Interaction of 11-cis-Retinal Dehydrogenase with the Chromophore of Retinal G Protein-coupled Receptor Opsin*

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Vertebrate opsin s in both photoreceptors and the retinal pigment epithelium (RPE) have fundamental roles in the visual process. The visual pigments in photoreceptors are bound to 11-cis-retinal and are responsible for the initiation of visual excitation. Retinol-like opsin s in the RPE are bound to all-trans-retinal and play an important role in chromophore metabolism. The retinal G protein-coupled receptor (RGR) of the RPE and Müller cells is an abundant opsin that generates 11-cis-retinal by stereospecific photoisomerization of its bound all-trans-retinal chromophore. We have analyzed a 32-kDa protein (p32) that co-purifies with bovine RGR from RPE microsomes. The co-purified p32 was identified by mass spectrometric analysis as 11-cis-retinol dehydrogenase (cRDH), and enzymatic assays have confirmed the isolation of an active cRDH. The co-purified cRDH showed marked substrate preference to 11-cis-retinal and preferred NADH rather than NADPH as the cofactor in reduction reactions. cRDH did not react with endogenous all-trans-retinal bound to RGR but reacted specifically with 11-cis-retinal that was generated by photoisomerization after irradiation of RGR. The reduction of 11-cis-retinal to 11-cis-retinol by cRDH enhanced the net photoisomerization of all-trans-retinal bound to RGR. These results indicate that cRDH is involved in the processing of 11-cis-retinal after irradiation of RGR opsin and suggest that cRDH has a novel role in the visual cycle.

The continual synthesis of rhodopsin by recombination of its apoprotein with the chromophore, 11-cis-retinal, is an essential process that maintains visual excitation (1, 2). It has long been known that formation of 11-cis-retinal for regeneration of rhodopsin is dependent on retinoid metabolic reactions in the retinal pigment epithelium (RPE),† where the majority of enzymes of the visual cycle are located (reviewed in Ref. 3). In the current model of the visual cycle, all-trans-retinal from bleached rhodopsin is reduced to all-trans-retinol by an all-trans-retinol-specific dehydrogenase (tRDH) located in photoreceptor outer segments (4–6). The all-trans-retinol is then delivered to the RPE, where it is converted by the lecithin-retinol acyltransferase to all-trans-retinyl ester (7, 8). A key step in the visual cycle is performed by an isomerohydrolase that catalyzes the formation of 11-cis-retinol from all-trans-retinyl ester (9–10). Alternatively, the isomerization of all-trans-retinol to 11-cis-retinol is achieved via an intermediate with an anhydro-like carbocation structure (11). An 11-cis-retinol-specific dehydrogenase (cRDH) is able to oxidize 11-cis-retinol to 11-cis-retinal (12–14), which is transferred to the outer segments of photoreceptors for recombination with opsin to form rhodopsin. Under light-adapted conditions, the rate of synthesis of 11-cis-retinal must be sufficient for regeneration of steady-state levels of visual pigments (15, 16).

Besides isomerohydrolase, another type of isomerase in the RPE may include the retinol-like visual pigment homologues peropsin or the RPE retinal G protein-coupled receptor (RGR) opsin (17, 18). In contrast to the visual pigments, RGR is bound in the dark to endogenous all-trans-retinal and is localized to intracellular membranes in RPE and Müller cells (19, 20). Upon illumination, all-trans-retinal bound to RGR is photoisomerized stereospecifically to the 11-cis isomer (20). These results provide evidence that RGR may function to generate 11-cis-retinal in vivo and participate in a light-dependent photic visual cycle.

A proposed mechanism of RGR function is that 11-cis-retinal dissociates from irradiated RGR and directly enters the pathway for regeneration of rhodopsin under photic conditions. A central hypothesis of the photoisomerase model is that exchange of the chromophore bound to RGR occurs and involves distinct geometrical isomers. Retinal must uncouple and bind anew to RGR through a stereospecific cycle that is driven by light energy. The observation that microsomal RGR can be labeled with 3H-labeled all-trans-retinal (21) and that ~50% of RGR isolated from RPE is in the form of the apoprotein (22) suggests that the binding of retinal is reversible and that the process of binding and dissociation of the chromophore occurs in vivo.

One of the factors that may control Schiff base hydrolysis and dissociation of 11-cis-retinal from RGR in vivo is specific protein interactions. We have observed that when RGR is isolated in digitonin solution from bovine RPE microsomes by immunoaffinity chromatography, it co-purifies consistently with a 32–34-kDa protein (p32) (22). It is possible that the co-purified protein forms a physical complex or associates functionally with RGR. Hence, the identification of the co-purified p32 protein may provide important insights into the mechanism of RGR function.

