J. Biochem. **123**, 953-958 (1998)

Isomerization of 11-*cis*-Retinol to All-*trans*-Retinol in Bovine Rod Outer Segments

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Received for publication, December 22, 1997

It is known that exogenous 11-*cis*-retinol inhibits the recovery of photosensitivity of bleached rod outer segments (ROS) and 11-*cis*-retinol exists in the interphotorecepter matrix. We examined the conversion of 11-*cis*-retinol with bovine ROS. ROS was incubated with 11-*cis*-retinol under dim red light. Retinoids were extracted from the reaction mixture with hexane and analyzed by HPLC coupled with a fluorescence spectrophotometer. Isomerization of 11-*cis*-retinol to all-*trans*-retinol was observed in the presence of ROS. This isomerization was not suppressed by heat treatment and did not have stereospecificity. In addition, we incubated purified rhodopsin and phospholipids extracted from ROS with 11-*cis*-retinol. Rhodopsin was found to isomerize 11-*cis*-retinol to all-*trans*-retinol as well as ROS, but phospholipids did not. In contrast, the phospholipids inhibited the isomerization of 11-*cis*-retinol to all-*trans*-retinol by the purified rhodopsin. Commercially available phospholipids, especially phosphatidylserine, also inhibited the isomerization. Our results suggest that rhodopsin has activity for the isomerization of 11-*cis*-retinol to all-*trans*-retinol and may play an important role in the detoxification of 11-*cis*-retinol in the ROS.

Key words: all-*trans*-retinol, 11-*cis*-retinol, isomerization, phospholipid, rhodopsin.

The isomerization of the chromophore of rhodopsin, 11-*cis*-retinal to all-*trans*-retinal by light is the initial reaction of the visual sense. In the visual cycle, all-*trans*-retinal is converted to all-*trans*-retinol by all-*trans*-specific retinol dehydrogenase (RDH) in the rod outer segments (ROS) (*1-6*). All-*trans*-retinol is converted to 11-*cis*-retinol through enzymatic esterification (*7-9*), de-esterification (*10*), and isomerization (*11-13*) in the retinal pigment epithelium (RPE) and 11-*cis*-retinol is converted to 11-*cis*-retinal by 11-*cis*-specific RDH in RPE (*2, 14, 15*). 11-*cis*-Retinal formed is delivered to ROS and regenerates rhodopsin.

The possible significance of phospholipid-retinal complex in photoisomerization of all-*trans*-retinal to 11-*cis*-retinal in ROS was first reported by Shichi and Somers (*16*). They suggested that phosphatidylethanolamine plays an important role in the isomerization of retinals through a protonated Schiff base. Groenendijk *et al.* found that the isomerization of retinals occurs even in the dark (*17*). On the other hand, the existence of isomerization of 11-*cis*-retinol to all-*trans*-retinol had been suggested previously (*17, 18*), but it has not been investigated extensively since the role of retinol isomerization was unclear. Recently, the toxic effect of 11-*cis*-retinol to ROS was reported by Jones *et al.* (*19*): ROS loses photosensitivity. Thus, the isomerization of 11-*cis*-retinol in ROS would be important for detoxification.

It is also known that rhythmic shedding of ROS and engulfment of the ROS tips by RPE occurs daily (*20-22*). It is likely that 11-*cis*-retinal in the engulfed ROS is converted to 11-*cis*-retinol by RPE-RDH since RPE-RDH has oxidoreductase activity and mediates the conversion of 11-*cis*-retinal to 11-*cis*-retinol under acidic conditions, such as in phagolysosomes, and subsequent isomerization of 11-*cis*-retinol to all-*trans*-retinol by the engulfed ROS may contribute to retrieval of the retinoid into the visual cycle. Therefore, we investigated the retinol isomerization with ROS and rhodopsin using exogenous 11-*cis*-retinol.

MATERIALS AND METHODS

Materials—Fresh bovine eyes were purchased from a local slaughterhouse. Retinas were collected from dark-adapted eyes and light-adapted eyes and stored at −80°C. Microsomes of RPE were fractionated by the procedure of Zimmerman and coworkers (*14*). Rod outer segments (ROS) were prepared from frozen retinas by sucrose density centrifugation (*23*). To extract lipids from ROS, a suspension of ROS containing 5 mg protein was homogenized under argon gas with 20 volumes of chloroform/methanol (2:1, v/v), using a conical all-glass homogenizer. After centrifugation at 500×*g* for 20 min, the extract was stored at −80°C under argon gas. Rhodopsin was extracted with 1.3% Emulphogene BC-720 from ROS prepared from dark adapted eyes and purified by chromatography on hydroxylapatite/Celite and concanavalin A-Sepharose (*24*). The A_{278}/A_{493} ratio of purified rhodopsin was about 2.2.

