Retinoid Specificity of Interphotoreceptor Retinoid-Binding Protein†

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ABSTRACT: Interphotoreceptor retinoid-binding protein (IRBP), the predominant protein in the interphotoreceptor matrix of retina, has been implicated in transfer of retinoids between retinal pigment epithelium and photoreceptor cells. In this work, the interactions of several retinoids with IRBP were studied in order to clarify whether the protein displays specificity toward particular forms of these ligands. The equilibrium dissociation constants of complexes of 11-*cis*- and *all-trans*-retinols and retinaldehydes with IRBP were measured. It was found that IRBP contains two binding sites for 11-*cis*-retinaldehyde and for *all-trans*-retinaldehyde and retinol. Binding affinities followed the order: 11-*cis*-retinaldehyde > *all-trans*-retinol > *all-trans*-retinaldehyde > 11-*cis*-retinol. The kinetic parameters of the dissociation of these retinoids from binding sites on IRBP were measured by monitoring the rate of transfer of the retinoids from IRBP to synthetic unilamellar vesicles. 11-*cis*-Retinaldehyde and *all-trans*-retinol were found to dissociate from the strong binding site of IRBP 3–4-fold slower than *all-trans*-retinaldehyde and 11-*cis*-retinol. The higher binding affinities and the slower rates of dissociation from IRBP displayed by 11-*cis*-retinaldehyde and by *all-trans*-retinol correspond to the physiological need to shuttle these particular retinoids between pigment epithelium and photoreceptor cells across the interphotoreceptor matrix as part of the visual cycle.

Interphotoreceptor retinoid-binding protein (IRBP)[1] is a large (140 kDa) glycoprotein that is the major protein component of the interphotoreceptor matrix (IPM), the aqueous compartment between photoreceptors and retinal pigment epithelium cells (RPE) in the eye. IRBP is known to bind various isomeric and chemical forms of retinoids as well as long chain fatty acids and vitamin E (Fong *et al.*, 1984; Bazan *et al.*, 1985). The function(s) of this protein is (are) not completely understood at present, but it has been suggested that it serves to facilitate transfer of retinoids between photoreceptors and the RPE through the IPM and/or to protect these labile compounds while in transit in this compartment (Adler & Martin, 1982; Lai *et al.*, 1982; Saari *et al.*, 1985; Flannery *et al.*, 1988; Jones *et al.*, 1989; Okajima *et al.*, 1989; Carlson & Bok, 1992).

Shuttling of retinoids across the IPM is a crucial part of the visual cycle. Vitamin A is stored in RPE cells in the form of *all-trans*-retinyl esters. Retinyl esters are isomerized to the 11-*cis* form and 11-*cis*-retinol is oxidized to 11-*cis*-retinaldehyde, which is transported from RPE to photoreceptor cells for rhodopsin regeneration. On exposure to light, 11-*cis*-retinaldehyde, bound to rhodopsin, is isomerized to *all-trans*-retinaldehyde. The *all-trans*-retinaldehyde chromophore is hydrolyzed to yield an opsin molecule and free *all-trans*-retinaldehyde, which is enzymatically reduced to *all-trans*-retinol and transferred back to the RPE cells for reoxidation and isomerization (Saari, 1990; Rando, 1991). Thus, the visual cycle includes continuous transfer of *all-trans*-retinol from the photoreceptors to the RPE and of 11-*cis*-retinaldehyde from RPE back to the neural retina. The mechanisms by which retinoids, which are poorly soluble in water, are transported through the IPM, which is an aqueous compartment, at rates that are sufficient to support visual function are not clear. It has been repeatedly suggested, however, that IRBP plays an important role in this process (Adler & Martin, 1982; Lai *et al.*, 1982; Saari *et al.*, 1985; Flannery *et al.*, 1988; Jones *et al.*, 1989; Okajima *et al.*, 1989, Carlson & Bok, 1992).

Little quantitative information is currently available regarding the binding of different forms of retinoids to IRBP or about the interactions between binding of the different types of ligands associated with this protein. We have recently studied the interactions of *all-trans*-retinol with IRBP and found that two binding sites with similar, but not identical, affinities for this ligand exist on this protein (Chen *et al.*, 1993). Binding at the stronger site is characterized by a dissociation constant of 0.1 μM, revealing a stronger affinity than previously reported (Adler *et al.*, 1985; Okajima *et al.*, 1989). The present study was undertaken in order to further investigate the interactions of retinoids with IRBP and to clarify whether this protein displays specificity in binding of different retinoids. Binding affinities of *all-trans*- and 11-*cis*-retinol and -retinaldehyde to IRBP were evaluated. In addition, the rate constants characterizing the dissociation of the different retinoids from IRBP were measured. The characteristics of the interactions of retinoids with IRBP were found to correspond to the physiological need to transport specific retinoids across the IPM. Of the four ligands tested, the highest affinities for binding to IRBP were displayed by 11-*cis*-retinaldehyde and by *all-trans*-retinol. These two retinoids also showed slower rates of dissociation from IRBP as compared to the other ligands tested.

MATERIALS AND METHODS

all-trans-Retinol and *all-trans*-retinaldehyde were obtained from Eastman Kodak (Rochester, NY). 11-*cis*-Retinaldehyde was obtained from the National Eye Institute. 11-*cis*-Retinol was prepared from 11-*cis*-retinaldehyde by reduction with

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[1] Abbreviations: IRBP, interphotoreceptor retinoid-binding protein; RBP, serum-retinol-binding protein; TTR, transthyretin; IPM, interphotoreceptor matrix; RPE, retinal pigment epithelium; ROS, rod outer segments; DOPC, dioleoylphosphatidylcholine; DTT, dithiothreitol; DHA, docosahexaenoic acid; NBD-DPPE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine; PY-PC, 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine; HEPES, *N*-2-hydroxyethylpipeazine-*N*′-2-ethanesulfonic acid.

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$NaBH_4$ (Akhtar *et al.*, 1968). Retinoids were routinely tested by HPLC (Carlson & Bok, 1992) to insure that they were free from degradation products and other isomers. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Fluorescence probes were purchased from Molecular Probes, Inc. (Eugene, OR). All experiments were carried out in dim red light at 20 °C. Buffers contained 20 mM HEPES, pH 7.0, 150 mM NaCl, and 0.1 mM DTT.

