Docosahexaenoic Acid Modulates the Interactions of the Interphotoreceptor Retinoid-binding Protein with 11-cis-Retinal*

(Received for publication, December 18, 1995, and in revised form, April 18, 1996)

Yong Chen‡, Leah A. Houghton, J. Thomas Brenna, and Noa Noy§
From the Division of Nutritional Sciences, Savage Hall, Cornell University, Ithaca, New York 14853-6301

Rapid transport of retinoids across the interphotoreceptor matrix is a critical part of the visual cycle, since it serves to replenish bleached rhodopsin with its chromophore 11-cis-retinal. The transport of retinoids in the interphotoreceptor matrix is believed to be mediated by the interphotoreceptor retinoid-binding protein (IRBP), a protein that, in addition to possessing two retinoid-binding sites, associates in vivo with long chain fatty acids. Here, the interrelationships between binding of the two types of ligands to IRBP were studied. The composition of fatty acids associated with IRBP in bovine retina was determined, and it was found that polyunsaturated fatty acids constitute a significant fraction of those. It was further found that docosahexaenoic acid, but not palmitic acid, induced a rapid and specific release of 11-cis-retinal from one of the protein's retinoid-binding sites. Based on these results and on the additional observation that a steep concentration gradient of docosahexaenoic acid exists between photoreceptor and pigment epithelium cells, a model for the mechanism by which IRBP may target 11-cis-retinal to photoreceptor cells is proposed.

Vitamin A is essential for vision, since one of its metabolites, 11-cis-retinal, serves as the chromophore for the visual protein rhodopsin. Vitamin A circulates in blood and enters the eye in the form of all-trans-retinol. This form is taken up from the circulation by retinal pigment epithelium (RPE) cells, which contain the enzymatic machinery necessary for conversion of all-trans-retinol to 11-cis-retinal. The later retinoid is transported to photoreceptor outer segments (ROS), where it associates with opsin to form rhodopsin. Exposure to light leads to isomerization of rhodopsin-bound 11-cis-retinal to the all-trans form, which is then hydrolyzed from the protein and reduced to all-trans-retinol. All-trans-retinol is transferred back to the RPE for reisomerization and oxidation (for review see Ref. 1). Thus, continuous shuttling of retinoids between photoreceptor and pigment epithelium cells across the aqueous compartment that separates them, the interphotoreceptor matrix (IPM), is a critical part of the visual cycle.

The hydrophobic nature of retinoids and their lability when dissolved in water raise the question of how their rapid transport across an aqueous compartment is accomplished. The details of this process are not clear at present, but it is generally believed to be mediated by a protein in the IPM, the interphotoreceptor retinoid-binding protein (IRBP) (see Ref. 2 for review). IRBP, which is a 140-kDa glycoprotein and is the major soluble protein component of the IPM, is known to bind various isomeric and chemical forms of retinoids as well as long chain fatty acids (3, 4). Participation of this protein in shuttling of retinoids in the IPM has been implied by the observations that its retinoid content is modulated by light (5) and that binding of retinoids to IRBP stabilizes them against degradation (6, 7). It was also reported that IRBP can take up 11-cis-retinal from RPE (8–10) and that it can efficiently deliver 11-cis-retinal to bleached ROS (11, 12). However, the exact role of IRBP in the transport process is not known. Theoretically, IRBP could serve simply as a storage compartment for retinoids in the IPM with the ability to bind and release these ligands according to their concentration gradients. Alternatively, IRBP could function to selectively target specific retinoids to particular locations in the eye by a yet unidentified mechanism (see Refs. 1 and 2 for discussion).

IRBP contains two binding sites for retinoids (3, 13, 14), and fluorescence studies of binding of retinoids to the protein indicated that the two sites are quite different. Binding of retinol in one of the sites results in a significant enhancement of the fluorescence of the ligand, a behavior indicative of a hydrophobic and/or restrictive nature of the binding pocket. In contrast, binding in a second IRBP retinoid-binding site does not affect the fluorescence of retinol, suggesting that the site is located at the surface of the protein and may interact with the ligand via its polar head group (14). Examination of the interactions of IRBP with several forms of retinoids revealed that the protein displays the highest affinity for the all-trans form of retinol and the 11-cis isomer of retinal (15). Thus, in agreement with the proposed role of IRBP in retinoid transport, the ligand selectivity of this protein corresponds to the physiological need to shuttle particular retinoids across the IPM.

It was reported that the second class of IRBP ligands, long chain fatty acids (FAs), interfere with retinoid binding by the protein. Of all the FAs tested, docosahexaenoic acid (c22:6 n-3; DHA) was the most effective in displacing retinoids from IRBP (14). This observation is of particular interest, since DHA is essential for vision and composes a large fraction of the acyl chains of phospholipids in the membranes of ROS (16–18). The previously reported studies were, however, confounded by endogenous FA that co-isolates with IRBP from retina. In the present work, protein preparations with a better defined FA content were used to further examine the interrelationships between the interactions of retinoids and FAs with IRBP. The

