RPE65 Operates in the Vertebrate Visual Cycle by Stereospecifically Binding All-*trans*-Retinyl Esters†

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ABSTRACT: RPE65 is a major protein of unknown function found associated with the retinyl pigment epithelial (RPE) membranes [Hamel, C. P., Tsilou, E., Pfeffer, B. A., Hooks, J. J., Detrick, B., and Redmond, T. M. (1993) *J. Biol. Chem. 268*, 15751−15757; Bavik, C. O., Levy, F., Hellman, U., Wernstedt, C., and Eriksson, U. (1993) *J. Biol. Chem. 268*, 20540−20546]. RPE65 knockouts fail to synthesize 11-*cis*-retinal, the chromophore of rhodopsin, and accumulate all-*trans*-retinyl esters in the RPE. Previous studies have also shown that RPE65 is specifically labeled with all-*trans*-retinyl ester based affinity labeling agents, suggesting a retinyl ester binding role for the protein. In the present work, we show that purified RPE65 binds all-*trans*-retinyl palmitate (tRP) with a $K_D = 20$ pM. These quantitative experiments are performed by measuring the quenching of RPE65 fluorescence by added tRP. The binding for tRP is highly specific because 11-*cis*-retinyl palmitate binds with a $K_D = 14$ nM, 11-*cis*-retinol binds with a $K_D = 3.8$ nM, and all-*trans*-retinol (vitamin A) binds with a $K_D = 10.8$ nM. This stereospecificity for tRP is to be compared to the binding of retinoids to BSA, where virtually no discrimination is found in the binding of the same retinoids. This work provides further evidence that RPE65 functions by binding to and mobilizing the highly hydrophobic all-*trans*-retinyl esters, allowing them to enter the visual cycle.

The vertebrate visual cycle encompasses those biochemical reactions which begin with the photoisomerization of the 11-*cis*-retinal Schiff base chromophore of rhodopsin and end with the resynthesis of 11-*cis*-retinal (Scheme 1) (*1*, *2*). While rhodopsin photobleaching and the reduction of all-*trans*-retinal into vitamin A occur in the retinal photoreceptors, the processing of vitamin A into 11-*cis*-retinal occurs in the retinal pigment epithelium (RPE)[1] (*1*−*3*). Two essential reactions in the RPE are catalyzed by lecithin retinol acyltransferase (LRAT), which generates all-*trans*-retinyl esters from vitamin A (*3*, *4*), and isomerohydrolase, which processes the esters into 11-*cis*-retinol (*5*−*8*).

Hydrophobic all-*trans*-retinyl esters, chiefly in the palmitate series, figure importantly in the visual cycle, both because they are the substrates for isomerohydrolase and because they may act as storage forms of vitamin A (*5*−*8*). The mobilization of hydrophobic molecules, such as all-*trans*-retinyl palmitate (tRP), presents substantial difficulties. Because of its limited solubility in aqueous solutions, tRP does not undergo intermembranous transfer at appreciable rates (*9*). Even if tRP is situated in RPE membranes in proximity to isomerohydrolase, it would still have to gain access to the active site of the enzyme, presumably through a binding protein. We had previously established that RPE65 binds all-*trans*-retinyl esters and suggested that this protein is essential for the mobilizing tRP and like molecules (*10*).

This protein, discovered in 1993, seems to play an important role in visual cycle function (*11*, *12*). This conclusion comes from knockout studies in mice, which show that RPE65 knockouts are unable to substantially produce 11-*cis*-retinoids (*13*). Most interestingly, all-*trans*-retinyl esters were found to accumulate in the RPE of these mice (*13*). No enzymatic activity whatsoever has ever been described for RPE65, and it is almost certainly not the isomerohydrolase (*14*, *15*). What function does the protein play then?

Previous studies have shown that RPE65 is specifically labeled by low concentrations of an all-*trans*-retinyl chloroacetate ester based affinity-labeling agent **1** (Scheme 2) and that the stoichiometry of incorporation is 2 (*10*). The design of labeling agent **1** was based on the structure of all-*trans*-retinyl bromoacetate **2** (RBA) (Scheme 2), a known affinity-labeling agent of RBPs (*16*, *17*). Affinity-labeling studies of RPE65 using **1** demonstrate that RPE65 can specifically bind all-*trans*-retinyl esters. Hydrophobic non-retinyl ester affinity-labeling agents were unable to bind, further demonstrating selectivity in binding (*10*). It is interesting to note that RPE65 shows very strong sequence homology to β-carotene 15,15′-dioxygenase, an enzyme that oxidizes β-carotene into all-*trans*-retinal (*18*, *19*). While RPE65 does