Unilamellar vesicles of phospholipids were prepared by sonication. DOPC and the appropriate fluorescent probe (NBD-DPPE or PY-PC) were comixed in chloroform, and the solvent was evaporated under a stream of nitrogen. Seven to eight milliliters of buffer was added and the suspension was sonicated in a Heat-System sonicator to clarity. Vesicles were centrifuged at 100000*g* for 15 min to pellet multilamellar vesicles. Concentrations of phospholipids were determined by phosphorus content (Dittmer & Wells, 1969).

Charcoal-coated dextran beads were prepared as described by Dokoh *et al.* (1981), lyophilized, and stored at −20 °C.

IRBP was purified from frozen bovine retina as described by Saari and Bredberg (1985). The protein was treated with dextran-coated charcoal as previously described (Chen *et al.*, 1993) to remove endogenous retinoids.

Fluorescence measurements were performed using a SPEX Industries (Edison, NJ) Fluorolog 2 DM1B spectrofluorometer.

Equilibrium dissociation constants of retinoid–IRBP complexes were measured by fluorometric titrations of IRBP with retinoids. Apo-IRBP (0.5–2 μM) was mixed with the appropriate retinoid dissolved in ethanol at different mole ratios. The final volume was 1 mL and the ethanol concentration was usually below 1% and never exceeded 2%. To ensure equilibration between protein and ligand, the fluorescence was monitored until a constant value was reached. Binding of ligands to IRBP upon titration with the various retinoids used in this study was monitored by following changes in the fluorescence of the ligand, changes in fluorescence energy transfer between the protein and the ligand, or changes in the intrinsic fluorescence of the protein upon ligand binding (see Results). The data were analyzed as described by Cogan *et al.* (1976) to yield the values of the dissociation constants and the number of binding sites.

Retinaldehydes are nonfluorescent compounds. In order to study the interactions or retinaldehydes with the strong binding site of IRBP, the dissociation constants of *all-trans*- and 11-*cis*-retinaldehyde were measured indirectly by monitoring displacement of *all-trans*-retinol from this site by retinaldehyde. IRBP was titrated with *all-trans*-retinol in the absence and in the presence of either 11-*cis*- or *all-trans*-retinaldehyde, and the increase in retinol fluorescence (excitation, 330 nm; emission, 480 nm) was monitored. Retinaldehyde and IRBP were mixed at retinaldehyde:IRBP mole ratios of 1:1 or 2:1 and the mixtures allowed to equilibrate for 5–10 min prior to titration with *all-trans*-retinol. Measurements of IRBP–retinaldehyde dissociation constants obtained by competition with retinol yielded information regarding binding of retinaldehydes in the IRBP strong binding site.

The data can be analyzed to yield the dissociation constant of the competitor (retinaldehyde) if the K_d of the observed ligand (retinol) is known (Connors, 1987). The later dissociation constant was previously measured (Chen *et al.*, 1993).

Rates of Transfer of Retinoids from IRBP to Unilamellar Vesicles of Phosphatidylcholine. These rates were measured either by utilizing fluorescent probes incorporated into acceptor vesicles or by monitoring changes in the intrinsic fluorescence

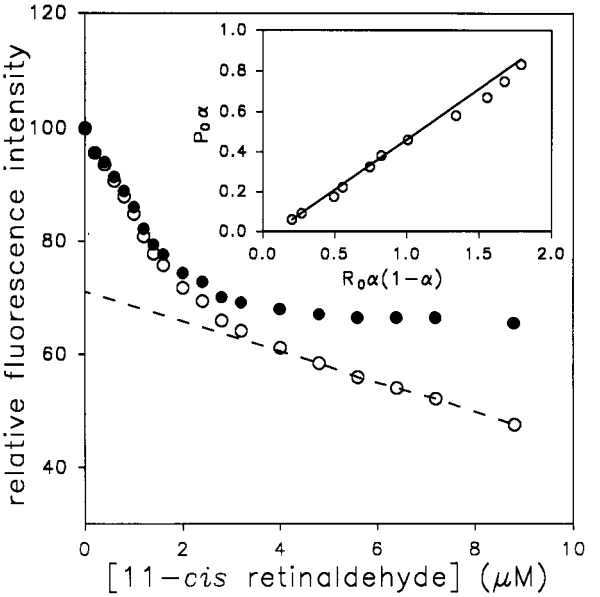

FIGURE 1: Fluorescence titration of IRBP with 11-*cis*-retinaldehyde by monitoring quenching of protein fluorescence by bound retinaldehyde. A representative titration curve is shown. The titration system consisted of 1 mL of 1.0 μM of apo-IRBP in a buffer containing 20 mM HEPES, pH 7.0, 150 mM NaCl, and 0.1 mM DTT. Retinaldehyde was added from a solution in ethanol. Excitation and emission wavelengths were 280 and 340 nm, respectively. The linear decrease in retinol fluorescence upon addition of retinaldehyde beyond protein saturation was similar to the slopes of retinol blanks and could be used for titration curve correction. Open and filled circles respectively show the raw data and the titration curve corrected for a nonspecific decrease in fluorescence. The inset shows analysis of the data plotted according to method of Cogan *et al.* (1976). The fit line was calculated by least square analysis.

of the protein upon ligand dissociation. Fluorescence probes were incorporated into synthetic unilamellar vesicles of DOPC by cosonication. To initiate transfer, protein complexed with the appropriate retinoid was mixed with vesicles using a Hi-Tech Scientific stopped-flow mixing accessory (Salisbury, England), and fluorescence was monitored continuously until equilibrium was reached.

Rates of transfer of retinoids from IRBP to unilamellar vesicles directly represented, in all cases, the rates of dissociation of retinoids from the protein. This was ascertained by the observations that the rate constants for transfer of all the retinoids studied were independent of the concentrations of protein or vesicles (data not shown). These observations excluded the possibility that transfer occurred by direct collisions between the protein and the vesicles. Instead, transfer proceeded by dissociation of retinoids into the aqueous phase followed by diffusion and association with the vesicles. The slowest, i.e. rate-limiting, step in this process is the dissociation from the protein. Thus, rates of transfer of retinoids from IRBP to vesicles represented the rate of dissociation from the protein. Such a transfer scheme was previously verified for a variety of retinoid-binding proteins including IRBP (Noy & Xu, 1990; Noy & Blaner, 1991; Ho *et al.*, 1989).