All-*trans*-retinal was obtained from Sigma. Photoisomerization of all-*trans*-retinal was performed according to the method of Bridges and Alvarez (*12*). Unless otherwise

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indicated, experiments with retinoids were carried out under dim red light (100 V, 20 W with filter, HANSA SAFE LIGHT GLASS No. 8) to prevent light-induced isomerization. Retinal isomers were isolated by means of high-pressure liquid chromatography (HPLC) using a Chemcosorb 5Si column (25×250 mm) according to Tsukida *et al.* (*25*). The mobile phase was diethyl ether/*n*-hexane (8:92) at 1 ml/min. Peaks were detected at 365 nm. All-*trans*, 9-*cis*, 11-*cis*, and 13-*cis* compounds were collected separately. All-*trans*, 11-*cis*, 9-*cis*, and 13-*cis*-retinol were prepared by $NaBH_4$ reduction of the corresponding retinals and purified separately by HPLC.

Phosphatidylethanolamine (egg), phosphatidylcholine (egg), phosphatidylserine (bovine brain), phosphatidylinositol (bovine liver), and sphingomyelin (bovine brain), phosphatidylserine dilauroyl, phosphatidylserine dimyristoyl, phosphatidylserine dipalmitoyl, and dioleoyl phosphatidylserine were obtained from Avanti Polar Lipids.

Methods—All experimental procedures with retinoids including reaction, extraction, and analysis were carried out under dim red light. The reaction of isomerization was started by mixing 50 μl of ROS solution containing 100 μg protein and 50 μl of substrate solution containing 0.2 mM 11-*cis*-retinol, 12% acetone (v/v), 1.2% Tween 80 (w/v), and 0.125 M Tris-HCl buffer, pH 7.0. Incubation was carried out at 37°C for 2 min. Immediately before use, ROS solution was subjected to thermal treatment at 80°C for 3 min to eliminate the effect of a known heat-labile enzyme. Unless otherwise mentioned, ROS preparation obtained from light-adapted eyes was used. The reaction was stopped with the addition of 0.1 ml of chilled ethanol and 1 ml of chilled *n*-hexane. The suspension was mixed and centrifuged at 12,000 rpm for 1 min at 4°C, then 0.9 ml of the upper layer was carefully collected and the lower layer was once more extracted with 0.5 ml of *n*-hexane. The combined upper layers were evaporated with nitrogen gas and resolved in dichloromethane/ethyl acetate/*n*-hexane (7.7:6.2:86.1). The extracted samples were analyzed by HPLC coupled with a fluorescence spectrophotometer (Ex: 320 nm, Em: 495 nm). The mobile phase was dichloromethane/ethyl acetate/*n*-hexane (7.7:6.2:86.1) at 4 ml/min.

To analyze further the isomerization of 11-*cis*-retinol to all-*trans*-retinol, we used purified rhodopsin or phospholipids extracted from ROS. Phospholipid suspensions were prepared from thin dry films obtained by evaporating the solvent with nitrogen gas and sonicated in 0.05 M Tris-HCl buffer, pH 7.0. We also made phospholipid liposomes and rhodopsin-containing liposomes from lipid extracts of ROS. To make liposomes, phospholipids were evaporated with nitrogen gas and dissolved in 0.5 ml of 10% sodium cholate, 0.15 M NaCl, 5 mM HEPES buffer, pH 7.0. The dissolved phospholipids were mixed with and without rhodopsin (mole ratio of rhodopsin:phospholipids was 1:100). Each mixture was dialyzed three times against 100 ml of 5 mM HEPES buffer, pH 7.0 (*26*). Phosphate was measured after acid hydrolysis (*27*).

In order to examine the effects of detergents, a mixture of ROS solution and detergent solution containing 1% detergents (sodium cholate, sodium dodecyl sulfate, dodecyl trimethyl ammonium bromide, *n*-octyl-β-D-glucoside, Emulphogene BC-720, and Triton X-100), 0.15 M NaCl and 0.05 M Tris-HCl buffer, pH 7.0 was incubated for 3 min at room temperature before adding 11-*cis*-retinol.

