Interactions of All-*trans*-retinol and Long-Chain Fatty Acids with Interphotoreceptor Retinoid-Binding Protein†

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ABSTRACT: Interphotoreceptor retinoid-binding protein (IRBP), a predominant protein in the interphotoreceptor matrix of the retina, has been implicated in transfer of retinoids between retinal pigment epithelium and photoreceptor cells. The interactions of IRBP with all-*trans*-retinol have been studied by three fluorescence-based methods and by measurements of binding of ^{3}[H]-labeled all-*trans*-retinol to this protein. It was found that IRBP contains two sites with similar but not identical affinities for all-*trans*-retinol. The dissociation constant of the all-*trans*-retinol–IRBP complex at the first site was 0.1 μM, which is about 10-fold lower than previously reported values. The second site had about 2.5-fold lower affinity for all-*trans*-retinol as compared to the first site. Long-chain fatty acids were found in this study to displace all-*trans*-retinol from the stronger retinol-binding site on IRBP. Displacement of all-*trans*-retinol was used to study the interactions of fatty acids with this protein. It was found that docosahexaenoic acid (DHA C22:6 $n-3$), an essential fatty acid which plays an important role in vision, had the highest apparent affinity for the site probed on IRBP of all the fatty acids studied.

Vitamin A is delivered from blood to retinal pigment epithelium (RPE) in the eye in the form of all-*trans*-retinol.[1] In these cells, retinol is esterified, isomerized to 11-*cis*-retinol, and oxidized to 11-*cis*-retinaldehyde, the form of vitamin A that regenerates rhodopsin. 11-*cis*-Retinaldehyde moves from RPE to photoreceptor cells for rhodopsin regeneration. On exposure to light, 11-*cis*-retinaldehyde, bound to rhodopsin, is isomerized to the all-*trans* form. The all-*trans*-retinaldehyde chromophore is then hydrolyzed to form an opsin molecule and free 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 shuttling of retinoids back and forth between RPE and photoreceptor cells through the interphotoreceptor matrix (IPM), the extracellular compartment between RPE and the neural retina. The mechanisms by which retinoids, which are poorly soluble in water, are transported through this aqueous compartment at rates that are sufficient to support visual function have not yet been clarified. It had been suggested that the interphotoreceptor retinoid-binding protein (IRBP), a soluble protein that is found almost uniquely in the IPM, serves to facilitate retinoid transfer and/or to protect these labile compounds while in the IPM (Adler & Martin, 1982; Lai et al., 1982; Saari *et al.*, 1985; Flannery *et al.*, 1988; Jones *et al.*, 1989; Okajima *et al.*, 1989). IRBP is a high molecular weight glycoprotein that is the major protein of the IPM and 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).

There is a discrepancy in the literature regarding the number of binding sites for retinoids on IRBP. This discrepancy may have arisen from utilization of different methodologies. Thus, one binding site with a dissociation constant of about 1 μM was observed when binding of all-*trans*-retinol to IRBP was investigated by monitoring the increase in retinol fluorescence which occurs upon binding (Adler *et al.*, 1985; Okajima *et al.*, 1989). On the other hand, a retinol/IRBP mole ratio of 2 was found following saturation of IRBP with radioactively labeled retinol (Saari *et al.*, 1985) or by spectral and HPLC analyses of ligands extracted from IRBP (Fong *et al.*, 1984).

Regarding the interactions of IRBP with long-chain fatty acids, it was reported that IRBP contains 6–7 mol of these ligands when purified from monkey retina. Of total fatty acids, 65% were found to be noncovalently attached, with the remainder covalently bound (Bazan *et al.*, 1985). It was suggested on the basis of these observations that IRBP may function as a carrier for fatty acids as well as for retinoids.

In the present study, some of the characteristics of binding of all-*trans*-retinol and of fatty acids to IRBP were investigated. It was found that IRBP contains two binding sites for retinol with similar but not identical binding affinities. Only the stronger of these sites could be observed by monitoring changes in retinol fluorescence upon binding, and the observed dissociation constant for this site was found to be 1 order of magnitude lower than previously reported (Adler *et al.*, 1985; Okajima *et al.*, 1989). It was further observed that the presence of long-chain fatty acids results in displacement of retinol from this site. This observation was used to study the interactions of various fatty acids with IRBP. The term "retinol" used below refers to all-*trans*-retinol unless specified otherwise.

MATERIALS AND METHODS

All-*trans*-retinol was obtained from Eastman Kodak (Rochester, NY). [^{3}H]Retinol was from Du Pont New England

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[1] Abbreviations: IRBP, interphotoreceptor retinoid-binding protein; RBP, serum retinol-binding protein; CRBP, cellular retinol-binding protein; IPM, interphotoreceptor matrix; RPE, retinal pigment epithelium; ROS, rod outer segments; PC, phosphatidylcholine; DTT, dithiothreitol; DHA, docosahexaenoic acid; retinol, all-*trans*-retinol.

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Nuclear (Boston, MA). *N*-Acetyl-L-tryptophanamide, charcoal (Norit A), and Dextran T-70 were purchased from Sigma Chemical Company (St. Louis, MO). Other chemicals were purchased from Baker (Phillipsburg, NJ). Scintillation liquid (Liquiscint) was obtained from National Diagnostics (Manville, NJ).