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[1] Abbreviations: BSA, bovine serum albumin; CRALBP, cellular retinaldehyde binding protein; RBP, retinoid binding protein; DMSO, dimethyl sulfoxide; RBA, all-*trans*-retinyl bromoacetate; RPE, retinyl pigment epithelium; PBS, phosphate-buffered saline; tRP, all-*trans*-retinyl palmitate; cRP, 11-*cis*-retinyl palmitate; tRol, all-*trans*-retinol; cRol, 11-*cis*-retinol; au, arbitrary units; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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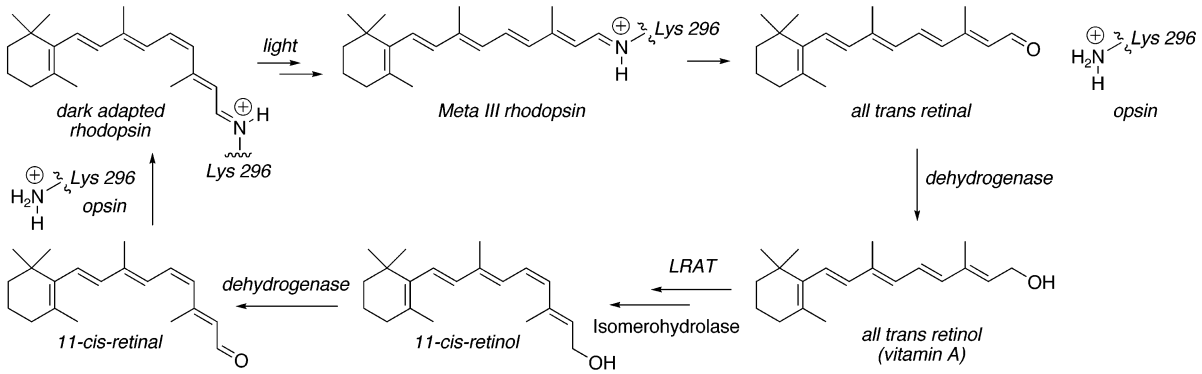
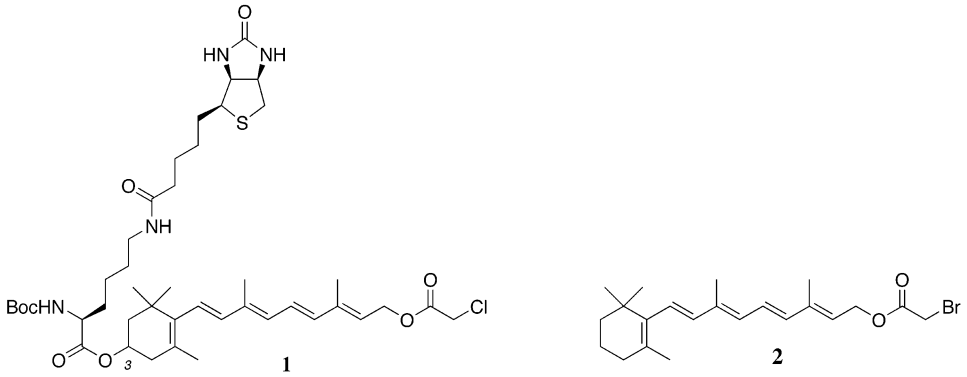
Scheme 1: Vertebrate Visual Cycle



Scheme 2: Retinyl Ester Based Affinity Labeling Agents



not show this activity, it is reasonable to assume that the protein does possess retinoid binding sites in common with the β-carotene oxygenase.

The affinity labeling of RPE65 by all-*trans*-retinyl ester based alkylating agents certainly establishes the retinyl ester binding capacity of this protein as one of its biochemical functions, but to extend this work quantitatively, a reversible binding method was sought. The retinols and retinyl esters are well suited to measurements of this type because they fluoresce (*20*−*22*). Furthermore, because their maximal UV−visible absorption spectra overlap with the main protein fluorescence (tryptophan) band of proteins, retinols/retinyl esters can also quench intrinsic protein fluorescence (*20*−*22*). Therefore, many possible fluorescence methodologies can be employed to quantitatively measure the binding of retinol and esters to a retinoid binding protein (RBP). Indeed, various fluorescence methods have already been used to access the binding of the retinols and congeners to known retinoid binding proteins such as IRBP (*23*). In the present study, the quenching of protein fluorescence is used to quantitatively demonstrate that all-*trans*-retinyl esters specifically bind to bovine RPE65 and further establish an RBP role for this protein.