We also examined the conversion of exogenous 11-*cis*-retinal to all-*trans*-retinol with ROS, RPE, and ROS plus RPE under an acidic condition. The samples were incubated with 10 nmol of 11-*cis*-retinal containing 12% acetone, 1.2% Tween 80, 1 mM NADH, 0.125 M sodium acetate buffer, pH 5.0 at 37°C for 30 min. The extraction and analysis of retinoids by HPLC were carried out as mentioned above.

RESULTS

A typical HPLC chromatogram of the reaction products is shown in Fig. 1a. Isomerization of 11-*cis*-retinol to all-*trans*-retinol with ROS was prominent and a peak of 13-*cis*-retinol associated with increase in all-*trans*-retinol formation was also detected. This peak of 13-*cis*-retinol was observed only after the formation of all-*trans*-retinol, so all-*trans*-retinol seemed to be the primary product. When 11-*cis*-retinol was removed from the substrate solution, no peak was detected (Fig. 1b). The incubation of 11-*cis*-retinol in the absence of ROS showed a single peak of

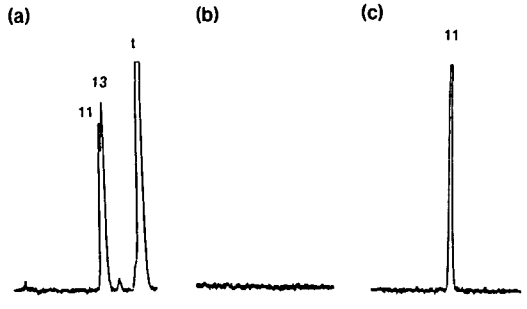

Fig. 1. **All-*trans*-retinol formation by the isomerization of 11-*cis*-retinol.** (a) Ten nanomoles of 11-*cis*-retinol was incubated with 0.1 mg of ROS. 11-*cis*-Retinol, 13-*cis*-retinol, and all-*trans*-retinol were detected. (b) Substrate solution without 11-*cis*-retinol was incubated with 0.1 mg of ROS. No peak was detected. (c) The incubation of 10 nmol of 11-*cis*-retinol and 0.01 M Tris-acetate buffer, pH 7.0. A single peak of 11-*cis*-retinol was detected. Peak identification: 11, 11-*cis*-retinol; 13, 13-*cis*-retinol; t, all-*trans*-retinol.

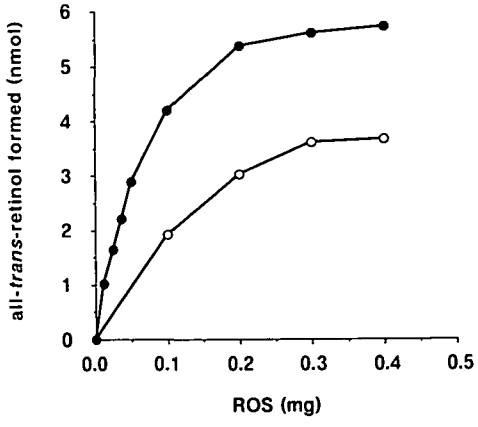

Fig. 2. **The effects of ROS concentration and heat treatment of ROS on the formation of all-*trans*-retinol.** Ten nanomoles of 11-*cis*-retinol was incubated with varying amounts of ROS. ●, boiled ROS at 80°C for 3 min before incubation; ○, untreated ROS.

11-*cis*-retinol (Fig. 1c). Eleven-*cis*-retinol is stable under our experimental conditions and no significant decrease was observed during incubation and extraction. Stereospecificity was not found, because ROS also converted 9-*cis*-retinol or 13-*cis*-retinol to all-*trans*-retinol (data not shown). The isomerizing activity was enhanced by heat treatment of ROS (Fig. 2). All-*trans*-retinol formation was in proportion to the concentration of 11-*cis*-retinol and to the concentration of ROS up to 0.1 mg protein/reaction tube (Figs. 2 and 3). The V_{max} for the isomerization of exogenous 11-*cis*-retinol was estimated to be 78.7 nmol/min/mg ROS protein (Fig. 3). The concentrations of all-*trans*-retinol and 13-*cis*-retinol isomerized from 11-*cis*-retinol approached equilibrium, which was determined to be approximately 75 and 25%, respectively. The time to reach equilibrium was 15 min (Fig. 4). The first-order rate constant was measured at the concentration of 0.3 mg of ROS/tube. It was 0.0097 s^{-1} and the half time was 71 s. Purified opsin preparation showed almost the same isomerization activity as ROS (Fig. 5a), whereas phospholipids extracted from ROS did not convert 11-*cis*-retinol to all-*trans*-retinol (Fig. 5b).