In this paper, we report the analysis and characterization of co-purified p32, which has been identified as 11-cis-retinol de-
hydrogenase of the RPE. We demonstrate enzymatic activity of the co-purified cRDH and discuss its potential role in RGR function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Digitonin was obtained from Calbiochem-Novabiochem. Hydroxylamine and all-trans-retinol were purchased from Sigma. 11-cis-Retinal was provided by Dr. Rosalie Crouch (Medical University of South Carolina, Charleston, SC). An 11-cis-retinol standard was prepared by reduction of 11-cis-retinal in the presence of NaBH₄ and was purified by high performance liquid chromatography (HPLC) as described previously (23). Organic solvents were HPLC grade. Dichloromethane and hexane were obtained from Fisher. Ethanol and methanol were from J. T. Baker Inc. Dioxane was from Burdick & Jackson.

**Isolation of RGR**—All isolation procedures were performed under dim red light. RPE cells were isolated from fresh bovine eyes, as described previously (22). The cells were washed in ice-cold 0.25 M sucrose, 50 mM sodium phosphate buffer, pH 6.5, and homogenized in a glass Dounce homogenizer. After a low speed centrifugation at 300 × g, the homogenate was centrifuged at 15,000 × g for 20 min at 4 °C. RPE microsomes in the supernatant were sedimented by centrifugation at 150,000 × g for 1 h at 4 °C and stored at −80 °C until later use. For isolation of RGR, the microsomal membranes were extracted three times for 1 h at 4 °C with 1.2% digitonin solution containing 10 mM sodium phosphate, pH 6.5, 150 mM NaCl, and 0.5 mM EDTA. The extracts were centrifuged each time at 100,000 × g for 20 min at 4 °C. The pooled supernatants were incubated overnight at 4 °C with Affi-Gel 10 resin (Bio-Rad) conjugated to monoclonal antibody 2F4, which is raised against the human RGR. The antibody was removed by washing and dried again under nitrogen. The residue was either stored in darkness at −80 °C or used directly for HPLC.

**HPLC Analysis of Retinoloximes**—The isomers of retinoloximes were analyzed by HPLC, as described previously (20). The extracted retinoloximes were dissolved in hexane and analyzed by a LiChrosorb RT Si60 silica column (4 × 250 mm, 5 μm) (Merck). The HPLC system was equipped with a Beckman model 126 solvent module and module 166 UV-visible detector (Beckman Instruments). The 30-μl samples were introduced into the column and resolved on a Resolve silica column (35 cm × 3.5 mm, 5 μm) (Waters Corp.). Identification of the retinoloxime isomers was based on the retention times of the known retinoloxime products. Absorbance was measured at 360 nm, and the absorbance peaks were integrated with the Gold Nouveau software (Waters Corp.). The retention times of the known anti- and syn-retinoloxime isomers was based on the following extinction coefficients (εmax in hexane): all-trans syn = 54,900, all-trans anti = 51,600, 11-cis syn = 35,000, 11-cis anti = 29,600, 13-cis syn = 49,000, and 13-cis anti = 52,100 (31, 32).

**HPLC Analysis of 11-cis-Retinol**—11-cis-Retinol was co-extracted with retinoloximes and analyzed by HPLC, as described above. In some experiments, the retinoids were separated on a Resolve silica column (3.9 × 150 mm, 5 μm) (Waters Corp., Milford, MA) using a Waters 2690 HPLC module. The samples were resolved in a running solvent consisting of hexane supplemented with 8% diethyl ether and 0.33% ethanol.

**11-cis-Retinol Dehydrogenase Assay with Exogenous Substrate**—The reduction activity of cRDH was measured as described previously (22, 28). The thio Barb solution was used to monitor the reduced derivative formed by retinal and thio Barbicar (29). The immunoaffinity-purified RGR or RPE microsomal proteins were used as the source of 11-cis retinal. For the reduction of exogenous substrate, the substrate solution contained 10 nmol of exogenous retinal isomers, 12% acetone, 1.2% Tween 80, 0.1% NaN₃ or NaDH, and 0.2% sodium acetate buffer, pH 5.0. Controls were performed by omitting the NADH or NaDH cofactor or by addition of a heat-inactivated protein sample. The reactions were initiated by the addition of 50 μl of substrate solution to 50 μl of protein solution. After incubation at 37 °C, the reaction was terminated by the addition of 0.5 ml of ethanol. Subsequently, 0.2 ml of perchloric acid 0.2 ml of toluene and 0.5 ml of trichloroacetic acid were added, and the samples were incubated for 30 min at room temperature. The solutions were centrifuged at 14,000 rpm on a tabletop centrifuge (Eppendorf Centrifuge 5415C, Germany) for 1 min. 50 μl of distilled water was added to each supernatant to prevent precipitation. Color development was measured at 530 nm. Ethanol was used as a reagent blank for the color development. The concentration of exoge-
RESULTS