MATERIALS AND METHODS

Materials

Frozen bovine eyecups devoid of retinas were purchased from W. L. Lawson Co., Lincoln, NE. Bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), DEAE-Sepharose, phenyl-Sepharose CL-4B, all-*trans*-retinol, all-*trans*-retinyl palmitate, and Trizma base were from Sigma-Aldrich. Dithiothreitol (DTT) was from ICN Biomedicals Inc. 11-*cis*-Retinol and 11-*cis*-retinyl palmitate were synthesized by following the procedure described elsewhere (*17*). Anagrade Chapso was from Anatrace. HPLC-grade solvents were from EMD Chemicals. An anti-RPE65 (NFITKVNPETLETIK) for western blots was obtained from Genmed Inc. The precast gels (4−20%) for SDS−PAGE and the BenchMark prestained molecular mass marker were from Invitrogen. DEAE-Sepharose was from Amersham Biosciences. Buffers were changed by dialysis in the request buffer overnight in a slide-a-lyser cassette from Pierce (10 kDa cutoff). RPE65 solutions were concentrated with an Amicon Ultra centrifugal filtration device (30 kDa cutoff) from Millipore Corp. All reagents were of analytical grade unless specified otherwise.

Methods

Extraction and Purification of RPE65. The method used here for the extraction and purification of RPE65 was the same as previously reported (*24*). Bovine retinal pigment epithelial membranes were solubilized in 20 mM Tris-HCl, pH 9.0, 2 mM DTT, 1 mM EDTA, and 2% Chapso. The solubilized protein was applied to a DEAE-Sepharose column using a linear gradient of buffers A and B (buffer A, 20 mM Tris-HCl, pH 9.0, 2 mM DTT, 1 mM EDTA, and 1% Chapso; buffer B, buffer A plus 1 M NaCl). The active fractions were collected and concentrated, followed by an application to a phenyl-Sepharose CL-4B column, eluted with a linear gradient of buffers B and A. The pure RPE65 fractions (assayed by SDS−PAGE, western blot) were collected and concentrated, and the purified RPE65 was stored at −80 °C.

One-Dimensional SDS−PAGE Analysis. Tris−glycine−polyacrylamide gel (4−20%) electrophoresis was carried out

using the standard buffer system (25 mM Tris, 192 mM glycine, and 0.1% SDS) (*25*). Protein denaturing was done by heating the proteins in sample buffer (2×) containing 20% glycerol, 0.004% bromophenol blue, 4% SDS, and 125 mM Tris-HCl (pH 6.8). Proteins bands were visualized by Coomassie staining (0.1%).

Western Blot Analysis. After protein separation by SDS−PAGE, the proteins were transferred to a poly(vinylidene fluoride) membrane for 30 min at 15 V using Tris−glycine buffer (25 and 192 mM glycine) and ethanol (20%) on a semidry transfer apparatus (Amersham). The membrane was blocked with 5% nonfat dried milk for 1 h at room temperature. Anti-RPE65 (1:4000 dilution, 1 h), anti-rabbit Ig-linked horseradish peroxidase (1:4000, 1 h), and the enhanced chemiluminescence (ECL-Amersham) system were used to detect the RPE65 band.

Preparation and Purification of Retinoids. All-*trans*-retinyl palmitate and all-*trans*-retinol were commercially available and were purified by HPLC prior to use. 11-*cis*-Retinol and 11-*cis*-retinyl palmitate were synthesized from all-*trans*-retinol by a known procedure (*17*) and were purified by HPLC. The retinoid solutions in *n*-hexane were stored under argon at −80 °C. Prior to fluorometric titration, the retinoid solutions were brought to 4 °C, the hexane was removed using a slow and constant flow of argon, and then the retinoids were dissolved in dimethyl sulfoxide (DMSO).

Fluorescence Methods. RPE65 in PBS and 1% Chapso, pH 7.4, was used for the fluorometric titration of RPE65. The concentration of the protein was measured by a modified Lowry method (*26*). All titrations were performed at 25 °C. In the case of BSA, the samples in PBS buffer were excited at 275 nm, and the fluorescence was scanned from 305 to 500 nm; for RPE65, the samples were excited at 280 nm and scanned from 305 to 500 nm. Fluorescence measurements, using 650 μL quartz cuvettes with a 0.5 cm path length, were made at 25 °C on a Jobin Yvon Instruments Fluoromax 2 employing the right-angle detection method.

The fluorescence of the protein solution was measured after equilibrating it at 25 °C for 10 min. The sample was then titrated with a solution of retinoid in DMSO. In each titration, to a 500 μL solution of the protein was added an equal amount of retinoid, typically 0.5 μL, and the resultant solution was thoroughly mixed before allowing it to equilibrate for 10 min prior to the recording of the fluorescence intensity. The addition of DMSO (0.1% per addition) did not have any effect on the fluorescence intensity. The binding constant (K_D) was calculated from the fluorescence intensity by using the equation (*20*):

$$P_0\alpha = \frac{R_0\alpha}{n(1-\alpha)} - \frac{K_D}{n}$$

where P_0 = total protein concentration, $\alpha = (F_{max} - F)/(F_{max} - F_0)$, n = number of independent binding sites, R_0 = total retinoid concentration at each addition, K_D = dissociation constant, F_{max} = fluorescence intensity at saturation, and F_0 = initial fluorescence intensity.