RESULTS

Equilibrium Dissociation Constants of IRBP–11-cis-Retinaldehyde Complex. The absorption spectra of retinaldehydes center at 380 nm and overlap with the intrinsic fluorescence of IRBP (excitation, 280 nm; emission, 340 nm). Binding of retinaldehydes to IRBP results in quenching of the intrinsic fluorescence of the protein. In Figure 1, a representative titration curve of IRBP with 11-*cis*-retinaldehyde

Table 1: Equilibium Dissociation Constants of IRBP–Retinoid Complexes[a]

retinoid	method	K_d (μM)	no. of sites probed
11-*cis*-retinaldehyde	displacement of ROH[b]	0.060 ± 0.012	1
	quenching of P F[c]	0.085	2.08
all-trans-retinaldehyde	displacement of ROH[d]	0.195 ± 0.014	1
11-*cis*-retinol	E transfer bet P & ROH[c]	0.31	0.72
all-trans-retinol	enhancement of ROH F[e]	0.11 ± 0.02	0.8
	E transfer bet P & ROH[e]	0.1 ± 0.02	0.71
	quenching of P F[e]	0.2 ± 0.014	1.7

[a] Dissociation constants of IRBP–11-*cis*-retinaldehyde complex were measured by monitoring the displacement of *all-trans*-retinol from IRBP strong binding site and by following quenching of protein fluorescence as described in the captions to Figures 1 and 2. The dissociation constant of IRBP–*all-trans*-retinaldehyde complex was measured by monitoring the displacement of *all-trans*-retinol as described in the caption to Figure 3. The dissociation constant of IRBP–11-*cis*-retinol complex was measured by monitoring the fluorescence energy transfer between IRBP and bound 11-*cis*-retinol as described in the caption to Figure 4. The dissociation constants and number of binding sites of IRBP–*all-trans*-retinol complexes were taken from Chen *et al.*, 1993. ROH, P, and F are *all-trans*-retinol, protein, and fluorescence, respectively. [b] $n = 6$. [c] $n = 2$. [d] $n = 5$. [e] Chen *et al.*, 1993.

obtained by measuring the fluorescence of IRBP at increasing concentrations of the ligand is shown. Protein fluorescence was progressively quenched upon addition of 11-*cis*-retinaldehyde until saturation was reached. The inset to Figure 1 shows the analysis of the data according to the method of Cogan *et al.* (1976). The data revealed the presence of two binding sites for 11-*cis*-retinaldehyde characterized by an apparent dissociation constant of 0.085 μM (Table 1). The line obtained by analyzing these data (Figure 1, inset) is linear, indicating that binding affinities for retinaldehyde in the two sites are similar.

The equilibrium dissociation constant of 11-*cis*-retinaldehyde bound in the IRBP strong site was measured by monitoring the competition between 11-*cis*-retinaldehyde and *all-trans*-retinol for binding at this site (Chen *et al.*, 1993; also see Materials and Methods). To do so, IRBP was titrated with *all-trans*-retinol in the presence of different mole ratios of 11-*cis*-retinaldehyde:IRBP. The presence of retinaldehydes in mixtures containing IRBP and *all-trans*-retinol resulted in displacement of *all-trans*-retinol from the protein's stronger binding site. The displacement could be observed by the decrease in *all-trans*-retinol fluorescence upon addition of a retinaldehyde to IRBP–retinol complexes. Significant decrease in *all-trans*-retinol fluorescence, required retinaldehyde:IRBP mole ratios of only 1–2:1, indicating that the binding affinities of IRBP for retinaldehyde were similar to the affinity for *all-trans*-retinol. Titrations of IRBP with *all-trans*-retinol, in the presence of 11-*cis*-retinaldehyde are shown in Figure 2. These titration data were used to obtain the dissociation constant of 11-*cis*-retinaldehyde for this site (Connors, 1987). The value of K_d obtained from these experiments was 0.06 μM, somewhat lower than the K_d obtain by monitoring quenching of IRBP fluorescence by 11-*cis*-retinaldehyde (Table 1), but in agreement with the conclusion that the binding affinities of the two sites are similar.

Equilibrium Dissociation Constant of IRBP–all-trans-Retinaldehyde Complex. Quenching of the intrinsic fluorescence of IRBP by *all-trans*-retinaldehyde is not efficient enough to reliably determine binding of this ligand. The origin of the difference between the efficiency of quenching by 11-*cis*- and *all-trans*-retinaldehyde is not clear and may have to

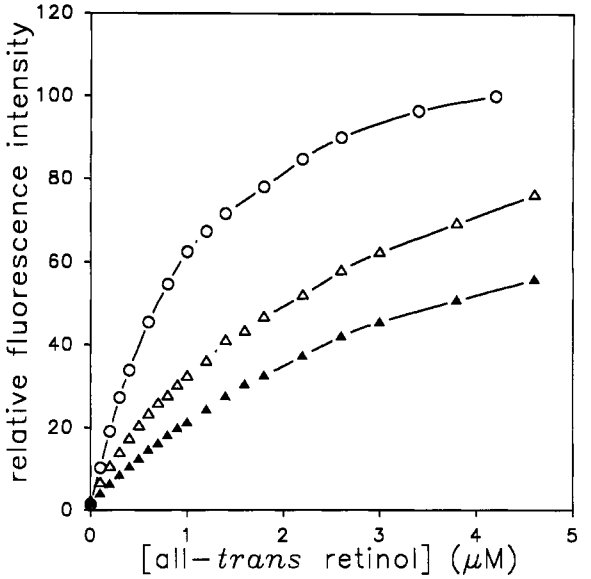

FIGURE 2: Fluorescence titration of IRBP with all-*trans*-retinol in the presence of 11-*cis*-retinaldehyde. IRBP (1.0 μM) was titrated with retinol and the titration monitored by following *all-trans*-retinol fluorescence as described in Materials and Methods. Excitation and emission wavelengths were 330 and 480 nm, respectively. Buffer composition was as described in the caption to Figure 1. Representative titrations are shown. Titrations were carried out in the absence of 11-*cis*-retinaldehyde (cop), in the presence of 1.2 μM of 11-*cis*-retinaldehyde (△), or in the presence of 2.0 μM of 11-*cis*-retinaldehyde (▲). 11-*cis*-Retinaldehyde was added from solutions in ethanol and equilibrated with IRBP prior to titration with *all-trans*-retinol.

