Blue and Ultraviolet Light-Absorbing Opsin from the Retinal Pigment Epithelium†

Wenshan Hao‡ and Henry K. W. Fong*,‡,§

Departments of Microbiology and Ophthalmology, University of Southern California School of Medicine, and Doheny Eye Institute, Los Angeles, California 90033

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ABSTRACT: The retinal pigment epithelium (RPE) contains an abundant opsin that is distinct from rhodopsin and cone visual pigments and is able to bind the retinaldehyde chromophore. The putative retinal G protein-coupled receptor (RGR) was isolated in digitonin solution from bovine RPE microsomes and copurified consistently with a minor 34-kDa protein. The absorption spectrum of RGR revealed endogenous pH-sensitive absorbance in the blue and near-ultraviolet regions of light. Membrane-bound RGR was incubated with exogenously added *all-trans*-retinal and formed two long-lived pH-dependent photopigments with absorption maxima of 469 ± 2.4 and 370 ± 7.3 nm. The effects of hydrogen ion concentration suggest that the blue and near-UV photopigments are tautomeric forms of RGR, in which an *all-trans*-retinal Schiff base is protonated or unprotonated, respectively. The RPE pigment was also demonstrable by its reactivity to hydroxylamine in the dark. The retinaldehyde−RGR conjugate at neutral pH favors the near-UV pigment and is a novel light-absorbing opsin in the vertebrate eye.

Retinal photoreceptors contain rhodopsin or cone pigments that consist of 11-*cis*-retinal (or 11-*cis*-3,4-dehydroretinal) as chromophore and a specific opsin as protein (Wald, 1968; Dratz & Hargrave, 1983; Nathans, 1987). Upon illumination, all visual pigments activate G proteins to initiate the process of photosensory transduction (Stryer, 1988; Pak & Shortridge, 1991). Recently, identification of nonvisual opsins has revealed additional evolutionary branches in the vertebrate opsin gene family. Visual pigment homologues have been detected in the chicken pineal gland (Okano et al., 1994; Max et al., 1995) and in mammalian retinal pigment epithelium (RPE)[1] and Müller cells (Jiang et al., 1993). The RPE opsin (RPE−retinal G protein-coupled receptor, or RGR) and pineal gland opsin (pinopsin, or P-opsin) are approximately 25% and 45% identical, respectively, in amino acid sequence to the vertebrate visual pigments.

RGR is also related distantly to retinochrome and shares amino acid sequence similarity with the invertebrate photoisomerase (Hara-Nishimura et al., 1990). The RPE opsin has been shown to be membrane-bound and located primarily within the cytoplasm of RPE and Müller cells (Pandey et al., 1994). RGR contains a conserved lysine residue that is homologous with the retinaldehyde attachment site in the visual pigments and is able to bind both *all-trans*- and 11-*cis* isomers of retinal in vitro (Shen et al., 1994). The ability of RGR to bind retinal in vitro and its status as a member of the opsin family support the prediction that RGR mediates its biological function through photoreception. Given the significant divergence between the amino acid sequences of RGR and the visual pigments and the absence of a conserved Schiff base counterion in RGR, it is probable that the retinal-bound RPE opsin possesses a unique absorption spectrum as a result of specific protein−chromophore interactions. In this study, we obtain an absorption spectrum for purified bovine RGR and analyze some of the spectral properties of RGR that is bound to *all-trans*-retinal.

MATERIALS AND METHODS

Isolation of Bovine RGR. After excision of anterior structures and the neural retina, RPE cells were removed from bovine eyes by gently scraping the epithelial cell monolayer with a spatula. Microsomal membranes from RPE cells were isolated under red or dim yellow light within 4 h of enucleation, as described previously (Shen et al., 1994). The subsequent purification of RGR was conducted under red light or in the dark. The membranes were twice extracted for 1 h at 4 °C with 1.2% digitonin (Eastman Kodak Co., Rochester, NY) in 10 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl and 0.5 mM EDTA. After centrifugation of the extract at 100000*g* for 20 min, the supernatant was mixed for 2 h at 4 °C with Affi-Gel 10 resin (Bio-Rad, Hercules, CA) conjugated to anti-bovine RGR monoclonal antibody 2F4 (Shen et al., 1994). The immunoaffinity resin was transferred to a column for washing with 25 bed volumes of 10 mM sodium phosphate buffer, pH 6.5, containing 0.1% digitonin, 150 mM NaCl, and 0.5 mM EDTA. The column was then loaded 10 times with 0.5 bed volume of wash buffer containing 100 μM bovine RGR carboxyl-terminal peptide (CLSPQRREHSREQ). The eluates were pooled and concentrated approximately 4-fold using a Centricon-3 concentrator (Amicon, Inc., Beverly, MA). Gel electrophoresis and immunoblot of proteins were performed, as described previously (Jiang et al., 1993; Pandey et al., 1994).