Isolation of RGR and Identification of Co-purified 32-kDa Protein—RPE microsomal membrane proteins were extracted in 1.2% digitonin solution at pH 6.5, and solubilized RGR was purified by immunoaffinity chromatography under dim red light. Under these conditions, a 32-kDa protein (p32) was found to co-purify reproducibly with RGR (Fig. 1). The p32 protein band was readily detectable in SDS-polyacrylamide gels by staining with Coomassie Blue. It was not recognized on Western blots by the antibody that was coupled to the immunoaffinity resin, even when the immunoblots were overdeveloped (results not shown). The relative amount of p32 that was co-purified with RGR was significantly increased by the inclusion of glycerol in the isolation procedure (Fig. 1A, lane 3). When the antibody binding sites of the immunoaffinity column were blocked by prewashing with excess peptide, neither RGR nor p32 was eluted from the column (Fig. 1B), and p32 did not appear isolatable as a nonspecifically adsorbed protein. The p32 band was excised from a SDS-polyacrylamide gel and analyzed by liquid chromatography MS/MS. When the resulting MS/MS spectra were correlated to the data base of known protein sequences using Sequest, peptides of p32 that matched the sequence of cRDH, 11-cis-retinol dehydrogenase (Accession no. A55429), are indicated by lines drawn above the corresponding portion of the sequence. Observed and calculated (in parentheses) m/z values for the doubly protonated molecular ion are given above the line. High resolution (Zoom) scan used to determine the accurate m/z value and charge state of the ion corresponding to the peptide LLWLPASYLPAR (m/z 519.9). The MS/MS data were correlated to the data base of known protein sequences using Sequest. The protein band was subjected to trypsin in-gel digestion, and the resulting MS/MS spectra were correlated to the data base of known protein sequences, a number of them giving positive matches to 11-cis-retinol dehydrogenase, a known critical enzyme.

Co-purification of Retinol Dehydrogenase Activity—An assay was performed to verify the presence of enzymatically active cRDH in preparations of RGR. Exogenous 11-cis-retinal was used as the substrate. The reduction of 11-cis-retinal was followed by its disappearance and was dependent on the amount of immunoaffinity-purified RGR added to the reaction (Fig. 3). The activity required the NADH cofactor. The redox properties of the dehydrogenase were tested in preparations of microsomal membranes. The enzyme in the microsomal membranes displayed substrate-specific activity with either cofactor, NADH or NADPH. These observed properties of microsomal cRDH were consistent with the results of previous studies (12–14).

Effect of cRDH on 11-cis-Retinal Bound to Irradiated RGR—Since cRDH reacts specifically with 11-cis-retinal, the role of co-purified cRDH in RGR function may be to reduce and re-isomerize of its all-trans-retinal chromophore. In previous experiments, cRDH reduction activity was not observed because the required cofactor was not added (20). RGR was kept in the dark or irradiated with blue light.
The predominant chromophore extracted from nonirradiated RGR was all-trans-retinal, as expected (Fig. 5A), and incubation with NADH had no effect on the all-trans chromophore in the dark (Fig. 5B). The irradiation of RGR and subsequent incubation in the dark without NADH resulted in stereospecific isomerization of ~55% of the bound all-trans-retinal to 11-cis-retinal (Fig. 5C); these results were in agreement with previous studies (20). When RGR was irradiated and then incubated in the dark in the presence of NADH, there was a significant decrease in total retinal. The decrease in total retinal was brought about by the selective loss of 11-cis-retinal with little effect on the amount of the all-trans isomer (Fig. 5D). Changes in the chromophore of RGR were most prominent when NADH was present during irradiation of RGR. Under conditions in which the cofactor is included and cRDH is active, irradiation of RGR resulted in significant loss of both 11-cis- and all-trans retinal (Fig. 6).