---

*This work was supported in part by National Institutes of Health Grant EY09296. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡Recipient of National Eye Institute Research Fellowship Award F32 EY06575.
§To whom correspondence should be addressed: Division of Nutritional Sciences, Cornell University, Ithaca, New York 14853.
1The abbreviations used are: RPE, retinal pigment epithelium; FA, long chain fatty acids; IRBP, interphotoreceptor matrix retinoid-binding protein; IPM, interphotoreceptor matrix; ROS, rod outer segments; DOPC, dioleoyl phosphatidylcholine; DHA, docosahexaenoic acid; NBD-DPPE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)l-α-phosphatidylethanolamine; PY-PC, 3-palmitoyl-2-(1-pyrendecanoyl)-L-α-phosphatidylcholine; GLC, gas-liquid chromatography.
Modulation of IRBP-Retinoid Interactions by DHA

FA content of bovine IRBP was analyzed, and it was found that the polyunsaturated fatty acids DHA and arachidonic acid comprise a significant fraction of the FAs endogenously associated with this protein. IRBP was depleted of endogenous FAs, and the interactions of all-trans-retinol and 11-cis-retinol with the fatty acid-depleted protein in the absence and in the presence of added FAs were investigated. The data revealed that DHA specifically modulates the interactions IRBP with 11-cis-retinol. Based on these results and on the observation that a steep concentration gradient of DHA exists between ROS and DHA, a model for the mechanism by which IRBP may function to target specific retinoids to their sites of action in the eye is proposed.

EXPERIMENTAL PROCEDURES

All-trans-retinol was purchased from Eastman Kodak Co. 11-cis- Retinal was obtained from the National Eye Institute. Lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Fluorescence probes were purchased from Molecular Probes, Inc. (Eugene, OR). Experiments were carried out in dim red light at 20 °C. Buffers contained 20 mM Hepes, pH 7.5–8.0, 150 mM NaCl, and 1 mM dithiothreitol.

IRBP—IRBP used for binding studies and for measurements of kinetic parameters was purified from frozen bovine retina. The FA composition of IRBP was determined using protein purified from eyes obtained within 3 h of slaughter. The retina was peeled from eyeballs and gently washed to obtain IPM components. The procedure was similar to that described in Ref. 4 except that stirring of the retina was avoided in order to minimize cell damage. IRBP from both sources was purified according to Ref. 19. Protein concentrations were determined by using its extinction coefficient (19). Purified IRBP was stored at −20 °C in buffer containing 50% glycerol.

Unilamellar Vesicles—Unilamellar vesicles of phospholipids were prepared by sonication. Dioleoylphosphatidylcholine (DOPC) and the appropriate fluorescent probe were co-sonicated in chloroform, and the solvent was evaporated under a stream of nitrogen. 7–8 ml of buffer was added, and the suspension was sonicated to clarity using a Heat System Inc. sonicator. Vesicles were centrifuged at 100,000 × g for 15 min to pellet multilamellar vesicles. Concentrations of phospholipids were determined by phosphorus content (20).

FA Content of IRBP—For these measurements, IRBP obtained by gently washing fresh retina as described above was used. FAs were extracted into an organic solvent, methylated, and analyzed by gas-liquid chromatography (GLC). Briefly, the organic solvent used to extract fatty acids from 3–10 nmol of IRBP was evaporated, samples were treated with 2 ml of 50% BF3 in methanol in a boiling water bath (5 min), 2 ml of hexane was added, and samples were boiled for an additional 10 min. 2 ml of saturated NaCl solution was added, and the hexane phase was collected. The extraction was repeated, the solvent was evaporated, and methyl esters were dissolved in a small volume of hexane (30–100 μl). A C17:0 fatty acid standard was added prior to extraction in order to allow for quantitation of extracts. GLC analyses were carried out using a RTX225 capillary column (Restek Corporation, Bellefonte, PA).

FA Acid Composition of Different Lipid Classes from Rod Outer Segments and RPE Cells—ROS were isolated from bovine retina (34). RPE cells were isolated from bovine eyes obtained from a local abattoir. Processing was carried out within 3–4 h of slaughter, and eyes were kept at 4 °C until treated. Cells were isolated from the eyes as described (35). ROS from 50 retina and RPE cells from 40 eyes were pooled prior to each determination. Lipids were extracted and separated into classes by thin layer chromatography using a solvent mixture containing hexane/ethyl ether/acetic acid (70/30/2.3). Areas corresponding to the appropriate lipid classes were scraped off the plates, and the FA composition of each was analyzed by GLC.

Delipidation of IRBP—Isolated IRBP was delipidated using the hydrophobic chromatography gel Lipidex-5000 (Packard). Lipidex-5000 (70 ml) was packed in a water-jacketed column and equilibrated at 37 °C. IRBP (50–70 nmol/20–35 ml) was slowly circulated through the column via a peristaltic pump for 30 min. Resulting protein solutions from three preparations contained 0.5–1 mol of FA/mol of IRBP as was ascertained by GLC.

Fluorescent measurements were performed using a SPEX Industries (Edison, N J) Fluorolog 2 DM1B spectrofluorometer.

Fluorometric Titrations of IRBP with Retinoids—Delipidated apolIRBP (1 μl) was titrated directly in the cuvette with the appropriate retinoid dissolved in ethanol. Ethanol concentration did not exceed 2%. To ensure equilibration between protein and ligand, the fluorescence was monitored until a constant value was reached. Ligand binding was monitored by following either the fluorescence of retinol (excitation, 330 nm; emission, 480 nm) or by following the intrinsic fluorescence of the protein (excitation, 280 nm; emission, 340 nm). Data were corrected for the contribution of free retinol to the overall fluorescence signals (when the fluorescence of retinol was monitored) or for inner filtering by the ligands (when the fluorescence of the protein was followed) as described (21). Corrected data were analyzed to extract the number of binding sites and the equilibrium dissociation constants.