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 previously (Saari & Bredberg, 1988). Endogenously bound retinoids were extracted from IRBP by treatment with charcoal-coated dextran beads. Holo-IRBP protein (50–200 nmol) was mixed with 16 g of charcoal-coated beads in a final volume of 60 mL buffer containing 20 mM HEPES, pH 7.0, 150 mM NaCl, and 0.1 mM DTT. The mixture was gently shaken for 1 h at 4 °C and filtered. Fine particles which were generated during the procedure were eliminated by centrifugation at 2000*g* for 5 min. The absorption spectrum (in the range 250–450 nm) as well as the fluorescence (excitation and emission wavelengths of 330 and 480 nm, respectively) of the resulting supernatant were examined to verify complete extraction of endogenous retinoids. The concentrations of IRBP and of retinol were determined from their extinction coefficients (Hubbard *et al.*, 1971; Horowitz & Heller, 1973; Saari & Bredberg, 1988). Experiments were performed at 20 °C.

Fatty acid contents of IRBP before and after charcoal treatment were measured by an enzymatic fluorometric method (Miles *et al.*, 1983). A standard curve was generated using oleic acid dissolved in a buffer containing 20% ethanol and 0.2% Triton X-100 (Miles *et al.*, 1983).

Binding of [^{3}H]Retinol to IRBP. ^{3}H-labeled retinol (25 000 cpm/nmol) was added to IRBP (0.5–1 μM) from a solution of 0.2 mM in ethanol to achieve mole ratios of retinol/IRBP in the range of 0.1–3. The final volume of each mixture was 0.43 mL. The buffer contained 20 mM HEPES, pH 7.0, 150 mM NaCl, and 0.1 mM DTT. Mixtures were incubated for 30 min, and 0.4 mL of each of the solutions was transferred to a clean tube to eliminate undissolved retinol which may have adsorbed to the walls of the initial test tubes. The total concentration of [^{3}H]retinol in each mixture was determined by counting that in a 0.05-mL sample. A 0.2-mL sample of 5% charcoal-coated dextran beads in buffer containing 150 mM NaCl, 0.015 M NaN_3, 20 mM HEPES, and 0.1% gelatin was added to the test tubes. Gelatin was added to the mixtures to better suspend the charcoal beads (Dokoh *et al.*, 1981). The mixtures were then incubated with gentle shaking for 4 min. Note that the charcoal concentration in the binding assays was lower and the incubation time shorter than in the procedure used for full extraction of retinoids from IRBP. The tube walls were rinsed by the addition of 0.45 mL of the same buffer to a final volume of 1 mL. Beads, containing free retinol, were pelleted by centrifugation at 3000*g* for 5 min, and the supernatant (containing the protein) was counted to obtain the concentration of bound [^{3}H]retinol. Data were analyzed using the Scatchard treatment (Connors, 1987).

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

Fluorometric Titrations of IRBP with Retinol. Apo-IRBP (0.6–2 μM) was titrated directly in the cuvette with retinol dissolved in ethanol. The final volume was 1 mL, and ethanol concentration was usually below 1% and never exceeded 2%. The buffer contained 20 mM HEPES, pH 7.0, 150 mM NaCl, and 0.1 mM DTT. To ensure equilibration between protein and ligand, the fluorescence was monitored until a constant value was reached. The titration was monitored by following (1) the fluorescence of retinol (excitation, 330 nm; emission, 480 nm), (2) energy transfer between IRBP and bound retinol (excitation, 280 nm; emission, 480 nm), or (3) the intrinsic fluorescence of IRBP (excitation, 280 nm; emission, 340 nm). The data were analyzed by the method of Cogan *et al.* (1976). In this treatment, the mass law equation (eq 1), is used to derive an equation for the evaluation of the apparent dissociation constant, K_d. In eq 1, [P], [R], and [PR] are the

$$K_d = (n[P][R])/[PR] \quad (1)$$

concentrations of free RBP, free retinol, and retinol bound to RBP, respectively; n is the number of binding sites for retinol per mole of RBP. When α is defined as the fraction of free binding sites on the protein molecule and $[P_0]$ and $[R_0]$ are the total protein and retinol concentrations, respectively, eq 1 can be written in the form of eq 2,

$$[P_0]\alpha = (1/n)([R_0]\alpha/(1-\alpha)) - K_d/n \quad (2)$$

A plot of $[P_0]\alpha$ *vs* $[R_0]\alpha/(1-\alpha)$ will have a slope of $1/n$ and an intercept of K_d/n. The value of α was calculated for every point on the titration curve of fluorescence intensity *vs* total retinol concentration using the relation

$$\alpha = (F_{max} - F)/(F_{max} - F_0) \quad (3)$$

where F_0, F, and F_{max} are the fluorescence intensities at $[R_0]$, at [R], and at saturation, respectively.

Calculations and corrections for the contribution of free retinol to the overall fluorescence signals were performed as in Cogan *et al.* (1976). In assays where fluorescence signals increased upon addition of retinol, the titration curves were corrected by subtracting a blank in which retinol was added to the buffer in the absence of protein. The slopes of such blanks were found to be similar to the slopes of the linear increase in fluorescence observed in the titration experiments following saturation of the protein (see Figure 3). Thus, in some experiments these slopes were used as a blank. In assays where fluorescence signals decreased upon addition of retinol, the titration curves were corrected by subtracting a blank in which retinol was added to a solution of *N*-acetyl-L-tryptophanamide with an initial fluorescence identical to that of the initial fluorescence of IRBP [see Cogan *et al.* (1976)]. The slopes of such blanks were found to be similar to the slopes of the linear decrease in fluorescence observed in the titration experiments following saturation of the protein (see Figure 4).