When ROS was incubated in the presence of 1% detergents, the isomerization activity decreased markedly to 5 to 10% of the control activity using untreated ROS (Table I). There was no difference among the detergents we used. The results suggest that phospholipids or other components of ROS may inhibit the isomerization reaction. To confirm this hypothesis, we examined the effects of exogenous phospholipids on the isomerization reaction.

When phospholipid solution was added to the opsin and 11-*cis*-retinol mixture, the isomerization reaction was inhibited (Table II). Opsin-containing liposomes showed reduced isomerization activity. We used phospholipids extracted from ROS and commercially available phosphatidylethanolamine, phosphatidylcholine, phosphatidylser-

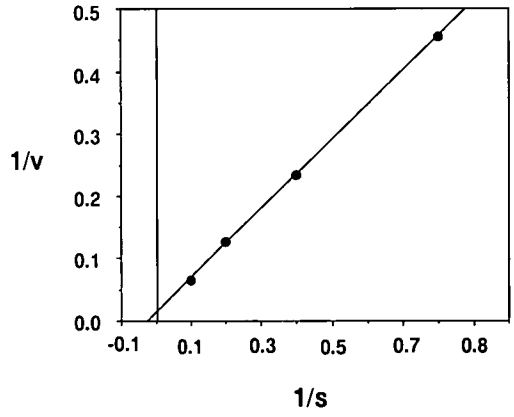

Fig. 3. **Determination of V_{max}.** ROS (0.1 mg) was incubated with varying concentrations of 11-*cis*-retinol for 2 min. V_{max} was determined by means of Lineweaver-Burk plots.

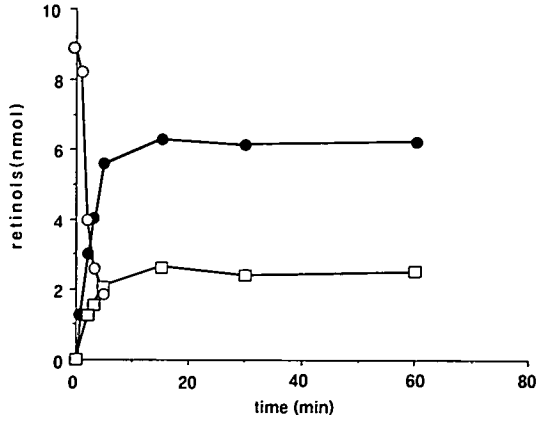

Fig. 4. **Time course of all-*trans*-retinol and 13-*cis*-retinol formation.** 11-*cis*-Retinol (10 nmol) and ROS (0.1 mg) were incubated for 0-60 min. ●, all-*trans*-retinol; □, 13-*cis*-retinol; ○, 11-*cis*-retinol.

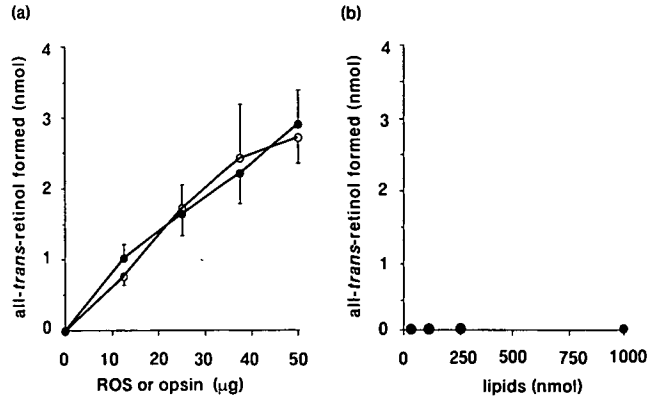

Fig. 5. **All-*trans*-retinol formation from 11-*cis*-retinol with ROS, opsin, and lipids from ROS.** a, ROS (●) or opsin (○); b, lipids extracted from ROS were incubated with 11-*cis*-retinol (10 nmol).