Analyses were carried out either by the linearization method described by Cogan et al. (21) or by fitting the titration curves to an equation derived from simple binding theory (22).

Rates of Transfer of Retinoids from IRBP to Unilamellar Vesicles of Phosphatidylcholine—Fluorescence probes were incorporated into synthetic unilamellar vesicles of DOPC by co-sonication at a probe/lipid molar ratio of 1–2% (15). To initiate transfer of retinoids from IRBP to the vesicles, protein complexed with the appropriate retinoid was mixed with vesicles using a Hi-Tech (Salisbury, UK) stopped-flow mixing accessory, and fluorescence was monitored continuously until equilibrium was reached. The rate constants of transfer from IRBP to unilamellar vesicles of all the retinoids studied were independent of the concentrations of protein or vesicles (data not shown, see Refs. 6 and 14). These observations excluded the possibility that transfer occurred by direct collisions between the protein and the vesicles and ascertained that it proceeded by dissociation of retinoids into the aqueous phase followed by diffusion and association with the vesicles. Measured rates thus directly represented the rates of dissociation of retinoids from the protein.

RESULTS

The Composition of FAs Endogenously Bound to Bovine IRBP—The composition of FAs that are noncovalently associated with IRBP in bovine retina was determined by GLC analyses (Table I). The FA:IRBP molar ratio of the protein was found to be 8:1.5. This value is about 2-fold lower than the value we previously reported based on a microfluorometric assay (14). However, we recently noticed that the microfluorometric assay is susceptible to several artefacts and may yield values that vary by up to 2–3-fold. We believe, therefore, that the data of the GLC analyses provided here yield better estimates of the FA content of the protein. The total amount of FA associated with bovine IRBP is in good agreement with a previous report that monkey IRBP contains about 4.5 mol of noncovalently attached endogenous FAs per mol of protein (4).

The major FAs found to be associated with bovine IRBP were palmitic, stearic, and oleic acid, which together comprised about 70% of FAs associated with the protein (Table I). In addition, the data in Table I show that bovine IRBP contains significant fractions of the polyunsaturated FAs arachidonic acid (7.7%) and DHA (8.6%). About 0.5 mol of each of these components was found to be bound to 1 mol of IRBP.

FA Composition of Phospholipids and Free FAs in Bovine ROS and RPE—Phospholipids of ROS from several species are significantly enriched in respect to DHA as compared with phospholipids of RPE (16–18). To the best of our knowledge, the compositions of free FAs in these cell types have not been
parameters were examined by fluorescence titrations. Dated IRBP, and the effects of the addition of FAs on these
irbp( the binding of this ligand to retinol-binding proteins including fluorescence. This phenomenon has often been used to monitor
native titration of IRBP with all-
tate and was completely devoid of unsaturated FAs. The affinity of IRBP was depleted of endogenous FAs by hydrophobic interaction chromatography on
lipidex-5000 (see "Experimental Procedures"), a procedure that yielded protein preparations containing 0.6–0.9 mol of FAs/mol of IRBP. We could not deplete IRBP of the final traces of FAs both in the phospholipids and in the free FA pool of both cell types. Palmitic acid composed a significantly larger fraction in RPE as compared with ROS. The difference in the concentration of palmitate between the two cell types was compensated for by DHA. DHA composed a major fraction of FAs in both the phospholipid and the free FA pool in ROS and was present in much smaller amounts in RPE. Thus, similarly to the well documented concentration difference between the concentration of DHA in phospholipids of ROS versus RPE, the data in Table II indicate that there exists a steep concentration gradient of free DHA between the two cell types.

Interestingly, comparison between the FA compositions of the cells (Table II) and of IRBP (Table I) reveals that the FA content of the protein represents an intermediate between FAs prevalent in the RPE and ROS. The only exception to this is oleic acid, which was present on IRBP in disproportionately larger amounts than in the cells. The origin for the latter observation is not clear to us. Nevertheless, the data on the other FAs seem to point to the conclusion that FAs bound to IRBP readily equilibrate with the FAs of ROS and of RPE (see "Discussion").

Effect of FAs on Binding of All-trans-retinol at the Hydrophobic Retinoid-biding Site of IRBP—IRBP was depleted of endogenous FAs by hydrophobic interaction chromatography on Lipidex-5000 (see "Experimental Procedures"), a procedure that yielded protein preparations containing 0.6–0.9 mol of FAs/mol of IRBP. We could not deplete IRBP of the final traces of FAs without denaturing the protein. However, GLC analyses revealed that the FA-depleted protein contained mainly palmitate and was completely devoid of unsaturated FAs. The affinities of binding all-trans-retinol and 11-cis-retinol to the delipidated IRBP, and the effects of the addition of FAs on these parameters were examined by fluorescence titrations.