**Synthesis of 11-cis-Retinol during Irradiation of RGR**—To confirm that 11-cis-retinal was reduced by the co-purified cRDH with retention of the expected isomeric configuration, we demonstrated the production of 11-cis-retinol upon irradiation of RGR in the presence of NADH. The retinol extracts from irradiated preparations of RGR were analyzed after exposure to light for different lengths of time. Absorbance was measured at 320 nm to optimize the detection of 11-cis-retinol. The irradiation of RGR in the presence of NADH resulted in significant decline of all-trans-retinal and a concomitant time-dependent increase in 11-cis-retinol (Fig. 7). The amount of 11-cis-retinol formed corresponded closely to the amount of all-trans-retinal lost at 5 min; however, the production of 11-cis-retinol lagged the more rapid decline in all-trans-retinal in the first 1–2 min of incubation. Since cRDH uses the 11-cis isomer specifically, we conclude that the increase in 11-cis-retinol is due to the reaction of cRDH on free or bound 11-cis-retinal, the photosomerization product of irradiated RGR.

**DISCUSSION**

The RGR opsin is a major all-trans-retinal-binding protein in the RPE. Physiological evidence that supports the processing of precursor all-trans-retinol and its incorporation into the chromophore of RGR in the dark has been obtained recently (33). Illumination of RGR results in stereospecific conversion of the bound all-trans-retinal to 11-cis-retinal. Since retinoid-binding proteins would be required to transfer the chromophore to or from RGR, the mechanism by which RGR functions may depend on specific protein interactions. In this paper, we demonstrate functional interaction in vitro between the RGR opsin and cRDH. Indeed, the isolation of highly purified RGR from bovine RPE results in consistent co-purification of cRDH in a manner that suggests that cRDH binds to RGR in a protein complex.

In the presence of glycerol, co-purification of cRDH was optimal and Coomassie Blue staining of cRDH was typically as intense and specific as that of RGR in polyacrylamide gels. A general effect of glycerol is to stabilize the protein complex and reduce nonspecific binding to the affinity resin (34). The co-purified enzyme was active and held several properties similar to those of microsomal cRDH (12–14), isolated cRDH (28), or recombinant cRDH (35, 36). It was efficient in the reduction of exogenous 11-cis-retinal and had little reactivity with the all-trans isomer. NADH was preferred over NADPH as a cofactor in the reduction reaction. In contrast, microsomal cRDH activity was observable with both NADH and NADPH (Fig. 4, C and D). Despite the presence in the RPE of cRDH activity that uses NADPH (12, 37), co-purification of the NADPH-dependent enzyme with RGR was not evident.

Most noteworthy was the substrate stereospecificity of cRDH.
in reactions with the chromophore of RGR. The co-purified enzyme was inert to all-trans-retinal bound to RGR in the dark but active toward endogenous 11-cis-retinal that was generated by irradiation of RGR. The cRDH was remarkably stable during co-purification with RGR over a period of 28 h, and its activity did not require purification of the enzyme in the presence of NADH, as reported previously (28).

The functional significance of RGR and cRDH interaction may lie in the mechanism of chromophore dissociation from RGR. Like squid rhodopsin and other invertebrate visual pigments (38, 39), the chromophore of RGR opsins does not readily dissociate after photoisomerization in vitro, and irradiation does not lead to bleaching of RGR, as determined by difference absorption spectra before and after light exposure (22). If RGR operates in vivo as a stereospecific photoisomerase to directly generate the 11-cis-chromophore in the visual cycle, then all-trans-retinal should be photoisomerized and 11-cis-retinal should be released from RGR catalytically. The previous experiments on irradiation of RGR were run without NADH and achieved at most 50% net conversion of all-trans- to 11-cis-retinal (20). We now can demonstrate more complete photoisomerization of the bound all-trans-retinal to the 11-cis isomer. When RGR is irradiated in the presence of NADH, isomerization of the chromophore is coupled to efficient reduction by cRDH. These results strongly suggest that 11-cis-retinol is generated in a photic visual cycle. Possible steps in further processing of 11-cis-retinol include its conversion to 11-cis-retinyl esters or binding to cellular retinaldehyde-binding protein. The results do not exclude an additional mechanism of dissociation and direct transfer of 11-cis-retinal from RGR to cellular retinaldehyde-binding protein or any effect of cellular retinaldehyde-binding protein on the interaction between RGR and cRDH.

