Retinol (pmol/mg/min) (<i>b</i>)	Ratio(<i>a/b</i>)
—	—	9.49	12.72	0.7
Methanol	CH ₃ OH	12.00	7.71	1.5
Ethanol	CH ₃ CH ₂ OH	10.15	3.84	2.6
<i>n</i> -Propanol	CH ₃ CH ₂ CH ₂ OH	6.72	2.64	2.5
2-Propanol		12.27	0.89	13.8
Isobutanol		10.52	0.72	14.6
Isopentanol		14.16	1.30	10.9
2,2,2-Trifluoroethanol	F ₃ CC ₂ H ₄ OH	11.86	2.67	4.4
Benzyl alcohol		3.05	0.89	3.4
2-(2-Ethoxyethoxy)ethanol	CH ₃ H ₂ C — O — CH ₂ H ₂ C — O CH ₂ CH ₂ OH	9.19	1.37	6.7
Geraniol		2.81	1.20	2.3