RESULTS

Binding of Retinoids to BSA. BSA is a well-known, albeit nonspecific binder of hydrophobic compounds (*27*). To establish the fluorescent assay used here, the binding of all-*trans*-retinyl palmitate (tRP) to defatted BSA was studied. A study of this type will also establish a baseline for the binding measurements of retinoids to RPE65 with respect to both affinity and specificity. The quenching of protein fluorescence as a consequence of the binding of tRP binding was followed. As shown in Figure 1A, the binding of tRP to BSA led to an exponential decay in protein fluorescence. This decay followed a saturable binding isotherm (Figure 1B) and yielded an average K_D for binding of approximately 2.1 nM. Similar affinities were also measured for the binding of vitamin A to BSA (Figure 2). These data are also close to published data on the binding of palmitate to BSA (K_D = 4.5 nM) (*27*). Thus, as expected, BSA binds to hydrophobic retinoids without substantial specificity. Binding data for BSA−retinoid interactions are compiled in Table 1.

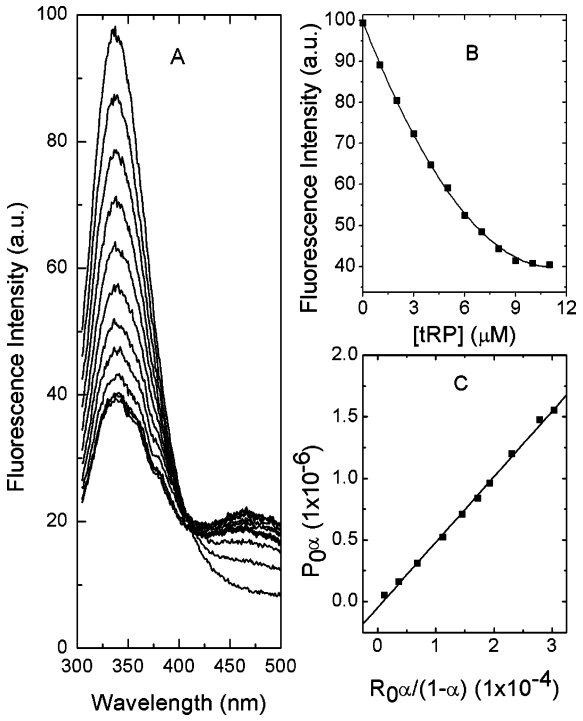


FIGURE 1: Fluorescence titration of BSA (1 μM) with all-*trans*-retinyl palmitate (tRP). The excitation wavelength was 275 nm, and the emission was observed through a 0.5 cm layer of solution. The titration solution consisted of 1 μM BSA in 100 mM phosphate-buffered saline (150 mM), pH 7.4. Panel A shows the emission spectra of BSA with increasing concentrations of all-*trans*-retinyl palmitate. Panel B shows the change in the fluorescence intensity at 340 nM with increasing concentrations of all-*trans*-retinyl palmitate. Panel C shows the linear square fit plots for eq 1, $P_0\alpha$ vs $R_0\alpha/(1 - \alpha)$, for the titration of BSA vs all-*trans*-retinyl palmitate.

Binding of Retinoids to RPE65. The data shown in Figure 1 show that fluorescence quenching of protein fluorescence by tRP can be used to quantitatively measure the binding of this molecule to BSA. The next step is to measure tRP binding to RP65. Membrane-associated bovine RPE65 was harvested and purified by a combination of hydrophobic interaction chromatography and ion-exchange chromatography (*24*). The purity of the protein is demonstrated by Coomassie staining and western blotting analysis using an anti-RPE65 peptide antibody (Figure 3). The binding of tRP to purified RPE65 was then measured using the protein fluorescence quenching technique described above, as shown in Figure 4. The data reveal readily saturable and high-