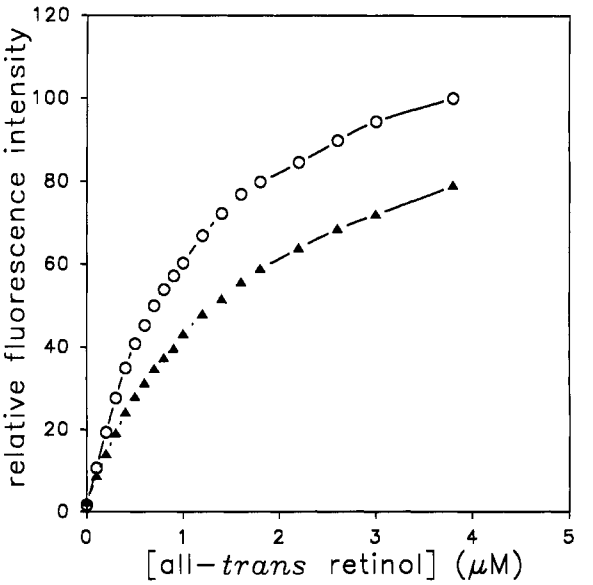

FIGURE 3: Titration of IRBP with *all-trans*-retinol in the presence of *all-trans*-retinaldehyde. IRBP (1.0 μM) was titrated with retinol and the titration monitored by following *all-trans*-retinol fluorescence as described in the Materials and Methods and in the caption to Figure 2. Titrations were carried out in the absence of *all-trans*-retinaldehyde (○) or in the presence 2.0 μM of *all-trans*-retinaldehyde (▲).

do with different orientations of the two ligands within the binding sites in relation to the tryptophane residue being quenched (see Discussion). The dissociation constant of the *all-trans*-retinaldehyde–IRBP complex was hence studied only by monitoring the displacement of *all-trans*-retinol from the strong binding site by this ligand. The competition titration curve is shown in Figure 3. The value of K_d obtained upon analysis of the data was 0.2 μM (Table 1). The affinity of

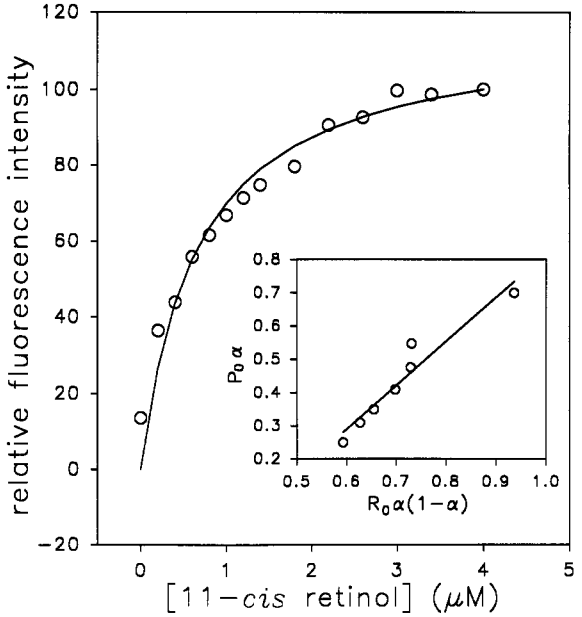

FIGURE 4: Titration of IRBP with 11-*cis*-retinol by following fluorescence energy transfer from protein to bound 11-*cis*-retinol. A representative titration curve is shown. The titration system consisted of 1 mL of 1 μM of apo-IRBP (see Materials and Methods). 11-*cis*-Retinol was added from a solution in ethanol. Excitation and emission wavelengths were 280 and 480 nm, respectively. Buffer composition was as described in the caption to Figure 1. The linear increase in retinol fluorescence upon addition of retinol beyond protein saturation was similar to the slopes of retinol blanks and could be used for titration curve correction. The inset shows analysis of the data plotted according to the method of Cogan *et al.* (1976). The fit line was calculated by least square analysis.

binding of *all-trans*-retinaldehyde in the strong binding site of IRBP is thus significantly weaker as compared to that of the 11-*cis* isomer.

Equilibrium Dissociation Constants of IRBP–11-cis-Retinol Complex. Upon binding in the strong IRBP site, fluorescence energy transfer can be observed between tryptophane residue(s) in the vicinity of the binding site and bound retinol (Chen *et al.*, 1993). Energy transfer results in retinol fluorescence upon excitation of the protein (excitation, 280 nm; emission, 480 nm) and can be used to monitor binding of retinol in IRBP's strong binding site. Analysis of titration curves (Figure 4) resulted in K_d = 0.31 μM (Table 1). The value of the K_d for binding of 11-*cis*-retinol in the strong binding site clearly indicates a weaker binding affinity for this ligand as compared with all other ligands studied (Table 1).

11-*cis*-Retinol is an extremely labile compound and rapidly degrades upon exposure to light. Exposure of mixtures to the fluorescence excitation beam in these experiments was therefore minimized, resulting in more scattered signals as compared to experiments with other ligands. HPLC analysis performed following exposure of 11-*cis*-retinol to the experimental conditions revealed isomerization of about 10%. In contrast, 70–80% of 11-*cis*-retinol was isomerized if the ligand was bound to bovine serum albumin in the absence of IRBP. These observations confirmed previous reports that binding to IRBP stabilizes retinoids (Ho *et al.*, 1989; Crouch *et al.*, 1993).

Equilibrium Dissociation Constants of IRBP–all-trans-Retinol Complex. The equilibrium dissociation constants of *all-trans* retinol from IRBP were measured and reported previously (Chen *et al.*, 1993). They are presented in Table 2.

Rates of Dissociation of Retinoids from IRBP. Mixing of IRBP complexed with a retinoid with unilamellar vesicles of phosphatidylcholine resulted in partitioning of the retinoid into the lipid bilayers of the vesicles. Rates of transfer of hydrophobic ligands between binding proteins and vesicles in many cases directly represent the rates of dissociation of ligands from the protein to the aqueous phase (Noy *et al.*, 1986; Noy & Xu, 1990; Noy & Blaner, 1991). This is so in cases where transfer proceeds by solvation of ligand from the protein, followed by diffusion of ligand through the aqueous phase and subsequent association with the lipid bilayers. The rate-limiting step for such a process is usually the dissociation from the protein. Such a transfer scheme was previously verified for the process of transfer of *all-trans*-retinol (Ho *et al.*, 1989) and other retinoids (see Materials and Methods) between IRBP and vesicles.