Binding constants of long-chain fatty acids to IRBP were measured indirectly by monitoring displacement of retinol from IRBP caused by the presence of these ligands. IRBP was titrated with retinol, and retinol binding was followed by monitoring the fluorescence of retinol as described above. Titrations of IRBP were carried out in the absence or presence of various fatty acids at a fatty acid/IRBP mole ratio of 10:1. Free fatty acids were added to IRBP (0.6–2 μM) from a concentrated solution in ethanol (1–2 mM), and the mixture was allowed to equilibrate for 5 min prior to the addition of retinol. A ratio of 10:1 fatty acid/protein was chosen following initial experiments which indicated that the binding affinities of fatty acids for IRBP were about 1 order of magnitude weaker than those for retinol. The data were analyzed by competitive fluorospectrophotometry (Connors, 1987) as briefly outlined below. The mass balance equations for the total concentration of the reacting species retinol (R), fatty acid (F), and IRBP (I) are given in eqs 4–6.

$$[R_t] = [R] + [RI] \quad (4)$$

$$[F_t] = [F] + [FI] \quad (5)$$

$$[I_t] = [I] + [RI] + [FI] \quad (6)$$

The binding constants of retinol (K_R) and of a fatty acid (K_F) are defined by eqs 7 and 8.

$$K_R = [RI]/[R][I] \quad (7)$$

$$K_F = [FI]/[F][I] \quad (8)$$

Equations 7 and 8 may be rearranged in the form of eq 9,

$$F_t/[P] = (K_R/K_F)Q + 1 \quad (9)$$

where $Q = 1/K_R[I]$ and $[P] = [R_t]K_F/(QK_R + K_F)$. Plotting the data as $F_t/[P]$ *vs* Q would result in a straight line with a slope corresponding to K_R/K_F and a y-intercept of 1. Thus, if the binding constant for retinol (K_R) is known, the binding constant for the fatty acid being studied (K_F) can be calculated.

RESULTS

Formation of Retinol–IRBP Complex Followed by Monitoring Changes in Retinol Fluorescence. Retinol is an efficient fluorophore, and its interactions with proteins are often accompanied by a marked increase in its fluorescence (excitation and emission wavelengths 330 and 480 nm, respectively). This phenomenon reflects movement of retinol from the aqueous solution to a less polar and more restrictive environment within the protein binding site and has been often used to monitor the binding of this ligand to retinol-binding proteins [e.g., Cogan *et al.* (1976), Ong & Chytil (1978), and Noy & Blaner (1991)]. Figure 1 shows a representative titration of IRBP with retinol. Analysis of the data according to eq 2 is shown in the inset to Figure 1. The apparent dissociation constant and the number of binding sites were 0.11 ± 0.02 μM and 0.77 ± 0.1 mol retinol/mol protein, respectively (Table I). Thus, as previously reported, one site for retinol on IRBP is revealed by this method. However, the value of the dissociation constant is 1 order of magnitude lower than in previous studies (Adler *et al.*, 1985; Okajima *et al.*, 1989), indicating a higher affinity.

Fluorescence Spectra of Apo- and Holo-IRBP. In Figure 2 are shown emission spectra of apo-IRBP (line 1) and of the retinol-IRBP complex (line 2). These spectra were obtained upon excitation at 280 nm, i.e., excitation of protein tryptophan and tyrosine residues. It is clear from these spectra that binding of retinol to IRBP results in significant quenching of the intrinsic fluorescence of the protein which peaks at 350 nm. An additional peak is seen at 480 nm, reflecting fluorescence of retinol. Clearly, energy transfer exists between a tryptophan or a tyrosine residue near the retinol binding site and bound retinol. These observations conflict with earlier studies in which neither protein quenching nor energy transfer between protein and bound retinol was observed following the interactions of retinol with IRBP (Adler *et al.*, 1985) but are in agreement with a recent report of the existence of energy transfer between IRBP and retinol (Hazard *et al.*, 1993). Quenching of protein fluorescence as well as energy transfer from the protein to bound retinol can be used to study the interactions of retinol with IRBP (see below).

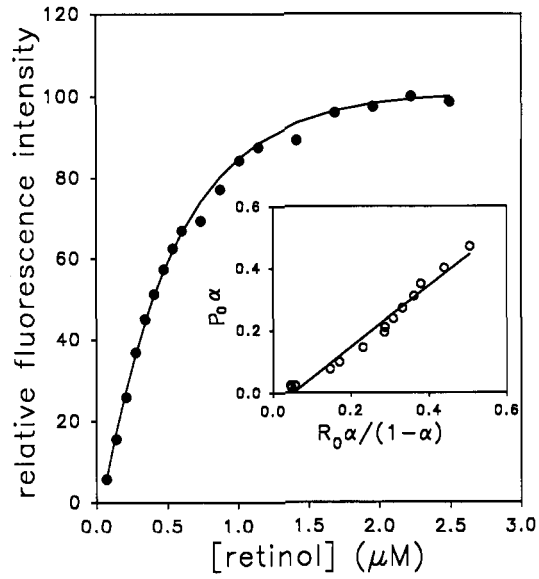

FIGURE 1: Titration of IRBP with retinol by following retinol fluorescence. A representative titration is shown. The titration system consisted of 1 mL of 0.6 μM apo-IRBP in 20 mM HEPES, pH 7.0, 0.15 M NaCl, and 0.1 mM DTT. Apo-RBP was obtained by extraction of endogenous retinoids by mixing with charcoal-coated dextran beads (see Materials and Methods). Retinol was added from a solution in ethanol. Excitation wavelength, 330 nm; emission wavelength, 480 nm. The inset shows analysis of the data plotted according to eq 2, $[P_0]\alpha$ *vs* $[R_0]\alpha/(1-\alpha)$ (see Materials and Methods). The fit line was calculated by least-squares analysis.