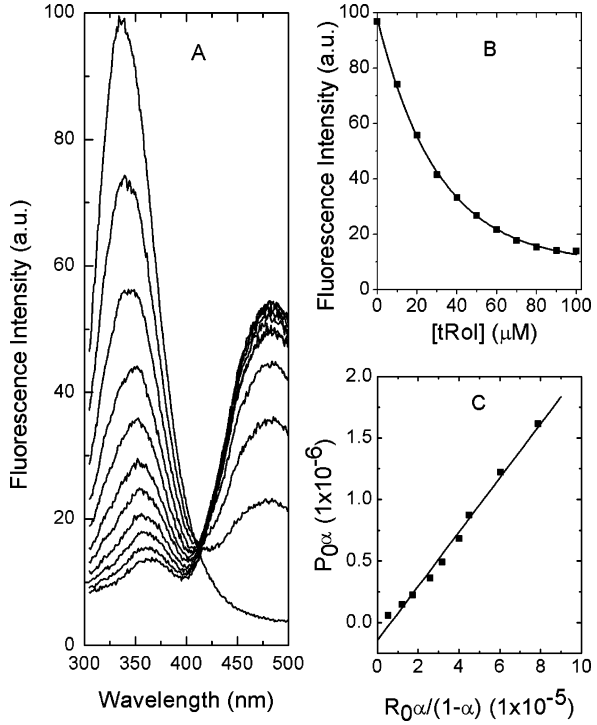


FIGURE 2: Fluorescence titration of BSA (1 μM) with all-*trans*-retinol (tROL). The excitation wavelength was 275 nm, and the emission was observed through a 0.5 cm layer of solution. The titration solution consisted of 1 μM BSA in 100 mM phosphate-buffered saline (150 mM), pH 7.4. Panel A shows the emission spectra of BSA with increasing concentrations of all-*trans*-retinol. Panel B shows the change in the fluorescence intensity at 340 nM with increasing concentrations of all-*trans*-retinol. Panel C shows the linear square fit plots for eq 1, $P_0\alpha$ vs $R_0\alpha/(1 - \alpha)$, for the titration of BSA vs all-*trans*-retinol.

Table 1: Binding Constants of Various Retinoids with RPE65 and BSA with 1% Chapso in 100 mM Phosphate Buffer with 150 mM Sodium Chloride

	retinoid binding proteins (RBP)	
retinoids	RPE65 (pM)	BSA (pM)
all-*trans*-retinyl palmitate	20 (6)	2143 (790)
11-*cis*-retinyl palmitate	14224 (3006)	2310 (611)
all-*trans*-retinol	1078 (144)	2316 (1216)
11-*cis*-retinol	3811 (100)	2143 (800)

affinity binding of tRP to RPE65. In this instance, the measured K_D for binding is 20 pM. These experiments demonstrate that tRP binds to RPE65 in a specific and high-affinity manner. To determine whether RPE65 binds to other retinoids with similar affinities, the binding of 11-*cis*-retinyl palmitate (cRP), 11-*cis*-retinol, and all-*trans*-retinol to RPE65 was studied. As shown in Figure 5, cRP only weakly binds to RPE65 as compared to tRP. Similar experiments were performed for the binding of 11-*cis*-retinol and all-*trans*-retinol to RPE65. Again, only weak binding is observed (data not shown). The data for the binding of tRP, cRP, 11-*cis*-retinol, and all-*trans*-retinol to RPE65 are summarized in Table 1. In aggregate, these experiments show that RPE65 selectively binds to tRP and that RPE65 is a RBP.

DISCUSSION

RPE65 is a major protein found associated with RPE membranes (*11*, *12*). The protein is largely, although not exclusively, expressed in the mammalian RPE, suggesting

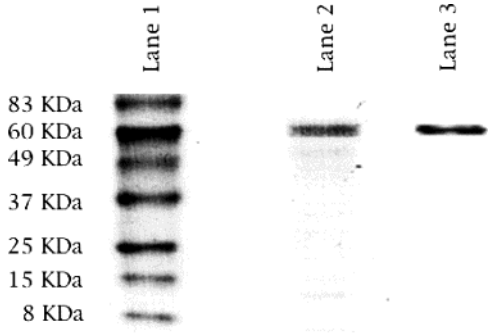


FIGURE 3: Purified RPE65. Lanes 1 and 2 show the Coomassie-stained SDS−PAGE (4−20%) gel of the molecular mass marker and purified RPE65 (42 ng of protein content). Lane 3 shows the western blot for the band shown in lane 2.