Table 2: Rate Constants of the Interactions of Retinoids with IRBP[a]

retinoid	k_{off} (s^{-1})	$t_{1/2(off)}$ (s)	k_{on} (M^{-1} s^{-1})
11-*cis*-retinaldehyde[b]	k_1 = 0.13 ± 0.02	5.33	2.2×10^6
	k_2 = 1.27 ± 0.23	0.54	1.1×10^7
all-trans-retinaldehyde[c]	k_1 = 0.51 ± 0.11	1.36	2.6×10^6
11-*cis*-retinol[c]	k_1 = 0.37 ± 0.064	1.87	1.2×10^6
all-trans-retinol[c]	k_1 = 0.126 ● 0.006	5.50	1.3×10^6

[a] The rate constants for dissociation of 11-*cis*-retinaldehyde from IRBP were measured as described in the captions to Figures 5 and 6. Rate constants for dissociation from IRBP of *all-trans*-retinaldehyde, *all-trans*-retinol, and 11-*cis*-retinol were measured as described in the captions to Figures 7, 8, and 9, respectively. The rate constants for association of the different ligands with IRBP (k_{on}) were calculated from the equilibrium distribution constants (K_d) given in Table 1 and the rate constants for dissociation (k_{off}) using the relation $K_d = k_{off}/k_{on}$. [b] $n = 6$. [c] $n = 4$.

Rate of Dissociation of 11-cis-Retinaldehyde from IRBP. To monitor transfer of 11-*cis*-retinaldehyde from IRBP to vesicles, the fluorescent lipid probe PY-PC was incorporated into vesicles of DOPC by cosonication. The fluorescence emission spectrum of PY-PC overlaps with the absorption spectrum of retinaldehyde, and the presence of retinaldehyde in close proximity to the probe will result in quenching of probe fluorescence. Thus, movement of retinaldehyde from IRBP to vesicles was followed by monitoring the time-dependent decrease in probe fluorescence (excitation, 330 nm; emission, 400 nm) which occurred following mixing the complex IRBP–retinaldehyde with probe-containing vesicles. A representative trace showing the time-dependent decrease in PY-PC fluorescence following mixing of the two phases and movement of 11-*cis*-retinaldehyde to the vesicles is shown in Figure 5.

In the experiment depicted in Figure 5, the initial mole ratio of ligand/protein was 0.5. The rate of dissociation of 11-*cis*-retinaldehyde from IRBP in this experiment was thus expected to predominantly represent dissociation of the ligand from the stronger IRBP site. The solid line in Figure 5a shows the fit of the data to a single first-order process. The residuals of the fit are shown in the inset to Figure 5a. Dissociation of 11-*cis*-retinaldehyde from IRBP agreed fairly well with a single first-order kinetics. In Figure 5b, the solid line represents a fit of the data to two first-order reactions. It can be seen that the fit line follows the data better in the initial part of the curve. Hence, it appears that 11-*cis*-retinaldehyde occupies two sites on IRBP, in agreement with the equilibrium studies (Table 1), and that the retinoid dissociates from the two sites at distinct rates. At the 0.5:1 mole ratio of ligand:protein used in this experiment, most of the reaction proceeded slowly and had a rate constant of 0.13 s^{-1}. The remaining fraction of 11-*cis*-retinaldehyde dissociated from the protein much

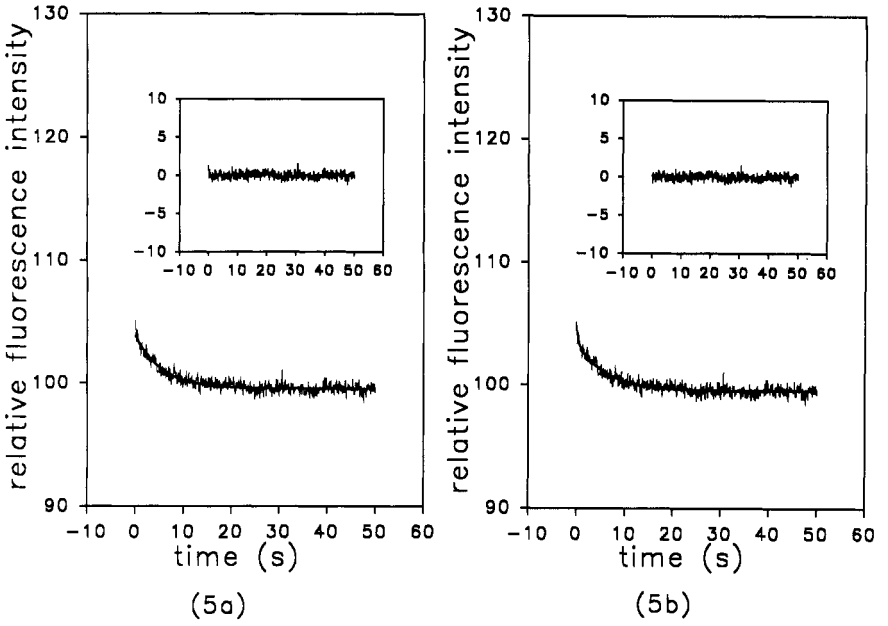

FIGURE 5: Transfer of 11-*cis*-retinaldehyde from IRBP to vesicles of DOPC at an initial 11-*cis*-retinaldehyde:IRBP mole ratio = 0.5. Unilamellar vesicles of DOPC were made by sonication and contained 2 mol % PY-PC. Vesicles were mixed with IRBP–11-*cis*-retinaldehyde using a stopped-flow accessory and fluorescence was monitored until equilibrium was reached. The final concentrations of protein and vesicles were 1.0 μM and 1 mM, respectively. Transfer of retinoid from IRBP to vesicles was followed by monitoring the quenching of PY-PC fluorescence by 11-*cis*-retinaldehyde upon its arrival at the vesicles. Excitation and emission wavelengths were 330 and 400 nm, respectively. Insets show the residuals corresponding to the fit of traces to a single first-order reaction (5a) or to two first-order reactions (5b).