The concentration of purified RGR was measured using the Bio-Rad Protein Assay reagents, following removal of the elution peptide from RGR by repeated dilution and

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* Address correspondence to this author at the Doheny Eye Institute, 1355 San Pablo St., Los Angeles, CA 90033.

‡ Department of Microbiology, University of Southern California.

§ Doheny Eye Institute.

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[1] Abbreviations: RPE, retinal pigment epithelium; RGR, RPE−retinal G protein-coupled receptor; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate.

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ultrafiltration through Centricon-3 concentrator tubes. The measurements were corrected for background using a control volume of the elution buffer that was concentrated and treated identically as the RGR sample. Bovine serum albumin was used as the standard for protein quantitation. The abundance of RGR in the RPE cell homogenate and microsomal membranes was determined from the relative signal intensities on Western blots, which were calibrated with varying amounts of each sample and purified RGR. The band intensities were quantitated using an Ultroscan XL laser densitometer (Pharmacia LKB, Bromma, Sweden).

Spectroscopic Measurements. The UV−visible absorption spectra of purified RGR were recorded with a Hitachi U-3000 scanning spectrophotometer on samples of 1.0-cm path length at room temperature. The reference sample consisted of the elution buffer−10 mM sodium phosphate (pH 6.5), 150 mM NaCl, 0.5 mM EDTA, 0.1% digitonin, and 100 μM carboxyl-terminal peptide. The reference showed no light absorbance in the visible region; however, digitonin and the peptide were capable of absorbing UV light. This absorbance by the buffer components introduced some distortion in the far-UV region of the absorption spectra of RGR; thus, the region below 300 nm was omitted from the spectra, and absorbance ratios were not used as a criterion of purity. The pH of the sample was raised by addition of 1 M Na_2HPO_3, or lowered by addition of 1 M NaH_2PO_3, 12 mM HCl, or 5 M trichloroacetic acid (TCA). The spectra were plotted from data files using Cricket Graph III software.

Incubation of Membrane-Bound RGR with all-trans-Retinal. *all-trans*-Retinal was purchased from Sigma (St. Louis, MO) and analyzed by normal phase chromatography using a LiChrosorb RT Si60 silica column (4 × 250 mm, 5 μm) (E. Merck, Darmstadt, Germany) and Bio-Rad HPLC system. Equivalent suspensions of RPE microsomes in 10 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl and 0.5 mM EDTA, were incubated in the dark with or without 50 μM *all-trans*-retinal for 2 h at room temperature. The membranes were recovered by centrifugation at 100000*g* for 20 min. The microsomal proteins were then solubilized with digitonin solution, and RGR was purified by an immunoaffinity procedure, as described earlier.

Effect of Hydroxylamine on Binding of all-trans-[^{3}H]-Retinal and Light Absorbance. *all-trans*-[11,12-^{3}H]Retinal was prepared and analyzed, as described previously (Shen et al., 1994). Equal aliquots of a suspension of RPE microsomes in 67 mM sodium phosphate (pH 6.5) were incubated in the dark for 2.5 h at room temperature with purified *all-trans*-[^{3}H]retinal (1 × 10^5 cpm, 50 Ci/mmol). Hydroxylamine (1 M NH_2OH in H_2O, pH 6.5) was then added to one sample to a final concentration of 0.25 M, and the incubation was continued for 30 min. After incubation, the membranes were collected by centrifugation at 40 000 rpm for 25 min at 4 °C using a Beckman SW55 rotor. The pellet was washed 3 times and resuspended in 1.0 mL of 67 mM sodium phosphate, pH 6.5. After adjustment of the buffer pH to 8.0 with 1 M NaOH, the membrane suspension was mixed with 38 mg of sodium borohydride (1 M $NaBH_4$, final concentration), and subsequent steps were carried out in the light. The membranes were recovered again by centrifugation, and labeled microsomal proteins were analyzed by fluorography after SDS−PAGE. For fluorography, the 12% polyacrylamide gel was saturated with Enlightning reagent (Dupont NEN Research Products, Boston, MA), dried, and exposed to Kodak X-omat AR film at −80 °C.