The functional coupling of RGR with cRDH extends the evidence that RGR and rhodopsin have evolved with distinct, yet parallel, features. The chromophores of RGR and rhodopsin are photoisomerized in opposite directions; hence, each is bound to 11-cis- and all-trans-retinal at a specific photochemical state of the opsin. After photoisomerization of the chromophore, 11-cis-retinal from RGR is reduced by cRDH, and all-trans-retinal from rhodopsin is reduced by tRDH to the respective alcohols. The cRDH and tRDH are highly homologous retinol dehydrogenases (6, 35, 36). The reduction of all-

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**FIG. 5. Interaction of co-purified 11-cis-retinol dehydrogenase with the chromophore of RGR.** Equal aliquots of RGR from the same preparation were irradiated or not for the first 5 min without cofactor. Subsequently, each sample (260 ng of RGR) was incubated in the dark for an additional 5 min in the presence or absence of NADH. Retinal isomers were then extracted by the hydroxylamine derivatization method and analyzed by HPLC. A, incubation in the dark for 10 min without NADH. B, incubation in the dark for 5 min and then incubation in the dark for another 5 min in the presence of 3 mM NADH. C, irradiation for 5 min and then incubation in the dark for 5 min without NADH. D, irradiation for 5 min followed by incubation in the dark for 5 min in the presence of 3 mM NADH. The incubations were performed at 37 °C, and the irradiated samples were exposed to 470-nm monochromatic light. RGR in this and all following experiments was isolated in the presence of 20% glycerol. 11, 11-cis syn-retinaloxime; all, all-trans syn-retinaloxime; 13, 13-cis syn-retinaloxime. In these experiments, the anti isomers of the retinaloximes were below the level of detection.

**FIG. 6. Irradiation of RGR in the presence of NADH-dependent 11-cis-retinol dehydrogenase activity.** Equal aliquots of RGR from the same preparation were irradiated with 470-nm monochromatic light for 5 min at 37 °C in the absence (upper trace) or in the presence (lower trace) of 3 mM NADH (260 ng of RGR/reaction). The retinal isomers were then extracted by the hydroxylamine derivatization method and analyzed by HPLC. 11, 11-cis syn-retinaloxime; all, all-trans syn-retinaloxime; 13, 13-cis syn-retinaloxime. In these experiments, the anti isomers of the retinaloximes were below the level of detection.
FIG. 7. Production of 11-cis-retinol by irradiation of RGR in the presence of NADH. Equal aliquots of RGR from the same preparation were irradiated with 470-nm monochromatic light for various lengths of time at 37 °C in the presence of 3 mM NADH (640 ng RGR/reaction). The reactions were stopped, and the retinal isomers were extracted at the indicated times by the hydroxylamine derivatization method. 11-cis-Retinol was co-extracted and analyzed simultaneously by HPLC. Two representative experiments are shown for the decline in all-trans-retinaloxime, corresponding to the all-trans-retinal chromophore of RGR (A), and synthesis of 11-cis-retinol (B). The accumulation of 11-cis-retinol did not occur without NADH. C, HPLC chromatograms of the absorbance (AU) of 11-cis-retinol at 320 nm at various time points of a single experiment. D, representative HPLC chromatograms of the 11-cis-retinol standard (5.5 pmol) and extracted retinoids after 0 and 7-min incubations. The retinoids were detected by absorbance at 320 nm using the Waters HPLC system and were predominantly all-trans syn-retinaloxime and 11-cis-retinol (arrows) after 0 and 7-min incubations, respectively.
trans-retinal participates in the inactivation of rhodopsin and is a rate-limiting step of the visual cycle at high light levels (5). The tRDH is tightly associated with the rod outer segments (40–43); however, its orientation in the rod outer segment membrane and possible binding to rhodopsin are unknown. In comparison, there is evidence that the catalytic domain of cRDH in the RPE is situated toward the lumen of the smooth endoplasmic reticulum (44). This membrane topology of cRDH suggests that 11-cis-retinal from irradiated RGR is reduced by cRDH and presented at the lumenal surface of the smooth endoplasmic reticulum for transport or further processing of the alcohol.

The physiological significance of the 11-cis-retinal reduction reaction involving cRDH and RGR needs to be corroborated by in vivo studies. Another reductase role for cRDH was first proposed by Lion et al. (12). Most models of the visual cycle since then have emphasized the oxidation reaction of cRDH instead, because no experiment to regenerate rhodopsin with cRDH has succeeded (45–47). On the other hand, cone pigments in excised retina can be regenerated with 11-cis-retinol (48). In this case, the reduction of 11-cis-retinal from RGR to 11-cis-retinol might be sufficient for chromophore synthesis by the RPE in a cone visual cycle. Alternatively, the 11-cis-retinol from RGR may be converted to the retinyl ester for storage or usage as a requisite intermediate in the synthesis of 11-cis-retinol. Our results provide a new argument for a reductase role of cRDH in addition to its role in the alcohol.

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