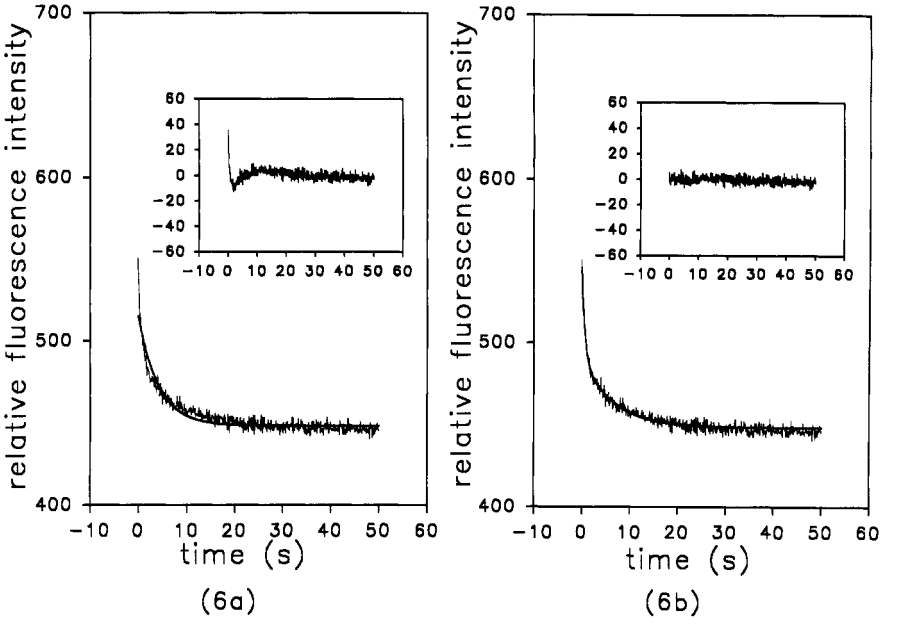

FIGURE 6: Transfer of 11-*cis*-retinaldehyde from IRBP to vesicles of DOPC at an initial 11-*cis*-retinaldehyde:IRBP mole ratio = 2. Experiments were carried out as described in the caption for Figure 5 using a higher initial mole ratio of ligand bound to the protein. Insets show the residuals corresponding to the fit of the trace to a single first-order reaction (6a) or to two first-order reactions (6b).

faster (k_{off} = 1.3 s^{-1}). The distribution of 11-*cis*-retinaldehyde between the slow and the fast pools was 70% and 30%, respectively. The binding affinity of the stronger site can thus be assessed to be about 2.3-fold stronger than the affinity of the weaker site.

To further ascertain that the two observed reaction reflected dissociation of 11-*cis*-retinaldehyde from two distinct sites, the rates of dissociation were also measured with an initial ligand:protein mole ratio = 2.0. Under these conditions, it is expected that a larger fraction of 11-*cis*-retinaldehyde will occupy the weaker site, resulting in a larger pool of ligand dissociating at the faster rate. The data shown in Figure 6 confirm the existence of two pools, as can be seen by the poor fit of the data to a single first-order reaction (Figure 6a) and the excellent fit observed when two first-order reactions are assumed (Figure 6b). The distribution of 11-*cis*-retinaldehyde between the slow and the fast dissociating pools under these conditions was 40% and 60%, respectively. Thus, a larger fraction of the ligand was associated with the weaker binding site when a ligand:protein ratio of 2:1 was used. The rate constants that best fit the data in experiments utilizing an initial ligand:protein mole ratio of 2:1 were identical to those obtained with an initial mole ratio of 0.5.

Rate of Dissociation of all-trans-Retinaldehyde from IRBP. The rate of dissociation of *all-trans*-retinaldehyde from IRBP also was measured by following transfer of this ligand between IRBP and vesicles of DOPC containing the fluorescent probe PY-PC. As can be seen in the trace shown in Figure 7,

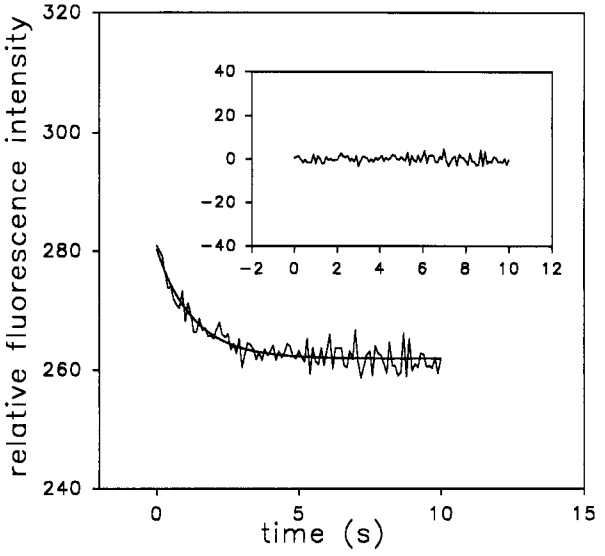

FIGURE 7: Transfer of *all-trans*-retinaldehyde from IRBP to vesicles of DOPC. The mole ratio of 11-*cis*-retinaldehyde:IRBP was 1.2:1.0. Unilamellar vesicles of DOPC were made by sonication and contained 2 mol % PY-PC. Vesicles were mixed with IRBP–*all-trans* retinaldehyde and transfer of the retinoid to the vesicles was followed as described in the caption to Figure 5. The final concentrations of protein and vesicles were 0.5 and 250 μM, respectively. Insets show the residuals corresponding to the fit of the trace to a single first-order reaction.

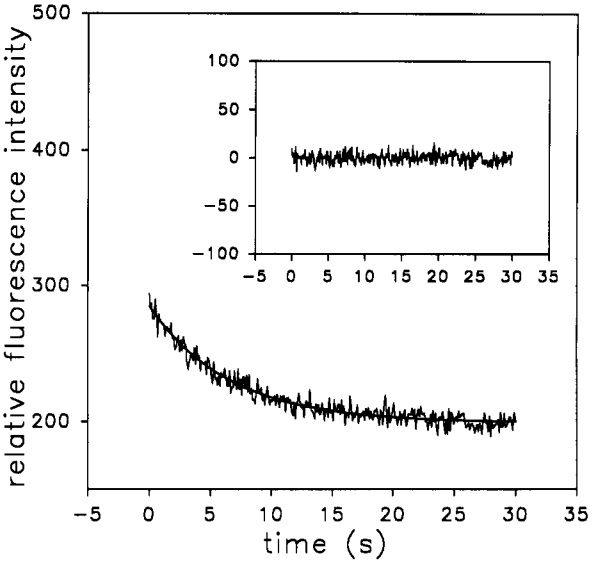

FIGURE 8: Transfer of *all-trans*-retinol from IRBP to vesicles of DOPC. The mole ratio of *all-trans*-retinol:IRBP was 2.0:1.0. Unilamellar vesicles of DOPC were made by sonication and contained 2 mol % NBD-DPPE. The final concentrations of protein and vesicles were 0.5 μM and 1.0 mM, respectively. Transfer of *all-trans*-retinol from IRBP to vesicles was followed by the time-dependent quenching of *all-trans*-retinol fluorescence by NBD in the vesicles. Excitation and emission wavelengths were 350 and 480 nm, respectively. Insets show the residuals corresponding to the fit of the trace to a single first-order reaction.