TABLE I. **The effects of various detergents on the isomerization of 11-*cis*-retinol with ROS.**

Detergent	Activity (%)
None	100
Sodium cholate	7.3±0.53
Sodium dodecyl sulfate	10.7±1.17
Dodecyl trimethyl ammonium bromide	6.5±0.30
n-Octyl-β-D-glucoside	7.4±0.63
Emulphogene BC-720	7.3±0.32
Triton X-100	4.7±0.66

Values are mean±SD (n=3). Activities are expressed as percent of the control activity using untreated ROS. 11-*cis*-retinol (10 nmol); ROS (0.05 mg); detergents [1% (w/v)]. Isomerization reaction was carried out as described in "MATERIALS AND METHODS."

TABLE II. **The effects of various phospholipids on the isomerization of 11-*cis*-retinol with opsin.**

Phospholipid	Activity (%)
None	100
ROS lipids	50.2±0.40
ROS lipids (liposome)	45.1±12.1
Phosphatidylserine	29.4±1.37
Phosphatidylethanolamine	56.1±5.85
Phosphatidylcholine	72.0±4.11
Phosphatidylinositol	37.6±6.19
Sphingomyelin	43.8±1.86
Opsin-containing liposome	17.7±0.76

Values are mean±SD (n=3). Activities are expressed as the percent of control activity obtained without phospholipids. 11-*cis*-retinol (10 nmol); opsin (0.36 nmol); phospholipids (36.3 nmol). Isomerization reaction was carried out for 2 min as described in "MATERIALS AND METHODS."

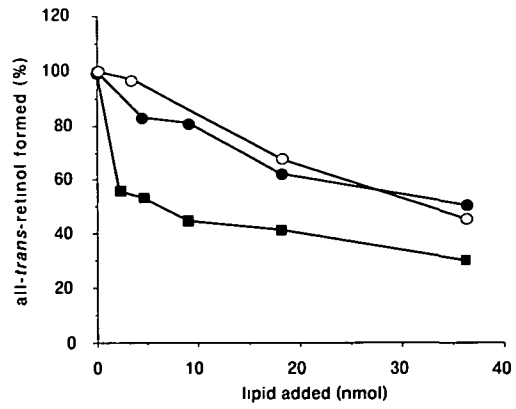

Fig. 6. **Inhibition of all-*trans*-retinol formation by lipids.** ROS (14.5 μg), 11-*cis*-retinol (10 nmol), and varying concentrations of three lipids were incubated for 2 min. ○, liposomes of ROS lipids; ●, ROS lipids sonicated in 0.05 M Tris-HCl buffer, pH 7.0; ■, phosphatidylserine sonicated in 0.05 M Tris-HCl buffer, pH 7.0.

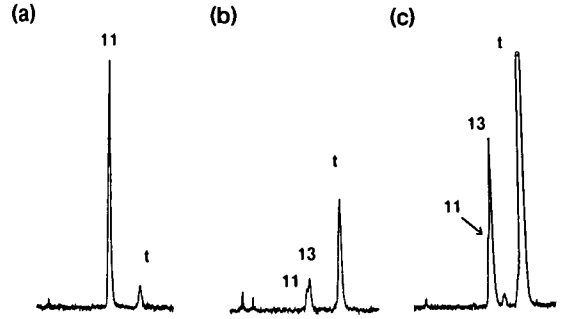

Fig. 7. **The retinol formation from 11-*cis*-retinal.** 11-*cis*-Retinal was incubated for 30 min with crude RPE homogenate (0.05 mg) (a), ROS (0.1 mg) (b), crude RPE homogenate (0.05 mg), and ROS (0.1 mg) (c). The reaction substrate was 10 nmol of 11-*cis*-retinal, 12% acetone (v/v), 1.2% Tween 80 (w/v), 1.0 mM NADH, 0.2 M sodium acetate buffer, pH 5.0. Peak identification: 11, 11-*cis*-retinol; 13, 13-*cis*-retinol; t, all-*trans*-retinol. Conditions for eluting the retinoids are described in the "MATERIALS AND METHODS."

ine, phosphatidylinositol, and sphingomyelin. All had inhibitory effects on the isomerization. This inhibitory effect was dose-dependent (Fig. 6). Phosphatidylserine showed the most potent inhibitory effect on the isomerization. Then we tried the effects of various phosphatidylserines having varying carbon chains (phosphatidylserine dilauroyl, phosphatidylserine dimyristoyl, phosphatidylserine dipalmitoyl, dioleoyl phosphatidylserine). These phosphatidylserines had similar inhibitory effects on the isomerization reaction (data not shown).