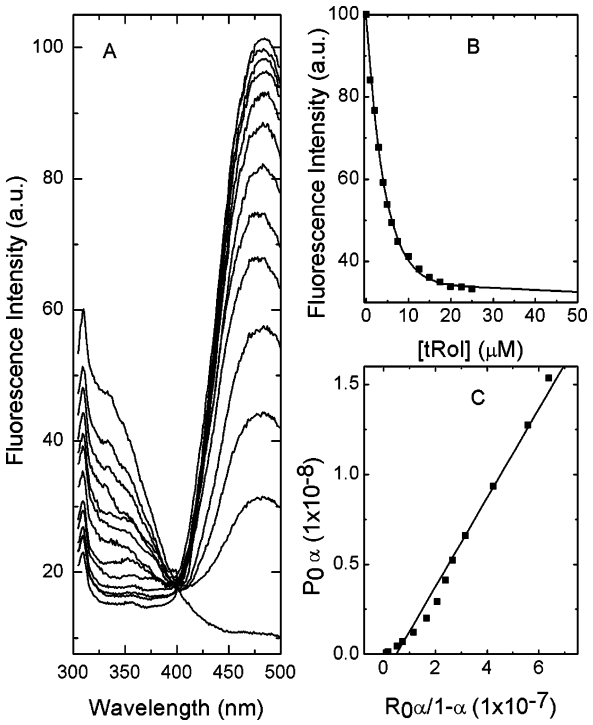


FIGURE 4: Fluorescence titration of RPE65 (0.17 μM) with all-*trans*-retinyl palmitate (tRP). The excitation wavelength was at 280 nm, and the emission was observed through a 0.5 cm layer of solution. The titration solution consisted of 0.17 μM RPE65 in 100 mM phosphate-buffered saline (150 mM), pH 7.4. Panel A shows the emission spectra of RPE65 with increasing concentrations of all-*trans*-retinyl palmitate. Panel B shows the change in the fluorescence intensity at 340 nM with increasing concentrations of all-*trans*-retinyl palmitate. Panel C shows the linear square fit plots for eq 1, $P_0\alpha$ vs $R_0\alpha/(1 - \alpha)$, for the titration of RPE65 vs all-*trans*-retinyl palmitate.

that it is essential for visual function (*11*, *12*). This point is clearly made in mouse knockout studies (*13*). RPE65 knockout mice are unable to synthesize 11-*cis*-retinoids, indicating a difficulty with retinoid processing in the visual cycle (*13*). It was found that all-*trans*-retinyl esters accumulate in these animals in oil-like droplets in the RPE (*13*). This further suggests that there is a defect in either the mobilization or enzymatic processing of all-*trans*-retinyl esters. RPE65 is probably not isomerohydrolase, first, because it does not show isomerohydrolase activity in vitro (*14*) and, second, because the extensive (90−95%) removal of RPE65 from RPE membranes does not materially affect

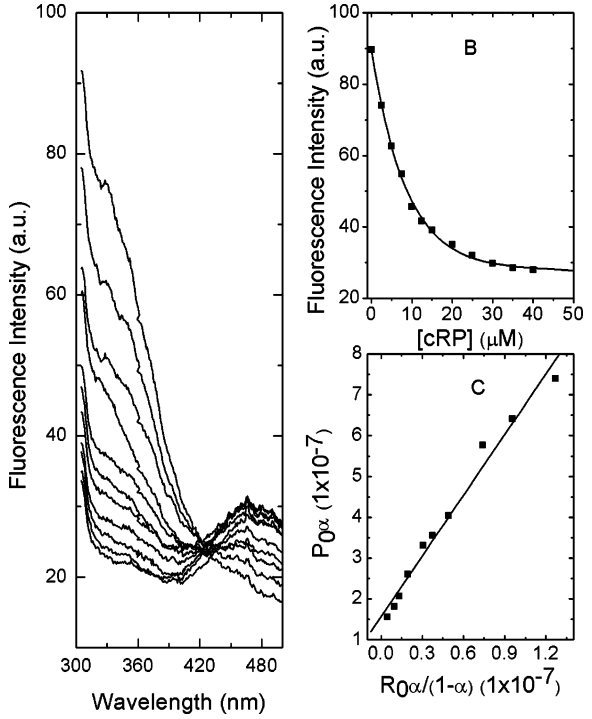


FIGURE 5: Fluorescence titration of RPE65 (0.17 μM) with 11-*cis*-retinyl palmitate (cRP). The excitation wavelength was 280 nm, and the emission was observed through a 0.5 cm layer of solution. The titration solution consisted of 0.17 μM RPE65 in 100 mM phosphate-buffered saline (150 mM), pH 7.4. Panel A shows the emission spectra of RPE65 with increasing concentrations of all-*trans*-retinyl palmitate. Panel B shows the change in the fluorescence intensity at 340 nM with increasing concentrations of all-*trans*-retinyl palmitate. Panel C shows the linear square fit plots for eq 1, $P_0\alpha$ vs $R_0\alpha/(1 - \alpha)$ for the titration of RPE65 vs all-*trans*-retinyl palmitate.

isomerohydrolase activity (*15*). In these experiments, isomerohydrolase activity was measured by incubating washed RPE membranes with vitamin A to generate all-*trans*-retinyl esters in situ, obviating the requirement of hydrophobic all-*trans*-retinyl ester mobilization from bulk solution (*15*).