dissociation followed a single first-order reaction. The dissociation rate constant was 0.51 s^{-1}, 4-fold faster than the rate of dissociation of 11-*cis*-retinaldehyde from the strong site of IRBP (Table 2). No additional reaction was observed even at higher ligand:protein mole ratios. The apparent absence of a second reaction with a different reaction rate, though two binding sites for 11-*cis*-retinaldehyde were found in the equilibrium studies (Table 1), could indicate that the rates of dissociation of this ligand from the two binding sites were similar or that the rate of the fast reaction, in this case, was too rapid to be observed under the conditions used.

Rate of Dissociation of 11-cis-Retinol from IRBP. To measure the rate of transfer of 11-*cis*-retinol between IRBP and vesicles, the observation that binding of retinol to IRBP results in quenching of the intrinsic fluorescence of the protein was utilized (see Materials and Methods). Thus, IRBP complexed with 11-*cis*-retinol was mixed with vesicles of DOPC devoid of external probes. Dissociation of 11-*cis*-retinol from the protein resulted in release of quenching of IRBP fluorescence, and movement of this ligand from IRBP to the vesicles could be followed by monitoring the time-dependent increase in protein fluorescence. A representative trace resulting from such an experiment is shown in Figure 8. The rate of dissociation of 11-*cis*-retinol from IRBP followed a single first-order kinetics with a rate constant of 0.37 s^{-1} (Table 2).

Rate of Dissociation of all-trans-Retinol from IRBP. To follow transfer of *all-trans*-retinol between IRBP and vesicles the fluorescent probe NBD-DPPE was used. The absorption spectrum of NBD overlaps extensively with the fluorescence emission spectrum of retinol, and energy transfer between retinol and this probe when both are incorporated in the same lipid bilayer results in quenching of retinol fluorescence (Noy, 1988; Noy & Xu, 1990). A representative trace depicting transfer of *all-trans*-retinol from IRBP to NBD-containing vesicles as observed by the time-dependent decrease in retinol fluorescence is shown in Figure 9. The data could be fitted well (Figure 9, solid line) by assuming a single first-order

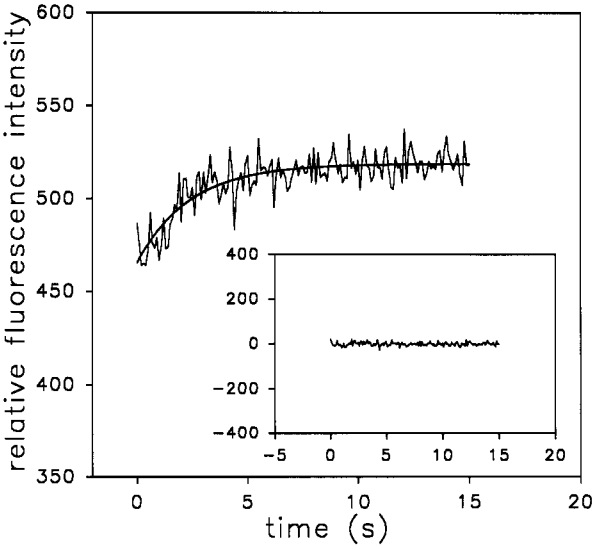

FIGURE 9: Transfer of 11-*cis*-retinol from IRBP to vesicles of DOPC. Unilamellar vesicles of DOPC were made by sonication. Vesicles were mixed with IRBP complexed with 11-*cis*-retinol at a 11-*cis*-retinol:IRBP mole ratio of 1.5. The final concentrations of protein and vesicles were 0.5 and 500 μM, respectively. Transfer of 11-*cis*-retinol from IRBP to vesicles was followed by the release of quenching of protein fluorescence upon the dissociation of ligand from IRBP. Excitation and emission wavelengths were 280 and 340 nm, respectively. Insets show the residuals corresponding to the fit of the trace to a single first-order reaction.

process with a rate constant of 0.126 s^{-1}. This value is in agreement with previous reports (Ho *et al.*, 1989). Similarly to the dissociation of *all-trans*-retinaldehyde and 11-*cis*-retinol from IRBP, no additional reaction could be observed either at high or low mole ratios of ligand:protein.

Rate Constants for Association of Retinoids with IRBP. The equilibrium dissociation constant, K_d, is governed by the rate constants for dissociation of retinoids from IRBP, k_{off}, and for association with the protein, k_{on}. The association rate

constants can be calculated from the measured equilibrium constants and k_{off}s by using the relation $K_d = k_{off}/k_{on}$. The values of the rate constants for association of retinoids with IRBP are shown in Table 2. For 11-*cis*-retinaldehyde, this parameter could be obtained for both binding sites as k_{off}s from both the weaker and the stronger sites were measured. The values of k_{on} revealed that association of 11-*cis*-retinaldehyde with the weaker site proceeded at a 5-fold faster rate *vs* association with the strong site, indicating an easier accessibility to this site.

DISCUSSION

It was recently reported that IRBP contains two binding sites for *all-trans*-retinol (Chen *et al.*, 1993). The dissociation constant characterizing binding of *all-trans*-retinol in one of these sites was 0.1 μM, on the same order as K_ds governing the interactions of this ligand with other binding proteins, e.g. plasma retinol-binding protein and cellular retinol-binding protein I (Noy & Xu, 1990; Noy & Blaner, 1991). Binding affinity for *all-trans*-retinol in the second IRBP binding site was about 2-fold lower. It was suggested in the above report that the strong IRBP site is hydrophobic in nature and that the second, weaker site may interact with the polar head group of *all-trans*-retinol rather than with the β-ionone ring. The studies reported here revealed the presence of two binding sites on IRBP for retinoids other than *all-trans*-retinol; analysis of fluorometric titrations of IRBP with either 11-*cis*- or *all-trans*-retinaldehyde resulted in identification of two binding sites for these ligands (Table 1). In addition, both retinaldehydes studied were found to compete with *all-trans*-retinol on binding in the hydrophobic IRBP site, suggesting that the same two sites serve to bind all retinoid forms and that the protein does not possess unique sites for different retinoids.