Retinol is an efficient fluorophore, and its interactions with proteins are often accompanied by a marked increase in its fluorescence. This phenomenon has often been used to monitor the binding of this ligand to retinol-binding proteins including IRBP (eg. Refs. 14, 21, 23, and 24). Fig. 1A shows a representative titration of IRBP with all-trans-retinol. Titration curves were corrected for the contribution of free retinol to the total fluorescence (21). Corrected data were analyzed both by a linearization method (21) and by fitting the data to an equation derived from simple binding theory. Values of number of binding sites and of equilibrium dissociation constants (K_d) extracted by the two methods agreed within 20%. Analyses of titrations of several protein preparations revealed the presence of 0.55–0.85 binding sites for retinol/mol of protein. Thus, as previously reported, monitoring the enhancement of retinol fluorescence upon binding to IRBP reports on interactions of the ligand with only one of the retinoid-binding sites of the protein. The K_d calculated from the data was 50 ± 15 nM (n = 5). This K_d is about 2-fold lower than we previously observed using protein preparations that were not depleted of endogenous FAs (15) and 20-fold lower than values reported by other groups (25, 26). The affinity of IRBP for retinol thus seems to be significantly stronger than previously suspected.

To examine the effects of FAs on the interactions of IRBP with all-trans-retinol, titrations were carried out in the presence of either palmitic acid or DHA at a FA:IRBP molar ratio of 5 (Fig. 1B). The studies focused on these two FAs, since the former was found to be a main constituent of IRBP-bound FA and the later was previously observed to be the most efficient FA in hindering the interactions of retinoids with IRBP. The K_d values calculated from the data (Table III) indicated that palmitate did not significantly affect the interactions of all-trans-retinol with the hydrophobic retinoid-binding site of IRBP. DHA somewhat interfered with binding at this site, but the effect was small and led to an increase of the K_d of all-trans-retinol by 2-fold. Interestingly, the K_d observed in the

| Table II | Fatty acid composition of phospholipids and free FAs in bovine ROS and RPE |
|---|---|
| | ROS | RPE |
| PL | FFA | PL | FFA |
| 14:0 | 0.3 | 4.0 | 0.7 | 1.1 | 0.4 | 1.4 | 1.6 |
| 16:0 | 25.2 | 23.2 | 11.9 | 15.2 | 29.9 | 4.0 | 1.6 |
| 18:0 | 22.5 | 18.8 | 23.5 | 23.5 | 18.8 | 19.9 | 16.4 | 15.5 |
| 18:1 | 3.7 | 3.8 | 9.0 | 4.4 | 10.4 | 5.3 | 10.9 | 7.8 |
| 18:2 | 1.2 | 1.1 | 3.0 | 1.4 | 1.6 | 3.7 | 9.0 | 4.4 |
| 20:4 | 4.8 | 5.1 | 14.2 | 7.8 | 14.2 | 7.8 | 4.6 | 3.7 |
| 22:5 | 3.1 | 3.1 | 9.0 | 4.4 | 10.4 | 5.3 | 10.9 | 7.8 |
| 24:0 | 6.1 | 6.3 | 2.6 | 6.3 | 0.5 | 3.6 | 0.0 | 1.5 |
| 22:6 | 22.2 | 24.1 | 8.5 | 22.3 | 1.3 | 9.3 | 0.4 | 3.7 |

* PL, phospholipids.  
* FFA, free fatty acids.
Modulation of IRBP-Retinoid Interactions by DHA

Effect of FAs on Binding of 11-cis-Retinal at the Hydrophobic Retinoid-binding Site of IRBP—Retinals are nonfluorescent compounds. Binding of 11-cis-retinal in the hydrophobic binding site of IRBP was thus studied indirectly by monitoring displacement of all-trans-retinol from this site by retinal. 11-cis-retinal and IRBP were mixed at a retinal:IRBP molar ratio of either 1 or 2, and the mixture was allowed to equilibrate for 5-10 min and titrated with all-trans-retinol. The increase in fluorescence of retinol was monitored. As can be seen in Fig. 2, 11-cis-retinal strongly curtailed the association of all-trans-retinol with the protein, indicating that the two retinoids compete for binding at this site. Using the Kd found above for the observed ligand (all-trans-retinol), the titration curves were analyzed to yield the dissociation constant of the competitor (11-cis-retinal) (Ref. 27, and see Ref. 14 for calculation details). The Kd of 11-cis-retinal calculated from the data yielded the same value regardless of the molar ratio of 11-cis-retinal to IRBP and showed a high binding affinity for 11-cis-retinal at this site (Table III). 11-cis-Retinal was found to interact with IRBP with a somewhat higher affinity than all-trans-retinol.

To examine the effect of FAs on the interactions of 11-cis-retinal at the hydrophobic site, competition fluorescence titrations were carried out in the presence of palmitic acid or DHA. The effects of 11-cis-retinal and of the FAs on the interactions of all-trans-retinol with IRBP at this site were additive. These observations and the similarity of the values of Kd for 11-cis-retinal obtained in the absence and in the presence of DHA (Table III), suggest that FAs and 11-cis-retinal inhibited binding of all-trans-retinol to IRBP independently. The data thus indicate that FAs do not alter the interactions of 11-cis-retinal with the hydrophobic retinoid-binding site of IRBP.

Effect of FAs on Binding of All-trans-retinol and 11-cis-Retinal in Both Retinoid-binding Sites of IRBP—The intrinsic fluorescence of IRBP decreases upon binding of retinoids in both of the retinoid-binding sites of this protein (14, 15). These observations are likely to reflect that a tryptophan or tyrosine residue(s) are located near both of the sites and that ligand binding leads to quenching of the fluorescence of these residues. Binding of retinoids in both retinoid-binding sites of IRBP can thus be investigated by monitoring changes in the intrinsic fluorescence of the protein that accompany retinoid binding. Since the binding affinities of retinoids in the two sites differ by less than an order of magnitude (15), the data can be analyzed by assuming the existence of two sites with the same binding constant (22), yielding information on the average binding affinities in the two sites.