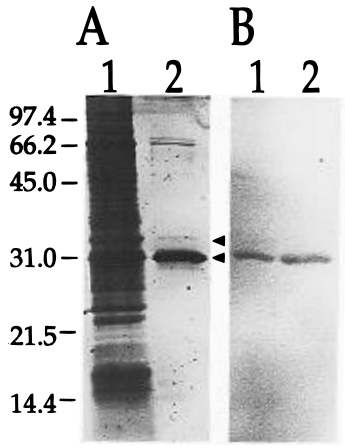

FIGURE 1: Isolation of RGR from the bovine retinal pigment epithelium. RGR was isolated from a digitonin extract of microsomal proteins by an immunoaffinity procedure and electrophoresed in a 12% SDS−polyacrylamide gel. The protein extract (14 μg) in lane 1 and purified RGR (0.7 μg) in lane 2 were analyzed by (A) protein silver staining and (B) immunoblot analysis using monoclonal antibody 2F4 directed against the carboxyl terminus of bovine RGR (Shen et al., 1994). Alkaline phosphatase-conjugated anti-mouse IgG and colorimetric substrates were used to detect the bound antibody. The arrows point to proteins that are approximately 31 and 34 kDa.

Hydroxylamine reactivity was also determined by measuring the absorption spectra of RGR. RPE microsomes were incubated with 50 μM nonradioactive *all-trans*-retinal, as described earlier, and RGR was purified at pH 6.5. The pH of the sample was adjusted to 4.2, and the absorption spectrum was obtained. Hydroxylamine was then added to a final concentration of 80 μM, and the absorption spectrum of the sample was obtained at various time intervals from 0.5 to 40 min at room temperature. No further change in the absorption spectrum occurred after 30 min. Smoothed difference spectra were computed by applying a 7-point moving average.

Illumination of RGR. Membrane-bound RGR was incubated with *all-trans*-retinal and isolated in the dark, as described earlier. The purified protein was then irradiated at room temperature by a 30-W fiber optic light source for 5 min at a distance of 10 cm. UV−visible absorption spectra were determined before and immediately after illumination.

RESULTS

Purification of RGR from Bovine RPE Cells. Bovine RGR was isolated from the microsomal membranes of RPE cells under red or dim yellow light. The RPE microsomes were enriched with RGR, but remained highly contaminated with pigmented material. The membranous proteins were extracted in 1.2% digitonin solution at pH 6.5, and solubilized RGR was purified by means of immunoaffinity chromatography. The purified fraction contained a major protein band that was approximately 31 kDa and reacted specifically to a bovine RGR antibody (Figure 1). Minor amounts of a 34-kDa protein and two other proteins between 70 and 76 kDa were also isolated. In a typical purification, approximately 26 μg of RGR was isolated from RPE cells from 20 fresh bovine eyes (Table 1). The data shown in Table 1 indicate that RGR represents about 3% of the initial RPE cell homogenate.

Table 1: Purification of RGR from Bovine RPE Cells

step	protein (mg)	RGR/protein (mg·mg^{-1})	purification (*x*-fold)
homogenate	41	0.030[a]	
microsomes	3.3	0.11[a]	3.7
immunoaffinity	0.026	~1	33

[a] The amount of RGR in crude extracts was determined by means of immunoblots using a purified RGR standard for calibration. The RPE cell homogenate and microsomal membranes were prepared from 20 eyes, as described previously (Shen et al., 1994).

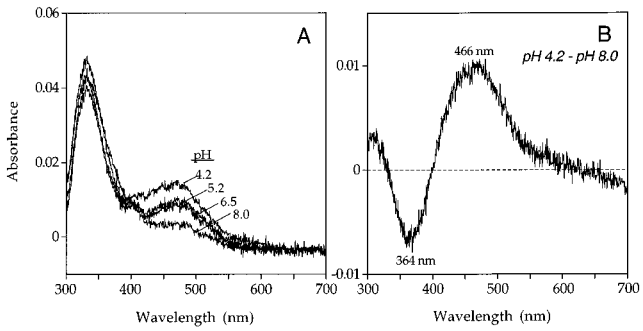

FIGURE 2: Absorption spectra of RGR. RGR from bovine RPE was purified in 10 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl, 0.5 mM EDTA, and 0.1% digitonin. (A) The spectrum of RGR was determined at pH 6.5, 5.2, 4.2, and 8.0. The absolute curves were superimposed at 660−680 nm to equalize the base lines. (B) The spectrum at pH 8.0 was subtracted from the spectrum at pH 4.2. The maximum and minimum for the absorption peaks lie at approximately 466 and 364 nm, respectively.