Given RPE65's abundance, coupled with the fact that no enzymatic activity has ever been reported for RPE65, the data suggest that RPE65 is not an enzyme but is a protein that functions stoichiometrically rather than catalytically. An RBP role for RPE65 presents itself as one possible function for this protein. Other RBPs are known to operate in the visual cycle and are important for its function (*28*−*33*). Interestingly, none of these other proteins function by binding to retinyl esters. In addition, the fact that RPE65 is significantly homologous to β-carotene 15,15′-dioxygenase also suggests the possibility that RPE65 may bind retinoids, since β-carotene is in essence two all-*trans*-retinoid moieties joined head to head (*18*, *19*). Finally, previous studies have already demonstrated that an all-*trans*-retinyl ester affinity-labeling agent **1** (Scheme 2) specifically labels RPE65 at C231 and C448, as determined by mass spectrometry (*10*). These experiments demonstrated that RPE65 specifically binds all-*trans*-retinyl esters, with a stoichiometry of 2. Since a chemically reactive all-*trans*-retinyl ester was employed in the studies mentioned above, quantitative information on the specificity of reversible retinoid binding to RPE65 is not readily obtained. This is because the efficiency of the irreversible binding of chemically reactive retinoids to RPE65 measures both probe chemical reactivity as well as binding affinity. To quantitatively reveal the reversible binding of retinoids to RPE65, the quantitative fluorescence method described here was developed.

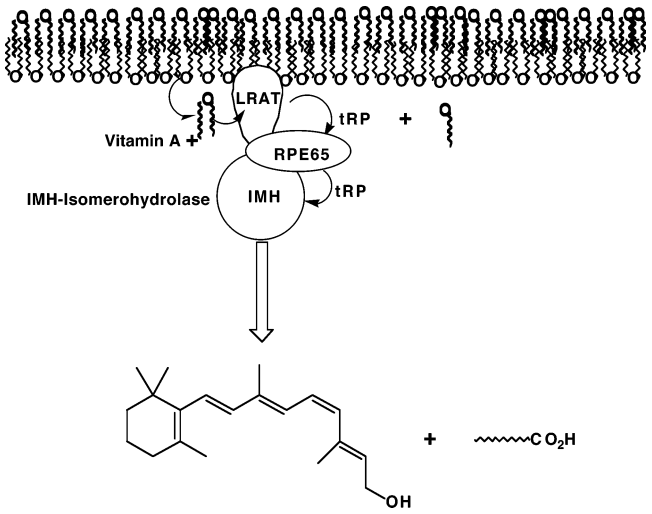
Fluorescence methodologies are ideally suited for measuring the binding of retinols and retinyl esters to proteins, because they are fluorescent (the retinals though are not significantly fluorescent) (*20*−*22*). A number of fluorescent assays are possible with retinyl esters and retinols. For example, the interaction of the protein with the retinol (ester) could enhance, quench, or shift the fluorescence emission spectrum of the retinoid (*20*−*22*). Conversely, the binding of the retinoid to the protein can quench the native (protein) fluorescence of the protein. This latter approach proved to be convenient in the current context and was chosen for study.

Initial measurements were performed on the binding of tRP to defatted BSA. These experiments demonstrated that binding could be readily detected and provided a binding constant for tRP of approximately 2.3 nM to a protein known to have a nonspecific capacity to bind hydrophobic molecules (*27*). A similar binding constant was also obtained for vitamin A. These values are quite similar to those previously determined for the binding of palmitate to BSA (*27*). The binding of retinoids to BSA is clearly nonspecific in the sense that a diverse group of hydrophobic compounds are capable of binding to the protein (*27*).

The situation with RPE65 proved to be quite different. Here, high affinity and specific binding for tRP is observed; RPE65 binds to tRP with a measured $K_D = 20$ pM. Interestingly, vitamin A binds only weakly ($K_D = 10.8$ nM) to RPE65 and shows a K_D approximately the same as found in the binding of vitamin A to BSA. This shows that the palmitate ester portion of tRP contributes to the binding of tRP. Furthermore, the binding of tRP to RPE65 is stereospecific since cRP binds with a $K_D = 14$ nM to the protein. Thus, there is nearly a 1000-fold discrimination observed between the affinities for tRP vs cRP. It is interesting to note that 11-*cis*-retinyl bromoacetate is not able to block the labeling of RPE65 by **1**, again demonstrating stereospecificity in retinyl ester binding to RPE65 (C. David and R. R. Rando, unpublished experiments). Thus, the fluorescence binding experiments reported here demonstrate that RPE65 is a RBP that specifically binds tRP with high affinity. The very high affinity of 20 pM for tRP is understandable in light of the great selectivity of RPE65 for tRP as compared to cRP. As previously mentioned, the stoichiometry of all-*trans*-retinyl ester binding to RPE65 has been established to be 2 by affinity labeling (*10*). Unfortunately, the fluorescence methods described here are not amenable to a precise determination of the stoichiometry of tRP binding to RPE65. Further experimentation is ongoing to establish the precise stoichiometry.