A representative titration of IRBP with all-trans-retinol in the absence (circles) or in the presence of 1 μM of 11-cis-retinol (squares) or in the presence of 1.0 μM of 11-cis-retinal and 5 μM DHA (triangles). Kd values for the 11-cis-retinal-IRBP complex in the absence and in the presence of the FA were calculated by analyzing the fluorometric competition data according to Connors (Ref. 27). The analysis of the data shown in the absence of DHA is depicted in the inset.

which ligand binding was monitored by following the intrinsic fluorescence of the protein is shown in Fig. 3. Analyses of titration curves utilizing several protein preparations indicated that the number of retinol-binding sites found by following the decrease in the fluorescence of the protein was always twice the number of binding sites extracted from titrations followed by monitoring the fluorescence of retinol. These observations verified that both retinoid-binding sites of IRBP are probed by the former procedure. The calculated average Kd for binding of all-trans-retinol to IRBP in both sites (Table III) indicated that the hydrophobic site has a somewhat higher affinity for this ligand as compared with the second site. Neither palmitic acid nor DHA affected the average affinity of the interactions of all-trans-retinol with IRBP (Fig. 3 and Table III).

Fluorescence titrations of IRBP with 11-cis-retinal (Fig. 4 and Table III) revealed that similarly to all-trans-retinol, binding of 11-cis-retinal in the second IRBP site is weaker as compared with binding in the hydrophobic site. Also similarly to all-trans-retinol, palmitic acid had no effect on the interactions of 11-cis-retinal with either of the retinoid-binding sites of IRBP.

In contrast, titrations that were carried out in the presence of DHA revealed that this FA efficiently hindered the interactions of 11-cis-retinal with IRBP (Fig. 4B). As shown above, DHA did not affect the interactions of 11-cis-retinal with the hydrophobic IRBP site. In addition, titration curves in the presence of DHA had a biphasic appearance. Thus, the data indicated that the difference between the Kd values for 11-cis-retinal at the two IRBP sites was quite large. These data were analyzed using the numerically based program BIOEQS, which allows for analysis of models containing multiple equilibria.

| Retinoid       | FA         | Apparent Kd  |
|----------------|------------|--------------|
|                | Palmitic   | DHA          | First site | Mean of two sites |
| All-trans-retinol | -         | -            | 50 ± 15^a | 140 ± 26^i |
|                 | +         | -            | 65^v       | 190^v       |
| 11-cis-Retinal  | -         | -            | 36^t       | 128 ± 46^i |
|                 | +         | -            | ND^e       | 116^f       |
|                 | +         | +            | 29 ± 3^g   | >1 NM        |

* a n = 5.
* b n = 2.
* c n = 3.
* d n = 4.
* e Not determined.
* f Second site.

---

Fig. 2. Effect of DHA on binding of 11-cis-retinal to the hydrophobic site of IRBP. IRBP (1.0 μM) was titrated with all-trans-retinol, and the titration was monitored as described in the legend to Fig. 1. Representative titrations are shown. Titrations were carried out in the absence (circles) or in the presence of 1 μM of 11-cis-retinal (squares) or in the presence of 1.0 μM of 11-cis-retinal and 5 μM DHA (triangles). Kd values for the 11-cis-retinal-IRBP complex in the absence and in the presence of the FA were calculated by analyzing the fluorometric competition data according to Connors (Ref. 27). The analysis of the data shown in the absence of DHA is depicted in the inset.
Modulation of IRBP-Retinoid Interactions by DHA

(28). Two $K_d$ values were extracted (Table III). One of these was remarkably similar to the $K_d$ for 11-cis-retinal in the hydrophobic site of IRBP. $K_d$ for the second binding site varied between different protein preparations but was always larger than 1 mM, demonstrating that in the presence of DHA, binding of 11-cis-retinal at the second binding site was extremely weak.

Effects of FAs on the Rates of Dissociation of IRBP-Retinoid Complexes—The data in Table III reveal that DHA specifically hinders the interactions of 11-cis-retinal with the second retinoid-binding site of IRBP without interfering with binding of this ligand in the hydrophobic site. The $K_d$ of a protein-ligand complex relates to the rate constants characterizing the dissociation of the complex ($k_{off}$) and the association of the two components ($k_{on}$) via $K_d = k_{off}/k_{on}$. $k_{on}$ values are too rapid to measure directly by the methodologies used here, but it is reasonable to expect that changes in the strength of the interactions of retinoids with IRBP will be reflected by changes in the rates of dissociation of ligand-protein complexes. To further investigate the effects of FAs on the interactions of retinoids with IRBP, the rate constants of the dissociation of IRBP-retinoid complexes were measured in the absence and in the presence of FAs.

To induce dissociation of retinoids from IRBP, unilamellar vesicles of phospholipids were used. Due to the high affinity of lipid bilayers for retinoids, mixing IRBP-retinoid complexes with lipid vesicles results in transfer of the retinoid from the protein to the vesicles. It was previously shown that the rates of transfer of retinoids from several retinoid-binding proteins, including IRBP, to vesicles, directly represent the rates of dissociation of retinoids from the protein (6, 15, 24, 29). Transfer reactions were monitored by incorporating fluorescent lipid probes into the bilayers of the vesicles. The probes were chosen such that their fluorescence was sensitive to the presence of retinoids within the bilayers, and the arrival of retinoids at the vesicles was followed by monitoring the change in the fluorescence of the probe. It should be noted that since, in this experimental setup, the measured parameter is the rate of arrival of retinoids at the vesicles, the observed rates reflect dissociation of retinoids from both IRBP retinoid-binding sites. If the rates of dissociation of a particular retinoid from the two IRBP sites are similar, the overall transfer will behave like a single first order reaction. In cases where there is a large difference between the rates of dissociation of a retinoid from the two sites, the transfer reaction will take the appearance of two first order reactions with different rate constants.