UV−Visible Absorption Spectra of RGR. The absorption spectrum of purified RGR showed endogenous pH-sensitive absorbance in the blue and near-ultraviolet (UV) regions of light (Figure 2A). When the pH was raised from 6.5 to 8.0, light absorbance by the native protein between 460 and 490 nm was nearly abolished. When the pH was lowered from 6.5 to 4.2, the absorbance in this region of blue light increased noticeably.

The large peak in the UV region ($\lambda_{max} \approx 330$ nm) was relatively unaffected by the pH. Nevertheless, some acid-sensitive changes in extinction could be detected within the near-UV region. Generally, increases in the hydrogen ion concentration reduced the light absorbance in the near-UV region and vice versa. The pH effect in the near-UV region can be illustrated by the difference spectrum that was obtained by subtraction of the spectrum at pH 8.0 from that at pH 4.2 (Figure 2B). The difference spectrum shows a pH-sensitive absorption peak at approximately 364 nm, as well as a peak at 466 nm.

Effect of all-trans-Retinal on the Absorption Spectrum of RGR. In an effort to improve the recovery and analysis of a photoreceptive RGR, the microsomes were incubated in the dark with exogenously added *all-trans*-retinal prior to solubilization and purification of the protein. RGR from the treated microsomes displayed a conspicuous and reproducible absorption peak between the wavelengths of 460 and 480 nm that was substantially enhanced in comparison to that of control RGR from RPE microsomes that had not been preincubated with *all-trans*-retinal (Figure 3A). The difference spectrum, which is dependent on exogenously added *all-trans*-retinal, showed two absorption peaks, one in the visible region with a λ_{max} of 466 nm and another in the near-UV region with a λ_{max} of 375 nm (Figure 3B).

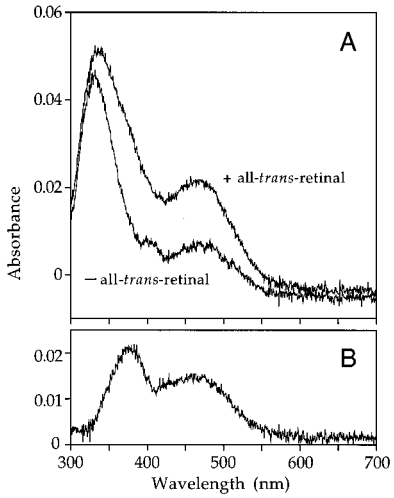

FIGURE 3: Effect of *all-trans*-retinal on the spectrum of RGR. (A) RPE microsomal membranes were incubated with or without 50 μM *all-trans*-retinal prior to purification in the dark and scanning the sample at pH 6.5 and at 24 °C. (B) Difference spectrum of RGR at pH 6.5. The spectrum of untreated RGR was subtracted from the spectrum of RGR that was incubated with *all-trans*-retinal. The maxima for the two prominent absorption peaks lie at 466 and 375 nm. After three separate experiments, mean wavelengths of 469 ± 2.4 and 370 ± 7.3 nm (±, standard deviation) were obtained for the absorption maxima.

The broad absorption peak in the blue visible region resembles that of *all-trans*-retinal ($\lambda_{max} = 389$ nm in aqueous digitonin); however, its absorption maximum is shifted to a longer wavelength, 466 nm (Figure 3B). The peak at 375 nm is also shifted slightly from the λ_{max} of *all-trans*-retinal, and in contrast to the free retinal, it was pH-sensitive. The *all-trans*-retinal difference spectrum was determined in three separate experiments, which indicated absorption maxima of 469 ± 2.4 nm and 370 ± 7.3 nm in the blue and near-UV regions, respectively. The retinal-dependent difference spectra did not exhibit the peak at 330 nm.