We compared the isomerization activity of rhodopsin with that of opsin which was obtained by illumination of rhodopsin solution for 10 min immediately before use. Opsin was 1.5 times more effective on the isomerization than rhodopsin. We also compared the activity of photobleached ROS and dark-adapted ROS. Bleached ROS was 1.5 times more effective on the isomerization than dark-adapted ROS.

ROS, RPE crude homogenate, and the RPE homogenate plus ROS were incubated in the presence of 11-*cis*-retinal to confirm the formation of all-*trans*-retinol under acidic conditions. Typical HPLC chromatograms of reaction products are shown in Fig. 7. RPE converted 11-*cis*-retinal to 11-*cis*-retinol in the presence of 1 mM NADH. Only small amounts of 11-*cis*, 13-*cis*, and all-*trans*-retinol were detected when 11-*cis*-retinal was incubated with ROS, whereas sufficient amounts of all-*trans*-retinol were formed from 11-*cis*-retinal by incubating it with ROS and RPE.

DISCUSSION

In the present study, we observed the dark isomerization of 11-*cis*-retinol to all-*trans*-retinol with ROS. Rhodopsin was found to isomerize 11-*cis*-retinol to all-*trans*-retinol as well as ROS, but phospholipids did not. Jones *et al.* reported a toxic effect of 11-*cis*-retinol on the recovery of photosensitivity in an electrophysiological study (*19*). Dark isomerization of 11-*cis*-retinol seems to be important, since it is also reported that 11-*cis*-retinol exists in the interphotorecepter matrix (IPM) (*28*) and mammalian ROS can not use 11-*cis*-retinol for rhodopsin regeneration (*29*), probably due to the lack of 11-*cis* specific retinol dehydrogenase. The origin of the 11-*cis*-retinol in the IPM is not well understood. Possibly, it is delivered from the RPE or retinal Müller cells. Some of the 11-*cis*-retinol binds to interphotoreceptor retinoid-binding protein (IRBP) in the IPM (*30-33*). In the case of partially bleached cattle eyes, approximately 7% of the binding sites of the IRBP were saturated with endogenous ligands (11-*cis*-retinol, 88%; all-*trans*-retinol, 12%) (*32*). As IRBP retards the transfer of retinoids (*34*), this binding may reduce the toxic effect of 11-*cis*-retinol to ROS. On the other hand, no binding protein has been found inside the ROS as yet. This implies that it would be beneficial for the visual system to convert 11-*cis*-retinol delivered into the ROS to a form of retinoid which is available to the visual cycle.

In a well-known visual cycle, all-*trans*-retinal released from photobleached rhodopsin is converted to all-*trans*-retinol by ROS retinol dehydrogenase (ROS-RDH) and reused in the RPE (scheme shown in Fig. 8). ROS-RDH, however, has low enzymatic activity in the ROS and all-*trans*-retinal accumulates at high bleach rates (*6*). Practically, the velocity for the reduction of exogenous all-*trans*-retinal to all-*trans*-retinol by ROS-RDH was 5.2 nmol/min/mg ROS in our previous study (*5*) while the velocity for the isomerization of exogenous 11-*cis*-retinol in ROS was 78.7 nmol/min/mg ROS in the present study.

Another possible role of the isomerization of 11-*cis*-retinol to all-*trans*-retinol by ROS is the recycling of 11-*cis*-retinal contained in the phagocytized ROS (scheme shown in Fig. 8). It is well known that RPE phagocytizes ROS tips daily (*20, 21*). As the phagolysosomes are acidic, 11-*cis*-retinal contained in the engulfed ROS is converted to 11-*cis*-retinol by 11-*cis* specific RDH of RPE. Then 11-*cis*-retinol could be converted to all-*trans*-retinol by the isomerization reported in this study and all-*trans*-retinol returns to the visual cycle and is reused for rhodopsin regeneration or retinoid storage. Incubation of 11-*cis*-retinal with ROS and RPE at pH 5.0 (Fig. 7) supports this hypothesis.