To measure the rate of transfer of all-trans-retinal from IRBP to vesicles, the fluorescent lipid probe NBD-DPPE was used. The absorption spectrum of NBD overlaps extensively with the fluorescence emission spectrum of retinal, and energy transfer between retinol and this probe when both are incorporated in the same lipid bilayer results in quenching of retinol fluorescence (29, 30). A representative trace depicting transfer of 11-cis-retinal to both retinoid-binding sites of IRBP is shown in Fig. 3. Data obtained in the absence of FAs were analyzed as described in the legend to Fig. 3. Data obtained in the presence of DHA were analyzed by the computer program BIOEQS, which is capable of analyses of models containing multiple equilibria (28). Solid lines through data points show the respective fits.

![Figure 3](image1)

**Fig. 3.** Effect of FAs on binding of all-trans-retinol to both retinoid-binding sites of IRBP. Representative titrations are shown. IRBP (1 μM) was titrated with all-trans-retinol added from a solution in ethanol, and the intrinsic fluorescence of the protein was monitored (excitation, 280 nm; emission, 340 nm). Titrations were carried out in the absence (circles) or in the presence of 5 μM palmitic acid (squares) or DHA (triangles). Data were fitted to yield the number of binding sites and the mean equilibrium dissociation constants of the two retinoid-binding sites of IRBP by assuming that the two binding sites display similar $K_d$ values (solid lines through data points).

![Figure 4](image2)

**Fig. 4.** Effect of FAs on binding of 11-cis-retinal to both retinoid-binding sites of IRBP. Representative titrations are shown. IRBP (1 μM) was titrated with 11-cis-retinal added from a solution in ethanol, and the intrinsic fluorescence of the protein was monitored (excitation, 280 nm; emission, 340 nm). Titrations were carried out in the absence (circles) or in the presence of 5 μM palmitic acid (A, squares) or DHA (B, triangles). Data obtained in the absence of FAs and in the presence of palmitate were analyzed as described in the legend to Fig. 3. Data obtained in the presence of DHA were analyzed by the computer program BIOEQS, which is capable of analyses of models containing multiple equilibria (28). Solid lines through data points show the respective fits.

To induce dissociation of retinoids from IRBP, unilamellar vesicles of phospholipids were used. Due to the high affinity of lipid bilayers for retinoids, mixing IRBP-retinoid complexes with lipid vesicles results in transfer of the retinoid from the protein to the vesicles. It was previously shown that the rates of transfer of retinoids from several retinoid-binding proteins, including IRBP, to vesicles, directly represent the rates of dissociation of retinoids from the protein (6, 15, 24, 29). Transfer reactions were monitored by incorporating fluorescent lipid probes into the bilayers of the vesicles. The probes were chosen such that their fluorescence was sensitive to the presence of retinoids within the bilayers, and the arrival of retinoids at the vesicles was followed by monitoring the change in the fluorescence of the probe. It should be noted that since, in this experimental setup, the measured parameter is the rate of arrival of retinoids at the vesicles, the observed rates reflect dissociation of retinoids from both IRBP retinoid-binding sites. If the rates of dissociation of a particular retinoid from the two IRBP sites are similar, the overall transfer will behave like a single first order reaction. In cases where there is a large difference between the rates of dissociation of a retinoid from the two sites, the transfer reaction will take the appearance of two first order reactions with different rate constants.

To measure the rate of transfer of all-trans-retinal from IRBP to vesicles, the fluorescent lipid probe NBD-DPPE was used. The absorption spectrum of NBD overlaps extensively with the fluorescence emission spectrum of retinal, and energy transfer between retinol and this probe when both are incorporated in the same lipid bilayer results in quenching of retinol fluorescence (29, 30). A representative trace depicting transfer of 11-cis-retinal to both retinoid-binding sites of IRBP is shown in Fig. 3. Data obtained in the absence of FAs were analyzed as described in the legend to Fig. 3. Data obtained in the presence of DHA were analyzed by the computer program BIOEQS, which is capable of analyses of models containing multiple equilibria (28). Solid lines through data points show the respective fits.

To induce dissociation of retinoids from IRBP, unilamellar vesicles of phospholipids were used. Due to the high affinity of lipid bilayers for retinoids, mixing IRBP-retinoid complexes with lipid vesicles results in transfer of the retinoid from the protein to the vesicles. It was previously shown that the rates of transfer of retinoids from several retinoid-binding proteins, including IRBP, to vesicles, directly represent the rates of dissociation of retinoids from the protein (6, 15, 24, 29). Transfer reactions were monitored by incorporating fluorescent lipid probes into the bilayers of the vesicles. The probes were chosen such that their fluorescence was sensitive to the presence of retinoids within the bilayers, and the arrival of retinoids at the vesicles was followed by monitoring the change in the fluorescence of the probe. It should be noted that since, in this experimental setup, the measured parameter is the rate of arrival of retinoids at the vesicles, the observed rates reflect dissociation of retinoids from both IRBP retinoid-binding sites. If the rates of dissociation of a particular retinoid from the two IRBP sites are similar, the overall transfer will behave like a single first order reaction. In cases where there is a large difference between the rates of dissociation of a retinoid from the two sites, the transfer reaction will take the appearance of two first order reactions with different rate constants.