Table I: Characteristics of Binding of Retinol to IRBP Revealed by Different Methods[a]

method	apparent K_d (μM)[b]	no. of sites
Fluorescence-based		
retinol fluorescence	0.11 ± 0.02*	0.77 ± 0.1
energy transfer	0.098 ± 0.02*	0.71 ± 0.12
quenching of protein F	0.2 ± 0.014*	1.68 ± 0.2
Direct measurement		
^{3}[H]-retinol + charcoal (see text)	0.31; 0.72**	2

[a] Apparent dissociation constants and number of binding sites were measured by fluorescence-based methods and by monitoring binding of ^{3}[H]-retinol as described in the legends for Figures 1, 3, 4, and 5. [b] Number of measurements: *, 4; **, means of 2.

Formation of Retinol–IRBP Complex Followed by Monitoring Energy Transfer from the Protein to Bound Retinol. IRBP was titrated with retinol, and the increase in retinol fluorescence (at 480 nm) upon excitation at 280 nm was monitored. A representative titration curve is shown in Figure 3. Analysis of the data (Figure 3, inset) yielded K_d = 0.098 ± 0.02 μM and 0.71 ± 0.12 (Table I). Thus, this method revealed the presence of one binding site with a dissociation constant which agrees well with the value of K_d obtained by following the increase in retinol fluorescence (Table I); hence, the results indicate that the same binding site was probed by the two methods.

Formation of Retinol–IRBP Complex Followed by Monitoring Quenching of the Intrinsic Fluorescence of the Protein. The emission spectrum of holo-IRBP (Figure 2) shows that binding of retinol to this protein results in quenching of the intrinsic fluorescence of the protein. Binding could thus be followed by titrating IRBP with retinol while monitoring the fluorescence of the protein (excitation and emission wavelengths, 280 and 340 nm, respectively). The data (Figure 4) revealed the presence of 1.68 ± 0.2 binding sites characterized by an apparent dissociation constant of 0.2 ± 0.014 μM (Table I). Thus, it was found by this method that IRBP contains two

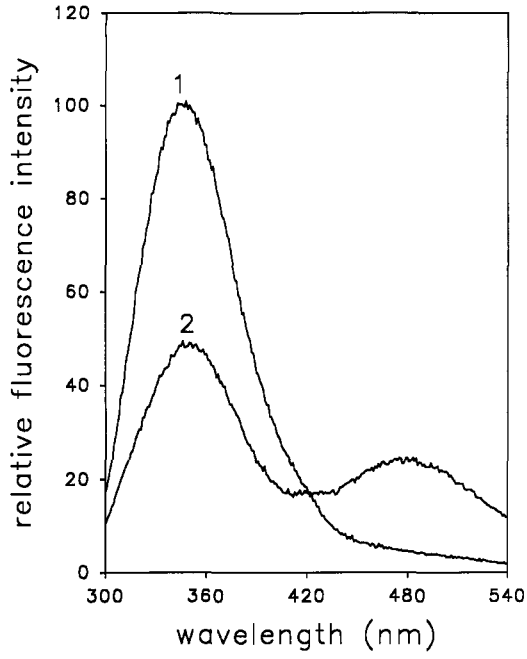

FIGURE 2: Emission spectra of IRBP. Spectra of apo- and holo-IRBP (lines 1 and 2, respectively) were obtained upon excitation at 280 nm. Solutions contained 1.3 μM IRBP with or without 2.4 μM retinol. Buffer composition was as in Figure 1.

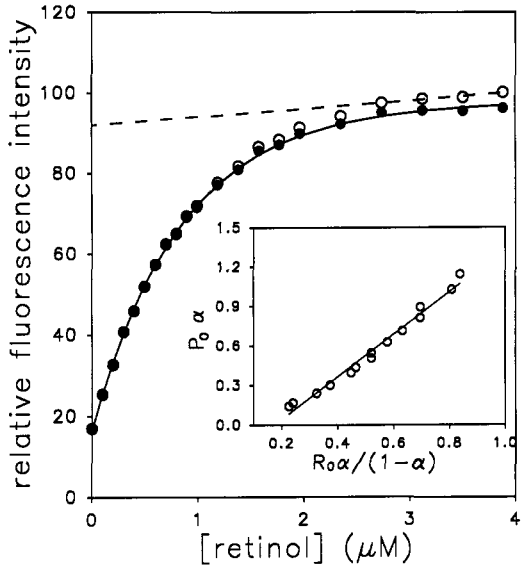

FIGURE 3: Titration of IRBP with retinol by following energy transfer from protein to bound retinol. A representative titration is shown. The titration system consisted of 1 mL of 1.28 μM apo-IRBP. Retinol was added from a solution in ethanol. Excitation and emission wavelengths were 280 and 480 nm, respectively. Buffer composition was as described in Figure 1. Open and filled circles show respectively the raw data and the corrected titration curve. As described in the methods section, the linear increase in retinol fluorescence upon additino 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 eq 2. The fit line was calculated by least-squares analysis.

binding sites for retinol. The line obtained by analyzing these data (Figure 4, inset) is seemingly linear, indicating the presence of two identical binding sites. However, the value of the dissociation constant derived from these data is significantly higher than the K_d obtained from the data in Figures 1 and 3 (Table I). This discrepancy most likely reflects that the binding constants are not identical but that the difference between them is not large enough to be visually distinguished by the analysis in the inset in Figure 4. The