pH-Dependent Absorption Spectra of RGR Preincubated with all-trans-Retinal. The λ_{max} at 466 nm in the difference spectra shown in Figure 3B is somewhat greater than that expected for a protonated *all-trans*-retinal Schiff base salt, which absorbs maximally at about 440 nm (Pitt et al., 1955). It is possible that the shift in maximal absorbance to 466 nm is produced through chromophore−protein interactions of a protonated *all-trans*-retinylidene bound to RGR. This hypothesis was tested by measuring the effect of pH and acid denaturation on the light absorbance by RGR that was isolated from microsomes treated with *all-trans*-retinal. At pH 8.0, the absorbance near 466 nm decreased greatly and appeared as a relatively small shoulder (Figure 4A). At pH 5.2 and 4.2, the absorbance increased with each addition of acid. Concomitantly, opposite changes in extinction were observed with respect to pH for the apparent absorbance in the near-UV region. The pH-dependent curves cross at an isosbestic point and indicate a pK_a close to 6.5 for an acid-binding group.

Subtraction of the spectrum at pH 8.0 from that at pH 4.2 gives a pH difference spectrum that suggests an interconversion of two pigments with absorption peaks at approximately 466 and 363 nm (Figure 4B). The pH difference spectra of retinal-treated and untreated RGR show virtually identical absorption peaks (Figures 2B and 4B), except that

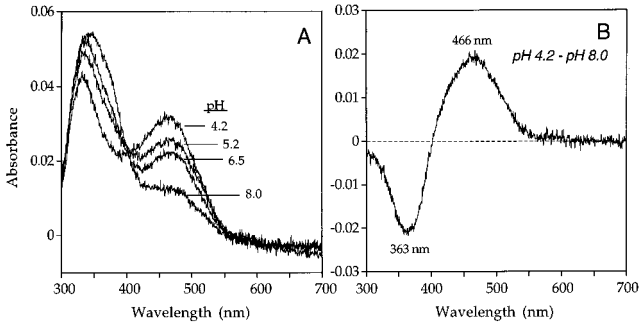

FIGURE 4: Absorption spectra and pH indicator property of RGR after incubation with *all-trans*-retinal. (A) The absorption spectrum of purified RGR at each pH was determined after incubation of RPE microsomal membranes with 50 μM *all-trans*-retinal. The absolute curves were superimposed at 660−680 nm to equalize the base lines. (B) pH difference spectrum for RGR incubated with *all-trans*-retinal. The spectrum at pH 8.0 was subtracted from the spectrum at pH 4.2. The absorption maximum and minimum for the interconverted peaks lie at approximately 466 and 363 nm, respectively.

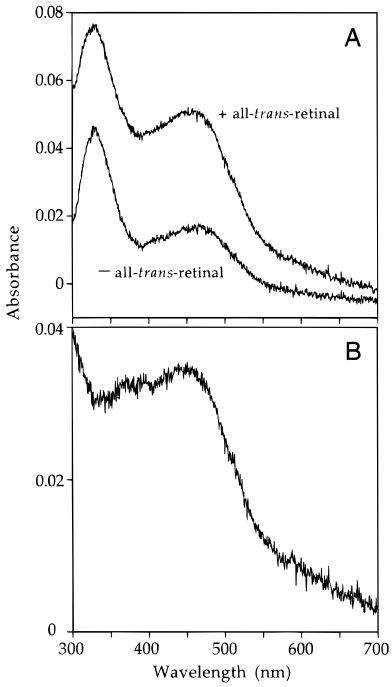

FIGURE 5: Effect of *all-trans*-retinal on the spectrum of RGR in 0.1 M TCA. RGR was purified in the dark at pH 6.5, and the solution was adjusted to 0.1 M TCA immediately before scanning. (A) Spectra of acid-denatured RGR from microsomal membranes that were incubated with or without 50 μM *all-trans*-retinal. (B) Difference absorption spectrum of RGR in 0.1 M TCA. The spectrum of untreated RGR was subtracted from the spectrum of RGR that was preincubated with *all-trans*-retinal. Instead of two main absorption peaks at 466 and 375 nm, as shown in Figure 3B, the absorption maximum appears at approximately 450 nm.

the absorbance by retinal-treated RGR is about 2-fold higher for the same amount of protein.