To measure the rate of transfer of all-trans-retinal from IRBP to vesicles, the fluorescent lipid probe NBD-DPPE was used. The absorption spectrum of NBD overlaps extensively with the fluorescence emission spectrum of retinal, and energy transfer between retinol and this probe when both are incorporated in the same lipid bilayer results in quenching of retinol fluorescence (29, 30). A representative trace depicting transfer of 11-cis-retinal to both retinoid-binding sites of IRBP is shown in Fig. 3. Data obtained in the absence of FAs were analyzed as described in the legend to Fig. 3. Data obtained in the presence of DHA were analyzed by the computer program BIOEQS, which is capable of analyses of models containing multiple equilibria (28). Solid lines through data points show the respective fits.
binding sites of IRBP were similar.

Alternatively, it was proposed that IRBP might function as a port mediated by such receptors can bypass the rapid fluxes of retinoids via the lipid regions of the membranes (30, 31). It was also hypothesized that IRBP may be recognized by receptors in the plasma membranes of photoreceptor cells and that upon binding to these receptors, 11-cis-retinal is released from RPE cells, perhaps by binding to receptors in the plasma membranes of photoreceptor cells and that upon binding to these receptors, 11-cis-retinal is released from the protein to the photoreceptor outer segments. How- no experimental evidence for the presence of IRBP receptors in plasma membranes of either pigment epithelium or photoreceptor cells has been reported, nor is it clear how transport mediated by such receptors can bypass the rapid fluxes of free retinoids via the lipid regions of the membranes (30, 31). Alternatively, it was proposed that IRBP might function as a

Table IV

| Retinoid          | FA    | DHA | $k_{off}$ | $t_{1/2}$ |
|-------------------|-------|-----|-----------|----------|
| All-trans-retinol | --    | --  | 0.108 ± 0.01$^c$ | 6.42     |
| --                | +     | --  | 0.113 ± 0.02$^c$ | 6.13     |
| --                | --    | +   | 0.103 ± 0.01$^c$ | 6.73     |
| 11-cis-Retinal    | --    | --  | 0.138 ± 0.01$^c$ | 5.02     |
| --                | +     | --  | 0.138 ± 0.01$^c$ | 5.30     |
|                   | --    | +   | 0.12 ± 0.01$^d$  | 5.10     |

$^a$ 10 μM FA.

$^b$ 5 μM FA.

$^c$ n = 4.

$^d$ n = 9.

the rates of dissociation of 11-cis-retinal from the two retinoid-binding sites of IRBP were similar.

Transfer of 11-cis-retinal from IRBP to vesicles was also followed in the presence of either palmitic acid or DHA. Palmitic acid did not influence the rate of dissociation of the complex (Table IV). In contrast, DHA had a profound effect on the kinetic parameters governing the dissociation of 11-cis-retinal from IRBP. As can be seen in Fig. 7, in the presence of DHA, transfer of 11-cis-retinal from IRBP to vesicles was clearly composed of two individual processes. Attempts to fit the data by assuming that the traces represented a single first order reaction resulted in poor fits. However, the traces could be well fit assuming that the transfer process was composed of two independent first order reactions. Two rate constants were thus extracted (Table IV). One rate constant was essentially identical to the rate constant obtained in the absence of DHA. Thus, in agreement with the conclusion that DHA does not alter the binding affinity of 11-cis-retinal in the hydrophobic IRBP site, the rate of dissociation of the ligand from one of the IRBP sites was not altered in the presence of DHA. The second rate constant calculated from the data was about 8-fold larger than the first, indicating that the presence of DHA dramatically facilitated the dissociation of 11-cis-retinal from the second IRBP site.

FIGURE 5. Effect of FA on rate of transfer of all-trans-retinol from IRBP to vesicles of DOPC. Unilamellar vesicles of DOPC were made by sonication and contained 2 mol % NBD-DPPE. Vesicles (2 mM) were mixed with all-trans-retinol-IRBP complex (1 μM at a ligand:protein molar ratio of 2) using a stopped-flow accessory. Transfer of all-trans-retinol from IRBP to vesicles was followed by time-dependent quenching of all-trans-retinol fluorescence upon its arrival at the vesicles (excitation, 350 nm; emission, 480 nm). Insets show the residuals corresponding to the fit of the trace to a single first order reaction. Measurements were carried out in the absence (A) or in the presence of 10 μM palmitic acid (B) or DHA (C).

FIGURE 6. Effect of palmitic acid on the rate of transfer of 11-cis retinal from IRBP to vesicles of DOPC. Unilamellar vesicles of DOPC were made by sonication and contained 2 mol % PY-PC. Vesicles (2 mM) were mixed with 11-cis-retinal-IRBP complex (1.0 μM at a ligand:protein molar ratio of 2) using the stopped-flow accessory, and fluorescence was monitored until equilibrium was reached. Transfer of retinoid from IRBP to vesicles was followed by monitoring the time-dependent quenching of PY-PC fluorescence by 11-cis-retinal upon its arrival at the vesicles (excitation, 330 nm; emission, 400 nm). Insets show the residuals corresponding to the fit of traces to a single first order reaction. Experiments were carried out in the absence (A) or in the presence of 5 μM palmitic acid (B).