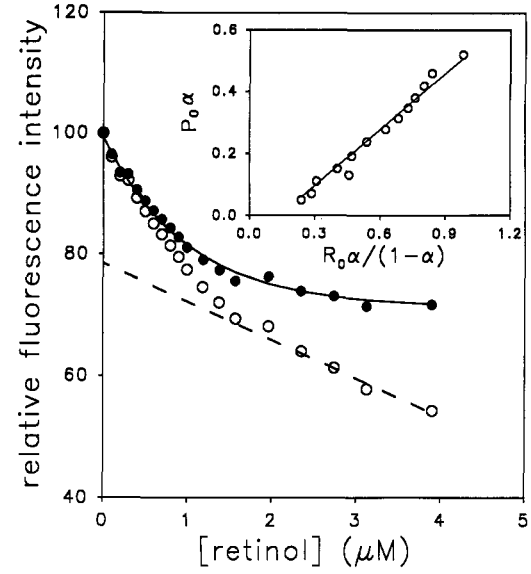

FIGURE 4: Titration of IRBP with retinol by quenching of protein fluorescence by bound retinol. A representative titration is shown. The titration system consisted of 1 mL of 0.7 μM apo-IRBP. Retinol was added from a solution in ethanol. Excitation and emission wavelengths were 280 and 340 nm, respectively. Buffer composition was as described in Figure 1. Open and filled circles show respectively the raw data and the corrected titration curve. As described in the methods section, the linear decrease 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 eq 2. The fit line was calculated by least-squares analysis.

apparent K_d thus represents a mean between the dissociation constants of a strong (K_{d1} = 0.1 μM) and a weaker site. Since the relative fractions of the observed fluorescence signals attributable to the different sites are not known, it is not possible to calculate the individual binding constants from these data. To do so, binding of retinol to IRBP was studied using ^{3}H-labeled retinol.

Formation of Retinol–IRBP Complex Followed by Studying the Binding of Radioactively Labeled Retinol to IRBP. IRBP was titrated with ^{3}H-labeled retinol. Bound ligand was separated from free, unreacted, retinol by treatment with charcoal-coated dextran beads as detailed under Materials and Methods and counted for the presence of [^{3}H]retinol. A representative titration curve is shown in Figure 5. Analyses of these data using two forms of Scatchard plots are shown in Figure 6. A plot of the most commonly used form of the Scatchard treatment points to the existence of two binding sites, and while some scatter is apparent, a linear line can be drawn through the data points. This indicates that the values of the two dissociation constants are different by less than 1 order of magnitude. The values of K_d of the two binding sites can be approximated by fitting the data using eq 10 (inset to Figure 6, solid line),

$$[ROH]_b/[IRBP]_{total} = ([ROH]_f/(K_{d1} + [ROH]_f) + ([ROH]_f/(K_{d2} + [ROH]_f) \quad (10)$$

where $[ROH]_b$ and $[ROH]_f$ denote the concentrations of bound and free retinol, respectively, $[IRBP]_t$ is the total concentration of protein in the assay mixtures, and K_{d1} and K_{d2} are the dissociation constants of the first and the second site, respectively. The values of the dissociation constants thus obtained from two different experiments were K_{d1} = 0.32 and 0.30 μM and K_{d2} = 0.78 and 0.66 μM. Thus, direct measurement of binding of retinol to IRBP revealed the existence of two binding sites, in agreement with data obtained

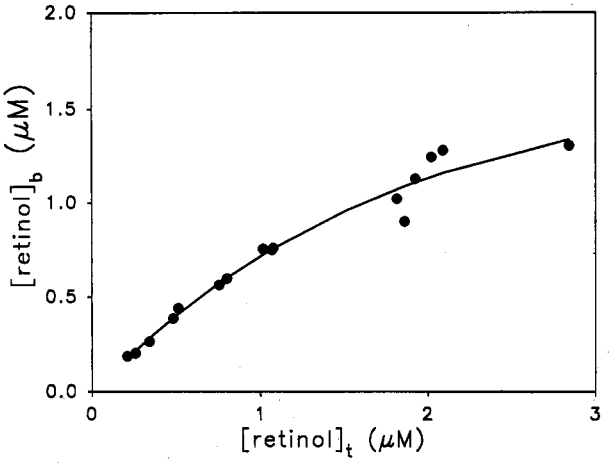

FIGURE 5: Binding of [^{3}H]retinol to IRBP. Tritiated retinol was added to IRBP (0.9 μM) from a solution in ethanol. The mixture was incubated, and free retinol was separated from IRBP–retinol complexes as described in the methods section. IRBP–retinol in the supernatant were counted for the presence of [^{3}H]retinol. Data points from two independent experiments are shown.

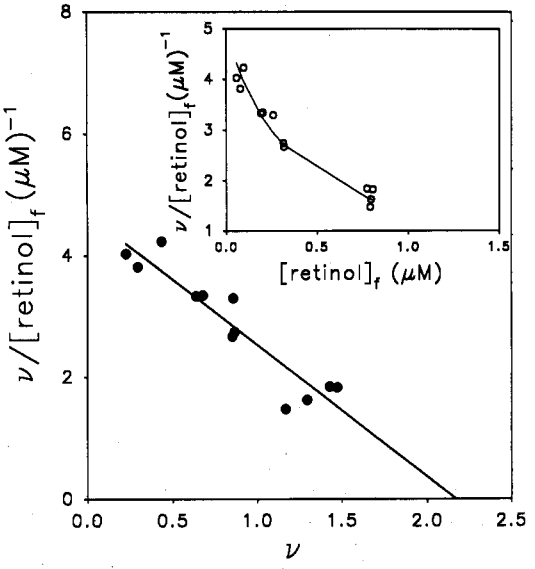

FIGURE 6: Scatchard plots for binding of retinol to IRBP. The data in Figure 5 were analyzed by two forms of the Scatchard plot. ν is the ratio [retinol]$_{bound}$/[retinol]$_{total}$. The slope of the plot of ν/[retinol]$_{free}$ *vs* ν is $1/K_d$, and the x-intercept is n/K_d. n is the number of binding sites and K_d the dissociation constant. To obtain a better estimate of the dissociation constants, the data were also plotted as ν/[retinol]$_{free}$ *vs* [retinol]$_{free}$ (inset) and fitted according to the Scatchard equation (see text).

by following the quenching of protein fluorescence (Figure 4). It is thus validated by two independent methods that IRBP contains two binding sites for retinol with dissociation constants which differ by 2–3-fold.