Acid denaturing conditions greatly affected the difference spectrum of RGR that results from the presence or absence of *all-trans*-retinal (Figure 5). RGR was purified, and its absorption spectrum was measured in 0.1 M TCA. Apparently, RGR in the digitonin micelle is not immediately precipitated in 0.1 M TCA at room temperature, and the spectra can be obtained. The shape of the difference spectrum in Figure 5B varied considerably from that seen in Figure 3B. The high acidity resulted in elimination of

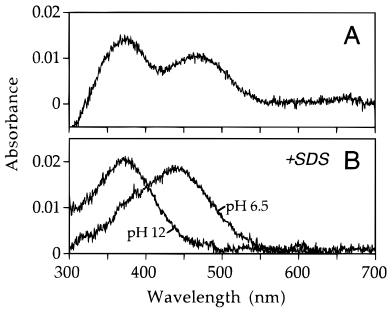

FIGURE 6: Effect of SDS on the *all-trans*-retinal difference spectrum of RGR. RGR was purified in the dark at pH 6.5 and then incubated at 20 °C for 30 min in the absence or presence of 2% SDS immediately before scanning. (A) *all-trans*-Retinal difference spectrum of control RGR at pH 6.5. The spectrum of untreated RGR was subtracted from the spectrum of RGR that was preincubated with *all-trans*-retinal, as in Figure 3B. (B) *all-trans*-Retinal difference spectrum of RGR in 2% SDS. The difference absorption spectra were determined from the SDS-treated samples, first at pH 6.5 and then at pH 12, after the addition of 5 M NaOH to a final concentration of 0.1 M.

the two absorption peaks at 466 and 375 nm and the appearance of a new absorption peak with a λ_{max} of 450 nm.

Purified RGR was also denatured with SDS, and the *all-trans*-retinal difference spectrum was determined at pH 6.5 and pH 12. In the absence of SDS, the two photopigments at approximately 468 and 375 nm were identified (Figure 6A). Treatment of RGR with 2% SDS resulted in loss of the 468-nm absorption peak and the emergence of a new peak at 442 nm (Figure 6B). The UV peak also was reduced at pH 6.5, but at pH 12 it was the predominant absorption peak ($\lambda_{max} \approx 372$ nm), following the addition of NaOH.

Hydroxylamine Reactivity of RGR. The stability of RGR against hydroxylamine was tested by binding of *all-trans*-[^{3}H]retinal and by measurement of absorbance. A complex mixture of RPE microsomal proteins was incubated in the dark with *all-trans*-[^{3}H]retinal before the addition of 0.25 M hydroxylamine, or a control buffer. Sodium borohydride was added last to the microsomes, and the labeling of proteins was analyzed by gel electrophoresis and fluorography. Hydroxylamine abolished completely the specific binding of *all-trans*-[^{3}H]retinal to a 31-kDa protein (Figure 7A). The single labeled protein was identified previously as RGR by specific immunoprecipitation (Shen et al., 1994).

Decomposition of RGR at pH 4.2 is shown via absorbance measurements (Figure 7B) that were made at various times after the addition of hydroxylamine. The decay of the RPE pigment was about 50% complete after 10 min and unchanged after 30 min. The absorption spectrum at 36 min was subtracted from each spectrum obtained at other time points or before the addition of hydroxylamine. The difference spectra indicate the loss of a pigment ($\lambda_{max} \approx 470$ nm) and formation of a new pigment ($\lambda_{max} \approx 364$ nm), which is consistent with retinaldehyde oxime.

Absorption Spectrum of RGR after Illumination. The absorption spectrum of RGR was determined before and after illumination (Figure 8). Irradiation did not lead to substantial bleaching of the blue-absorbing pigment; however, it did cause a decrease in the absorbance in the near-UV region. The difference spectrum showed an absorption peak at approximately 368 nm. The effect of light in the near-UV region was highly reproducible and occurred without treat-

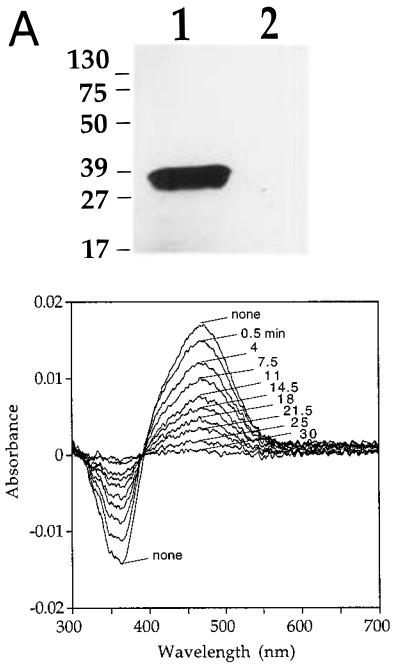

FIGURE 7: Instability of RGR toward hydroxylamine. (A) The binding of *all-trans*-[^{3}H] retinal to RGR was abolished by 0.25 M hydroxylamine. Each lane contained approximately 0.2 mg of protein from RPE microsomes. The X-ray film was exposed for a period of 5 days. (B) Hydroxylamine (80 μM) was added to purified RGR at pH 4.2. The decay of absorbance is shown by smoothed difference spectra, which were obtained by subtraction of the spectrum at 36 min from the spectra at the indicated time points.