Of all the retinoids studied, 11-*cis*-retinaldehyde had the highest affinity for IRBP. 11-*cis*-Retinaldehyde bound in the IRBP strong site displayed an equilibrium dissociation constant of 0.06 μM (Table 1). Experiments designed to probe binding of this ligand in both binding sites showed an average dissociation constant of 0.085 μM. It can be estimated from these data that K_d for 11-*cis*-retinaldehyde in the weak site was 0.11 μM, i.e. that the difference in binding affinity in the two sites was about 1.8-fold. A similar differential affinity for binding of 11-*cis*-retinaldehyde in the two IRBP sites was found in kinetic studies of the dissociation of 11-*cis*-retinaldehyde from IRBP. 11-*cis*-Retinaldehyde bound to IRBP was found to be distributed between a slow and a fast dissociating pool. 70% *vs* 30% of this ligand was associated with the slow *vs* the fast dissociating pool under conditions where none of the sites were saturated, indicating a 2.3-fold difference in binding affinity.

The affinity for binding of *all-trans*-retinol to IRBP in both sites was about 2-fold lower than that of 11-*cis*-retinaldehyde, and binding constants of *all-trans*-retinaldehyde and 11-*cis*-retinol to the strong IRBP site were 2–2.5-fold weaker still. Overall, IRBP displayed the following ligand preference: 11-*cis*-retinaldehyde > *all-trans*-retinol > *all-trans*-retinaldehyde > 11-*cis*-retinol. To further investigate the interactions of the various retinoids with IRBP, kinetic studies of the dissociation of ligands from the protein were undertaken. In experiments with 11-*cis*-retinaldehyde, two pools of ligand with distinguishable rates of dissociation were observed. In contrast, the dissociation of other retinoids from IRBP could be well fit to a single first-order kinetic scheme. The apparent absence of a second reaction, despite the observations that IRBP possesses two binding sites for all the retinoids studied, could indicate that the rates of dissociation of these ligand from the two binding sites were similar. Alternatively, and more likely, the rates of dissociation of ligands other than 11-*cis*-retinaldehyde from the weak IRBP site were too rapid to be observed under the conditions used. $t_{1/2}$ for dissociation of retinoids from the strong site of IRBP followed the order 11-*cis*-retinaldehyde = *all-trans*-retinol > *all-trans*-retinaldehyde = 11-*cis*-retinol. Thus, 11-*cis*-retinaldehyde and *all-trans*-retinol were found to have a higher affinity for IRBP and to dissociate from the protein at a slower rate as compared to the other retinoids studied. It is interesting to note that the rates by which retinoids interact with IRBP are much more rapid than rates of interactions of these ligands with other binding proteins even though the equilibrium binding affinities are similar. For example, $t_{1/2}$s for dissociation of retinol from complexes of *all-trans*-retinol with bovine serum-retinol-binding protein (RBP), rat cellular retinol binding protein (CRBPI), and bovine IRBP are 370, 230, and 5 s, respectively (Noy & Xu, 1990; Noy & Blaner, 1991; and the present work). The rapid dissociation of retinoids from IRBP may hint at a different function for IRBP, or at a physiological need to supply retinoids at a faster rate in the eye as compared to rates of utilization of retinol in other tissues.

In considering the interactions of retinoids within the IRBP strong binding site, it was found that there is a preference, within this site, for binding of retinoids with an aldehyde head group of 11-*cis* configuration and for binding retinoids with a hydroxyl head group of *all-trans* configuration. The binding site thus appears to have two regions for retinoid binding, with a possible "switch" between them. This possibility is supported by the observation that quenching of the intrinsic fluorescence of IRBP by *all-trans*-retinaldehyde is not as efficient as by 11-*cis*-retinaldehyde. The origin of the difference between the efficiency of quenching by the two retinaldehydes is not clear, but it is likely to stem from different orientations of the two ligands in relation to tryptophane residue(s) within the binding pocket. Additional information regarding the interactions of retinoids with IRBP can be obtained by considering the rate constants for association of these ligands with the protein. The values of k_{on} for the association of 11-*cis*-retinaldehyde with the two binding sites on IRBP (Table 2) showed a significantly faster interaction of this ligand with the second, weaker site. This observation agrees with our previous suggestion that this site is superficially located at the protein's surface (Chen *et al.*, 1993). k_{on}s for association of the different retinoids with the IRBP strong site (Table 2) showed that retinaldehydes associated with this site faster than retinols, indicating the a smaller energy barrier for association with IRBP of retinoids containing an aldehyde *vs* an alcohol head group.

The studies reported here reveal that the retinoid specificity of IRBP corresponds to the proposed physiological role of this protein in transfer of retinoids between retinal pigment epithelium and photoreceptor cells across the interphotoreceptor matrix. Movement of two retinoid species across the IPM is believed to be required for the visual cycle; these species are 11-*cis*-retinaldehyde and *all-trans*-retinol. The former is generated and secreted by the RPE for transfer across the IPM to photoreceptors, where it serves to regenerate rhodopsin. The later is liberated from photoreceptors following exposure to light and moves across the IPM to pigment epithelium cells for oxidation to retinaldehyde and reisomerization to the 11-*cis* form (Saari, 1990; Rando, 1991). The data presented above showing that the two retinoid forms which move across the IPM as an integral part of the visual cycle are those that

display the highest affinity for IRBP further support the suggestion (Adler & Martin, 1982; Lai *et al.*, 1982; Saari *et al.*, 1985; Flannery *et al.*, 1988; Jones *et al.*, 1989; Okajima *et al.*, 1989, Carlson & Bok, 1992) that IRBP plays a role in transfer of retinoids between pigment epithelium and photoreceptor cells. The exact function of IRBP in this process is not known at present (see Saari, 1994, for a detailed discussion). Retinoids could distribute between binding sites on IRBP and the membranes of cells by a series of rapid equilibria with net transfer taking place through the aqueous phase and driven by concentration gradients. Alternatively, IRBP could play a more specific role in the transport process, for example by extracting retinoids from either RPE or photoreceptor cells. Thus, the mechanism(s) by which retinoid transfer across the IPM is accomplished at a fast enough rate to sustain visual function and the step(s) assisted by binding of retinoids to IRBP remain to be clarified.

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