Fatty Acid Content of IRBP. An enzymatic microfluorometric assay (Miles *et al.*, 1983) was used to measure the amount of endogenous fatty acids associated with IRBP used in these studies. It was found that bovine IRBP contained 10.5 mol of noncovalently bound FA/mol protein, which is about 2.5-fold higher than the fatty acid content of IRBP from monkey retina reported by Bazan *et al.* (1985). Treatment of the protein with charcoal-coated dextran beads removed a small fraction of the fatty acids and resulted in a preparation that contained about 8 mol FA/mol protein. Hence, IRBP used in these experiments contained a "background" of tightly bound endogenous fatty acids. Since fatty acids inhibit binding of retinol to IRBP (see below), it is possible

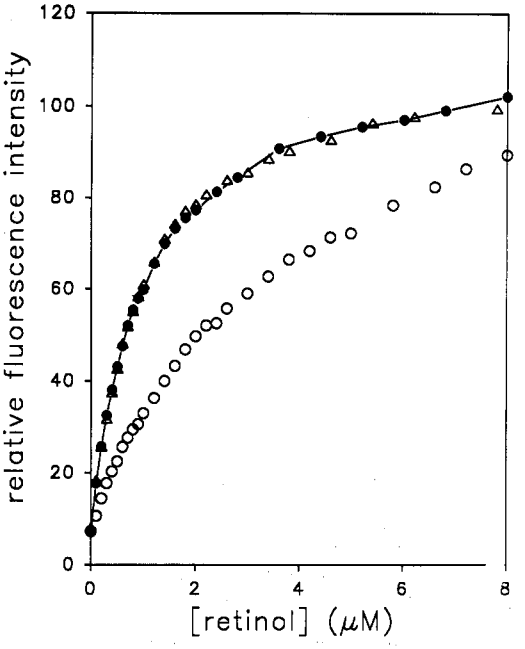

FIGURE 7: Titration of IRBP with retinol in the presence of long-chain fatty acids. IRBP (1.1 μM) was titrated with retinol and the titration monitored by following retinol fluorescence as described in Figure 1. Representative titrations are shown. Titrations were carried out in the absence of fatty acids (●) or in the presence of 11 μM of DHA (○) or palmitic acid (△). Fatty acids were added from solutions in ethanol and equilibrated with IRBP prior to titration with retinol.

that the binding affinity of IRBP for retinol is stronger than that observed in this study. The values of the dissociation constants of the retinol–IRBP complexes presented in Table I thus represent upper limits for the actual values (see Discussion).

Interactions of Fatty Acids with IRBP. It was found that the presence of fatty acids in a mixture containing IRBP and retinol results in displacement of retinol from the protein's stronger binding site. Displacement could be observed by the decrease in retinol fluorescence upon addition of fatty acids to IRBP–retinol complexes. The decrease in retinol fluorescence, which was directly related to the concentration of fatty acid added, was used to study the interactions of fatty acids with IRBP. Significant decreases in retinol fluorescence required an excess of fatty acids over IRBP and retinol, indicating that the binding affinities of IRBP for fatty acids are significantly lower than for retinol. IRBP was thus titrated with retinol in the presence of different fatty acids at a 10:1 mole ratio of fatty acid/IRBP. Retinol–protein association in the stronger site was followed under these conditions by monitoring retinol fluorescence as shown in Figures 7 and 8. The polyunsaturated fatty acid DHA (C22:6 $n-3$, Figure 7, ○) had the largest, while the saturated palmitic acid (C18:0, Figure 7, △) had the smallest effect of all the fatty acids tested. Measurements of the extent of displacement of retinol by fatty acids from the IRBP binding site were used to obtain the dissociation constants of different fatty acids for this site (Connors, 1987; see also Materials and Methods). Figure 9 shows the analysis of the data obtained with DHA. Apparent dissociation constants for all fatty acids studied are given in Table II.

DISCUSSION

The data presented above show that IRBP contains two binding sites for retinol. Binding of retinol in one but not in the other of these sites results in a large increase in the

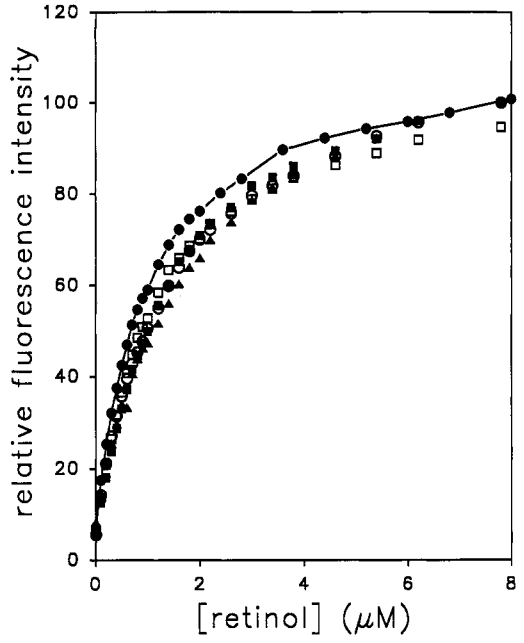

FIGURE 8: Titration of IRBP with retinol in the presence of long-chain fatty acids. IRBP (1.1 μM) was titrated with retinol and the titration monitored by following retinol fluorescence as described in Figure 1. Representative titrations are shown. Titrations were carried out in the absence of fatty acids (●) or in the presence of 11 μM oleic acid (○), linolenic acid (□), arachidonic acid (■), or retinoic acid (▲). Fatty acids were added from solutions in ethanol and equilibrated with IRBP prior to titration with retinol.