The binding experiments described here, of course, allow one to understand the phenotype observed in the RPE65 knockout in mice (*13*). Here, all-*trans*-retinyl esters were found to accumulate as a consequence of the knockout, and 11-*cis*-retinoids were prevented from forming (*13*). This result is now readily understandable. In the absence of a functioning RPE65, the highly hydrophobic all-*trans*-retinyl esters apparently become insoluble and thus not accessible to isomerohydrolase for further processing. Thus, 11-*cis*-

Scheme 3: Three-Component System for the Biosynthesis of 11-*cis*-Retinol in the RPE



retinoids are not formed. It is interesting to note that despite clear-cut evidence demonstrating the fact that all-*trans*-retinyl esters are the isomerization substrates in the visual cycle (*2*, *7*, *8*), it has been very difficult to demonstrate substantial processing of these esters into 11-*cis*-retinol in in vitro systems. This is doubtless because the retinyl esters are unavailable to the isomerohydrolase in the absence of RPE65. The low solubilities of molecules such as tRP are well-known. For example, while vitamin A undergoes rapid intermembranous transfer, under the same conditions, tRP is inert to transfer (*9*). Intermembranous transfer requires a minimal solubility in the aqueous milieu which tRP apparently does not achieve. It is interesting that in the RPE65 knockout, tRP does not appear to remain in RPE membranes but becomes insoluble in extramembranous droplets (*13*). The chemical makeup of these droplets is unknown at the present time, so it is not known whether tRP interacts with additional components, which causes the complex to be membrane insoluble. Certainly, the binding of tRP to RPE65 prevents this from occurring, rendering tRP accessible to isomerohydrolase.

Given the essential tRP binding role of RPE65, the biosynthesis of 11-*cis*-retinol in the RPE ought now be considered to involve a three-component system, rather than a two-component system, as originally envisaged (*1*, *2*). The minimal three-component system includes LRAT, RPE65, and isomerohydrolase (Scheme 3). LRAT catalyzes the esterification of vitamin A by lecithin, producing tRP as the major retinyl ester (*1*). The tRP is then bound to RPE65 where it is directed to isomerohydrolase and transformed into 11-*cis*-retinol. There is already evidence in the literature suggesting that LRAT and RPE65 interact (*34*). In fact, tetravidin chromatography of RPE65 labeled with **1** in RPE membranes shows that the two proteins interact (C. David and R. R. Rando, unpublished experiments). After formation by isomerohydrolase, the 11-*cis*-retinol probably binds to CRALBP (*29*), facilitating its oxidation by 11-*cis*-retinol dehydrogenase to generate 11-*cis*-retinal, the visual chromophore. This completes the enzymatic processing in the visual cycle. The delivery of 11-*cis*-retinal to the photoreceptors, probably by IRBP, results in rhodopsin regeneration (*31*).

The stereospecificity of RPE65 is noteworthy, although not surprising, given the homology between this protein and β-carotene 15,15′-dioxygenase (*18*, *19*). The β-carotene substrate for this enzyme contains only trans double bonds. One can ask the question as to whether the observed stereospecificity of RPE65−retinyl ester binding has physiologic consequences. The role of RPE65, as noted above, is probably to promote the mobilization and delivery of tRP to isomerohydrolase. However, 11-*cis*-retinyl esters, such as cRP, can also form in the RPE as a consequence of the action of LRAT on 11-*cis*-retinol (*35*). The question is where does cRP go and, furthermore, are there any particular reasons why it would be deleterious to the operation of the visual cycle if it bound to RPE65? First, if cRP competed with tRP for binding to RPE65, this would diminish the rate of isomerization because some RPE65 would be nonproductively bound to cRP. Second, 11-*cis*-retinoids are powerful feedback inhibitors of isomerohydrolase (*36*). If low concentrations of cRP could bind to RPE65 and thus be mobilized, there would be nothing to prevent the inhibition of isomerohydrolase by this molecule under conditions where isomerohydrolase activity should be maintained. With respect to where cRP is located, whether bulk cRP remains free in RPE membranes is unknown at the present time. However, it would not be surprising if additional retinyl ester binding protein(s) exist(s) whose role is to stereospecifically bind 11-*cis*-retinyl esters.

It now seems clear that RBPs have important roles to play in the operation of the visual cycle. Of the extant group of RBPs which are part of the visual cycle, RPE65 is the only one which stereospecifically binds retinyl esters. CRALBP stereospecifically binds 11-*cis*-retinol(al) and has an important role to play inasmuch as its knockout leads to significantly delayed rhodopsin regeneration (*37*). IRBP does not specifically bind retinoids; nevertheless, it seems to have a role to play in the shuttling of vitamin A and 11-*cis*-retinal between the RPE and photoreceptors (*33*). Knockout studies alluded to above show that of the known rod visual cycle RBPs, only RPE65 seems to play an essential role in visual processing that cannot be substituted for otherwise.

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