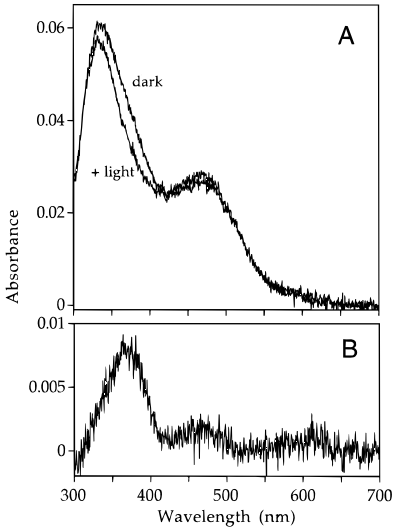

FIGURE 8: Effect of light on the absorption spectrum of RGR. (A) Absolute spectrum of RGR before and after illumination. (B) Difference between dark and postillumination spectra shown in panel A. The difference spectrum showed a change in absorption at approximately 368 nm.

ment of RGR with exogenous *all-trans*-retinal (result not shown).

DISCUSSION

We have isolated a novel pigment from RPE cells and characterized some of its absorbance properties. The shape of the absorption peaks and biochemical properties of this pigment are consistent with those of a retinylidene Schiff base chromophore and reveal the existence of two pH-dependent species with absorption maxima at approximately 466 and 364 nm (Figure 2). The results provide strong evidence that RGR is conjugated in vivo to an endogenous retinaldehyde chromophore. The RPE opsin differs from the visual pigments in its facile ability to form a stable photopigment by recombination with the *all-trans* isomer. Its absorption maxima and pH sensitivity are quite distinct from those of rhodopsin.

A few minor proteins were copurified with RGR. In particular, a distinct 34-kDa protein was consistently visible by protein staining. None of the minor protein bands were detected on immunoblots with an RGR antibody, even when the immunoblots were overdeveloped; nor were these microsomal proteins labeled covalently with [^{3}H]retinals. It is possible that one or more of the coisolated proteins forms a complex or associates functionally with RGR, although the identity of these proteins is not yet known. Interestingly, the 34-kDa protein approximates the size of cellular retinaldehyde binding protein (Crabb et al., 1988) and G protein α and β subunits.

Purified RGR displayed a pH indicator property, not unlike that of retinochrome (Hara & Hara, 1965), squid metarhodopsins (Hubbard & St. George, 1958), or counterion mutants of bovine rhodopsin (Zhukovsky & Oprian, 1989; Sakmar et al., 1989, 1991; Nathans, 1990). High pH favors the formation of a UV-absorbing pigment; low pH, its conversion into a blue-absorbing pigment. This pH dependence is the inverse of that reported for bovine metarhodopsins, MII (λ_{max} = 380 nm) and MI (λ_{max} = 480 nm), which are favored by lower and higher pH, respectively (Matthews et al., 1963), and it indicates that the RPE pigment is not a rhodopsin contaminant, which would be the most likely visual pigment contaminant.

Although the structure of the endogenous chromophore is unknown, RGR binds *all-trans*-retinal readily. The binding of *all-trans*-retinal generates a pair of long-lived pigments comprised of blue and UV light-absorbing conjugates. The two absorption maxima in the *all-trans*-retinal difference spectrum (Figure 3B) agree closely with those calculated from the pH difference spectra (Figures 2B and 4B). The similarity in λ_{max} measurements strongly supports the notion that the blue and UV light-absorbing pigments are tautomeric forms of RGR, in which the retinylidene Schiff base is protonated or unprotonated, respectively. It is known that protonation of a retinylidene Schiff base leads to an increase in the absorption maximum of the pigment from about 370 nm to a wavelength of 440 nm or greater (Pitt et al., 1955; Loppnow et al., 1989).