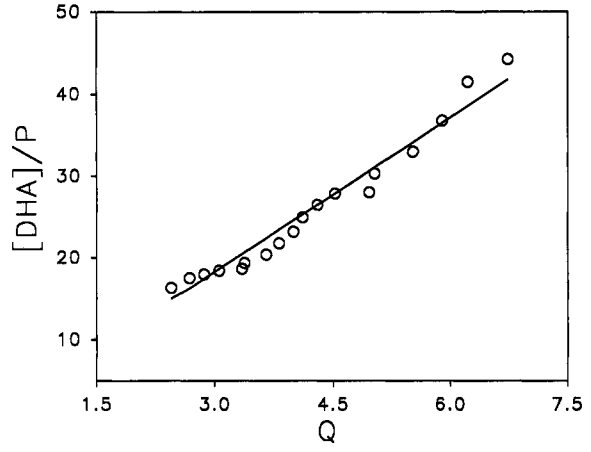

FIGURE 9: Binding of DHA to IRBP. Data from Figure 7 were analyzed according to Connors (1987) as described in the methods section. $Q = 1/K_R[I]$ and $[P] = [R_t]K_F/QK_R + K_F$), where K_R and K_F are the binding constants for retinol and the fatty acid, respectively, and [I] and $[R_t]$ are the concentrations of free IRBP and total retinol, respectively. The slope of this plot corresponds to K_F/K_R.

fluorescence of retinol as compared to its fluorescence in the aqueous phase (Figure 1). Such a change in retinol fluorescence can be ascribed to transfer of the ligand from the aqueous phase to a less polar and a more restrictive environment within the protein binding site [see, for example, Ross *et al.* (1978)]. An increase in retinol fluorescence accompanies its association with other retinol-binding proteins, for example, RBP (Futterman & Heller, 1972; Cogan, 1976) and CRBP (Ong & Chytil, 1978; Noy & Blaner, 1991). The association of retinol with RBP is driven solely by hydrophobic interactions within the binding site (Noy & Xu, 1990a; Manor *et al.*, 1993), while in CRBP, electrostatic interactions stabilize ligand–protein association (Jakoby *et al.*, 1993). Thus, the fluorescence of retinol is found to increase following transfer from water to a binding site in varied binding-site environments

Table II: Dissociation Constants of Fatty Acid–IRBP Complexes Measured by Monitoring Displacement of Retinol from the Protein[a]

fatty acid	apparent K_d (μM)
docosahexaenoic acid* (C22:6 $n-3$)	0.78
oleic acid** (C18:1)	1.45
retinoic acid**	1.53
arachidonic acid* (C20:4 $n-6$)	1.64
linolenic acid** (C18:3 $n-3$)	2.40
palmitic acid** (C16:0)	4.15

[a] IRBP (1 μM) was titrated with retinol in the presence of different fatty acids. The mole ratio of fatty acid/IRBP was 10. Data were collected as described in the legend to Figure 7 and analyzed as detailed in the methods section. [b] *, mean of three measurements. **, mean of two measurements. All values were within 10% of the means.

and types of interactions of the protein with the ligand. The lack of changes in the fluorescence of retinol upon binding to the second site on IRBP can be taken to indicate that retinol, in the second site, is located in a polar, nonrestrictive environment. The observations thus suggest that the second site may bind retinol while the ligand is retained essentially in the aqueous phase. Such a scenario can be envisioned, for example, for a binding site which is superficially located at the surface of the protein molecule and in which retinol–protein associations are stabilized by interactions with the hydroxyl head group and not with the acyl chain or the β-ionone ring of this ligand.

Increase in retinol fluorescence upon binding to IRBP was reported and used previously to measure K_d of the interaction of retinol with IRBP (Adler *et al.*, 1985; Okajima *et al.*, 1989). However, the value of K_d derived from the measurements in the present work is 1 order of magnitude lower than in the above reports, indicating that the affinity of IRBP for retinol is stronger than previously assumed. In one of the retinol sites, a tryptophan or a tyrosine residue is located in a manner that allows for efficient energy transfer to bound retinol (Figure 2). Measurement of the formation of retinol–IRBP complex by following this energy transfer resulted in a value of dissociation constant in good agreement with K_d obtained by following the fluorescence of retinol (Table I), indicating that the same site was probed by the two methods. K_d for this site was thus found to be 0.1 μM, which is similar to the dissociation constant for the complex of retinol with bovine RBP (0.07 μM; Noy & Xu, 1990b). Hence, the affinity of binding of retinol to IRBP is on the order of binding affinities of this ligand to other binding proteins. The origin of the discrepancy between the results described here and previous data (Adler *et al.*, 1985; Okajima *et al.*, 1989) is not clear. As shown in the present work, fatty acids inhibit binding of retinol in the IRBP site which displays energy transfer from protein to bound ligand. The presence of fatty acids in IRBP preparations will thus result in a lower affinity of IRBP for retinol as well as in loss of energy transfer. However, IRBP preparations in the present study contained about 8 mol of fatty acids per mole protein which could not be extracted by treating the protein with charcoal-coated beads (see Materials and Methods). It is thus likely that fatty acid-free IRBP will show a value of K_d for retinol which is even lower than the value reported here. It is also possible that the value of K_d for retinol depends on the chemical composition of the fatty acids bound to IRBP, a parameter that was not controlled either in the present or in previous studies.