The mechanisms of isomerization in the ROS have not been fully elucidated. Groenendijk *et al.* (*17*) demonstrated dark isomerization of retinals with ROS and they concluded that the dark isomerization of retinals was accounted for by the formation of a Schiff base between

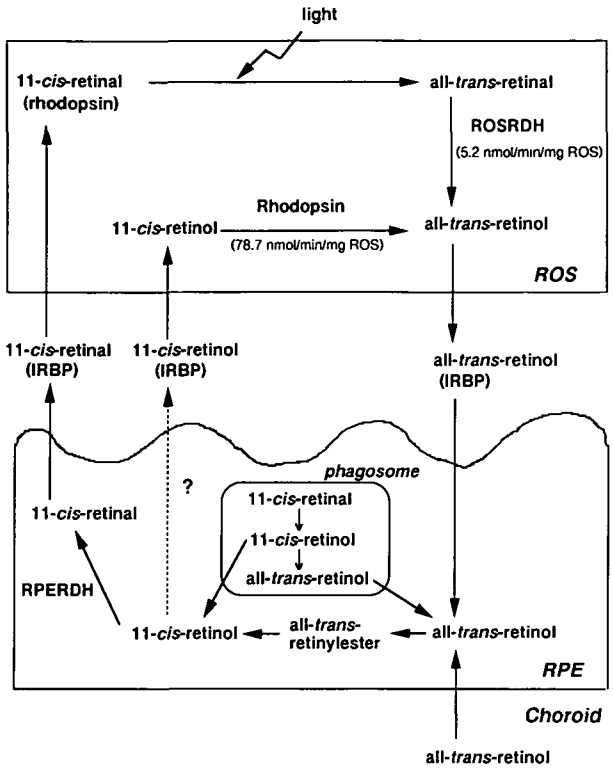

Fig. 8. **Possible roles of 11-*cis*-retinol isomerization in the ROS and phagosomes of the RPE.** In the visual cycle, all-*trans*-retinal released from photobleached rhodopsin is converted to all-*trans*-retinol by ROS retinol dehydrogenase and reused in the RPE. Rhodopsin may play further roles in removing 11-*cis*-retinol from the ROS and recycling it to the visual cycle in the phagosomes.

phosphatidylethanolamine and retinals. This mechanism would not be applicable to retinol, which can not form a Schiff base. Our results indicated that phospholipids do not mediate the isomerization but may inhibit the reaction, whereas rhodopsin showed the isomerization activity. Conformational change of rhodopsin to opsin by light or by thermal treatment increased the isomerization activity. The isomerizing activity of rhodopsin in ROS was decreased by washing out all-*trans*-retinal with NH_2OH (data not shown). These results indicate that the conformational change of rhodopsin may affect the isomerization reaction.

The velocity of the isomerization reached a plateau at the concentration of 0.4 mg ROS/0.1 ml substrate solution or more, as shown in Fig. 2. The rhodopsin content of 0.4 mg ROS is approximately 8.0 nmol using 40,000 as the molecular weight and based on the report that over 80% of the protein in bovine ROS is rhodopsin (*23*). As 11-*cis*-retinol added was 10 nmol, maximal isomerization was obtained at a rhodopsin/11-*cis*-retinol mole ratio of approximately 0.8:1. The results suggest that one molecule of rhodopsin interacts with one molecule of 11-*cis*-retinol in ROS.

The isomerizing activity of ROS was markedly reduced by treatment with various detergents. The inhibitory effect of the detergents on isomerization was considered to be primarily due to released phospholipids, but not to the denaturation of rhodopsin, since the rhodopsin extracted from ROS by 1.3% Emulphogene BC-720 showed similar isomerization activity to ROS. The inhibition of isomerization by phospholipids was dose-dependent. But the isomerization activities of ROS and rhodopsin were similar even though the molar ratio of phospholipid to rhodopsin of ROS membrane is higher than that of purified rhodopsin [110 for ROS membrane, 15 for purified rhodopsin (*35*)]. There is an asymmetric arrangement of phosphatidylserine in the disk membrane (*36*). Eighty-two percent of the phosphatidylserine is located on the cytoplasmic surface. Active sites of the isomerization may be located in the intradiscal monolayer, so phosphatidylserine does not affect the isomerization in the native ROS. When the asymmetry was destroyed by the detergents, phosphatidylserine could approach the active sites and reduce the isomerization activity.

In conclusion, isomerization from 11-*cis*-retinol to all-*trans*-retinol by ROS could be beneficial for the detoxification of 11-*cis*-retinol and recycling of the retinoids to the visual cycle. However, further investigation is needed to clarify the mechanism of the isomerization and its relation to phospholipids.

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