The pH-dependent equilibrium between two forms of RGR may result from the absence of an endogenous counterion for the protonated Schiff base. The protonated group on RGR has a pK_a at about 6.5, which is far lower than the pK_a of the retinylidene Schiff base in rhodopsin and suggests that the Schiff base nitrogen in RGR is not located near a stabilizing counterion. Glutamate is not conserved in RGR at the position corresponding to the counterion residue of rhodopsin (Zhukovsky & Oprian, 1989; Sakmar et al., 1989, 1991; Nathans, 1990); instead, histidine is found in the homologous position in bovine and human RGR (Jiang et al., 1993; Shen et al., 1994). Upon acid denaturation, RGR absorbed maximally at 450 nm. This result supports the proposition that the *all-trans*-retinal is bound to the protein.

The opsin shift in blue-absorbing RGR is about 19 nm (900 cm^{-1}).

As in the treatment of RGR with acid or exogenous *all-trans*-retinal, the incubation of RGR with hydroxylamine also demonstrated a blue-absorbing pigment ($\lambda_{max} \approx 470$ nm) in the difference spectrum. The hydroxylamine-dependent loss of [^{3}H]retinal binding to a specific 31-kDa protein corresponded to the disappearance of the 470-nm absorption peak. RGR was unstable in the dark against hydroxylamine at concentrations as low as 80 μM. The high reactivity with hydroxylamine suggests that the Schiff base bond is readily accessible to small molecules. Since the opsin shift of blue-absorbing RGR is weak, the chromophore itself may be bound near the surface of RGR, and consequently, it may be particularly accessible to other retinaldehyde-binding proteins in the RPE or Müller cells.

Hydroxylamine did not affect the absorption peak at 330 nm, which is present in the absolute spectrum of purified RGR. As indicated by difference spectra, the 330-nm peak was not pH-sensitive and was not generated by incubation with exogenous *all-trans*-retinal. The 330-nm peak may be derived from another RPE pigment that is copurified with RGR or is a residual impurity. The identity and biochemical properties of the 330-nm component remain to be determined.

There is an absence of electrophysiological or microspectrophotometrical data on the absorption spectrum of RGR, and the biological function of the RPE opsin is not yet understood. Thus, it is unknown whether the absorption spectrum of RGR that we observe corresponds to the native protein structure or not. The presence of retinal on the protein is unlikely to be a simple artifact of the preparative procedure. During its isolation, RGR was not exposed to an extreme pH condition, which would enhance the production of an indicator yellow compound (Morton & Pitt, 1955). When crude RPE microsomes were incubated with [^{3}H]-retinal, RGR was the only detectable protein that bound the radiolabeled isomer. This observation argues that the chromophore is bound specifically to RGR, insofar as it is unlikely that random Schiff base linkages could form exclusively in only one of the numerous microsomal proteins. Similar conditions were used for incubation of RGR with unlabeled *all-trans*-retinal. Even without treatment of RGR with *all-trans*-retinal in excess, the absorbance by the pH-sensitive photopigment was about 50% of the absorbance of retinal-treated RGR, and the absorption maxima from the pH difference spectra were not materially altered by incubation with exogenous retinal.

Clearly, additional work must be done to affirm and elucidate the biophysical basis for the absorption spectrum of RGR. Preliminary evidence indicates that the purified RGR apoprotein retains the ability to bind *all-trans*-retinal and regenerate the observed pigments in vitro. Other tests for RGR with a native structure may include circular dichroism spectroscopy, an efficient photoresponse with distinctive dark reactions, and a functional assay. Since there are two forms of RGR at neutral pH, analysis of the effects of irradiation may be complex. Illumination did not lead to bleaching of the blue-adsorbing pigment. As for many invertebrate visual pigments, such as squid and *Drosophila* rhodopsins (Hubbard & St. George, 1958; Hillman et al., 1983), retinal may not dissociate readily from RGR in the presence of light. In the near-UV region, a decrease in extinction was always observed after irradiation. This change in absorption may indicate the formation of a new species of photopigment with a lower extinction coefficient. With purified RGR, it will be possible to investigate the light reactions of the RPE opsin in greater detail.

From the results of this paper, we conclude that RGR binds retinal in vivo and propose that its biological function in the eye entails photoisomerization of the chromophore by blue or UV light. At neutral pH, the naturally occurring UV form would predominate, and thus, RGR may have a physiological role in UV light reception.

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