The existence of two sites for retinol on IRBP was revealed by studying binding of radioactively labeled retinol to IRBP as well as by titrating IRBP with retinol while monitoring the intrinsic fluorescence of the protein. The latter observations,

showing quenching in the intrinsic fluorescence of the protein upon binding of retinol in two distinct sites (Figure 4), indicated that tryptophanyl or tyrosinyl residues are located near both binding sites of IRBP for this ligand. Apparently, in the second site, the relative orientation of the transition dipoles of the fluorescent residue being observed and of retinol does not favor energy transfer between the two fluorophores.

It was reported some 8 years ago that 1 mol of IRBP, purified from monkey retina, contains 6–7 mol of long-chain fatty acids, two of which were covalently attached to the protein and the rest noncovalently associated (Bazan *et al.*, 1985). The binding properties and the structural specificities of these sites have not, to our knowledge, been characterized as yet. Recently, fatty acids labeled with the fluorophore anthracene were used to study fatty acid binding by IRBP (Putilina *et al.*, 1993). It was found that IRBP possesses one binding site with a dissociation constant of 0.36 μM for the fluorescent fatty acid. This site was shown to be hydrophobic in nature. In the present study, it was found that fatty acids inhibit binding of retinol to its stronger site on IRBP. These observations were used to measure apparent binding affinity of several fatty acids to IRBP. It cannot be established from these experiments whether fatty acids compete with retinol on binding to the same site or whether fatty acids bind to IRBP in a different location, causing changes in the protein (e.g., by inducing a conformational change) which are followed by displacement of retinol. The linearity of the curves resulting from analyzing the displacement data (Figure 9) establishes, however, a direct, competition-like correlation between binding of the two ligands. These plots also indicate that in the present study only one site for fatty acids on IRBP was probed. The apparent binding affinities to IRBP of the various fatty acids studied were found to be 10–50-fold weaker *vs* the affinity of binding of retinol to this protein (Table II). In addition, a 10-fold molar excess of fatty acid to protein had to be used to observe significant displacement of retinol from IRBP (Figures 7 and 8). Since IRBPs possess more than one binding site for fatty acids (Bazan *et al.*, 1985), it is possible, depending on the relative affinities of the different sites for fatty acids, that added fatty acids distributed between the various sites and their concentrations at the site probed were lower than assumed. Further, since the pattern of distribution of the different fatty acids between the different sites is not known, it is possible that their relative efficiency for displacing retinol reflects, at least in part, their availability for the site under study. Quantitative interpretation of the data in Table II is further complicated by the presence of tightly bound endogenous fatty acids in IRBP preparations. The values of the dissociation constants in Table II are thus likely to represent an upper limit for these parameters, and the actual binding affinities of fatty acids for IRBP, at this site, may be stronger than observed.

Somewhat surprising was the observation that retinoic acid, which, except for a carboxyl rather than a hydroxyl polar end, is identical to retinol, did not have the largest effect on displacement of retinol from its binding site. This may imply that fatty acids do not compete with retinol on the same site but that they exert their effect indirectly by binding at a different location as discussed above. The apparent dissociation constants in Table II show that the presence of unsaturated bonds is important for binding of fatty acids to IRBP at the site studied. Thus, palmitic acid (C16:0) had a weak effect, while DHA (C22:6) had a large effect. However, oleic acid (C18:1), which possesses only one unsaturation in its chain, had an effect comparable to that of retinoic and arachidonic acids. The structural requirements of the site under study, hence, are not clear yet, and other possible parameters (e.g., the three-dimensional structures of these ligands) are being explored.

Of all the fatty acids tested, DHA was the most effective in displacing retinol from IRBP. DHA comprises approximately 50% of the acyl chains of phospholipids in the membranes of photoreceptor cells (Fliesler & Anderson, 1983; Bazan & Reddy, 1985). This polyunsaturated fatty acid cannot be synthesized in animal tissues and must be supplied in the diet or derived from the essential fatty acid linolenic acid (C18:3 $n-3$). The retina has the ability to retain DHA even during prolonged periods of dietary deprivation of essential fatty acids (Fleisler & Anderson, 1983; Connor & Neuringer, 1988); it was recently suggested that following phagocytosis of photoreceptor membranes by retinal pigment epithelium, the lipids of these membranes are hydrolyzed, and DHA is then recycled back to ROS (Stinson *et al.*, 1991; Chen *et al.*, 1992). Similarly to retinoid shuttling, the pathways by which recycling of DHA may occur are unknown. One important consideration in this regard is the multiple unsaturated bonds present in DHA which may need to be stabilized against degradation while in transit through an aqueous compartment. The data presented here show that IRBP binds DHA in preference to other fatty acids, at least in the site probed. IRBP thus may serve as a specific vehicle for DHA in the IPM.

The observation that the ability of IRBP to bind retinoids is curtailed in the presence of fatty acids indicates that *in vivo* retinoid-binding by this protein will be affected by the amount and identity of the fatty acids associated with it. The role of IRBP as a retinoid transport vehicle may thus have to be reexamined. Further studied are needed to clarify the mechanisms by which IRBP may contribute to the process of transfer of retinoids and of DHA in the IPM and the physiological significance and relationships between binding of the two types of ligands to this protein.

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