DISCUSSION

IRBP is believed to participate in the visual cycle by mediating rapid shuttling of retinoids between photoreceptors and RPE cells (1, 2). It is not known, however, how specific retinoids are targeted to the particular cells in the eye that are their sites of action or whether IRBP plays an active role in directing retinoid fluxes. It was postulated that the protein might be able to selectively withdraw 11-cis-retinal from RPE cells, perhaps by binding to receptors in the plasma membranes of these cells. It was also hypothesized that IRBP may be recognized by receptors in the plasma membranes of photoreceptor cells and that upon binding to these receptors, 11-cis-retinal is released from the protein to the photoreceptor outer segments. However, no experimental evidence for the presence of IRBP receptors in plasma membranes of either pigment epithelium or photoreceptor cells has been reported, nor is it clear how transport mediated by such receptors can bypass the rapid fluxes of free retinoids via the lipid regions of the membranes (30, 31).
reservoir for retinoids in the interphotoreceptor matrix and may simply bind and release retinoids in response to changes in local concentrations of particular species. According to this hypothesis, "targeting" is accomplished passively and follows retinoid concentration gradients that are formed by metabolic and light-generated activities in particular cells.

In the present work, the interrelationships between binding of two classes of ligands that are known to associate with IRBP in the eye, retinoids and long chain FAs, were studied. Analyses of the FA composition of bovine IRBP indicated that the major constituents of endogenous FAs associated with the protein are palmitic, stearic, and oleic acid, which together composed 70% of the total FAs bound to the protein. It was previously reported that these three components compose 85% of FAs bound to monkey IRBP (4). Interestingly, in contrast with the report that IRBP isolated from monkey retina contains only an insignificant amount of polyunsaturated FAs (4), the data in Table I indicate that arachidonic acid and DHA composed 7.7% and 8.6% of total FAs associated with bovine IRBP, respectively. In fact, 0.5 mol of each of these FAs was found associated with 1 mol of IRBP.

To obtain a better understanding of the source of FAs that are endogenously bound to IRBP, the FA contents of the phospholipids and free FAs pools in RPE and ROS were determined. In agreement with previous reports (16–18) it was found that DHA is enriched in lipids of ROS as compared with those of RPE. The compositions of the free FA pools in ROS and RPE have not, to our knowledge, been previously documented. The data (Table II) show that the compositions of free FA correlate well with the FA compositions of phospholipid fractions in the two cell types. In both the phospholipids and the free FA pools, the major FA constituents in RPE were palmitic, stearic, and oleic acid. ROS contained a lower fraction of palmitate and were significantly enriched in respect to DHA. These data demonstrate that there exists a steep concentration gradient of free DHA across the interphotoreceptor matrix.

Long chain FAs flow through membranes rapidly. They were shown to traverse lipid bilayers at rates on the order of milliseconds (32) and to dissociate from membranes at rates in the 0.02–1-s range (33). These considerations imply that free FAs will rapidly move between the intracellular pools and the interphotoreceptor matrix. Thus, FAs bound to IRBP transiently located near a particular cell will rapidly equilibrate with FAs of this cell. It follows that IRBP that is located near ROS can be expected to contain a larger fraction of arachidonic acid and DHA as compared with IRBP in the vicinity of RPE, which will contain a larger fraction of palmitic acid but only a small amount of polyunsaturated FA. Since IRBP isolated from retina was originally distributed across the IPM, the above considerations would predict that the observed FA content of the protein will represent an average of IRBP pools at different locations within the matrix. Inspection of the FA content of IRBP shows that, indeed, the composition of protein-bound FAs is intermediate between the concentrations of FAs in ROS and RPE. For example, DHA composes about 20% and 3.5% of lipids in ROS and RPE, respectively. IRBP-bound DHA composes 8.6% of total bound FAs. The palmitate content of lipids in ROS and RPE is 19 and 43%, respectively, while IRBP-bound palmitate composes 26% of FAs in the protein-bound pool. The only exception to this trend is oleic acid, which constitutes a large fraction of IRBP-bound FAs, while its intracellular content in both ROS and RPE is relatively small. The origin of the selective enrichment of IRBP in respect to oleic acid is not clear. However, it should be noted that the FA content of IRBP would

**Fig. 7.** Effect of DHA on rate of transfer of 11-cis-retinal from IRBP to vesicles of DOPC. Experiments were carried out as described in the legend to Fig. 6. in the presence of 5 μM (A and B) or 10 μM (C and D) DHA. Insets show the residuals corresponding to the fit of the traces to a single first order reaction (A and C) or to two first order reactions (B and D).
be determined, in addition to the concentrations of FAs, by the affinity of the protein for specific FAs. No information is currently available regarding the relative affinities of IRBP for specific FAs, but it is possible that the affinity for oleic acid is higher than that for other FAs, leading to the observed enrichment in this particular FA.

The data in Table III show that DHA efficiently curtails binding of 11-cis-retinal in the hydrophilic retinoid-binding site of IRBP. The data in Table IV demonstrate that weakening of binding affinity of the ligand stems, at least partially, from facilitation of the dissociation of 11-cis-retinal from this site. It should be noted that previous studies utilizing nondelipidated protein showed that the rates of dissociation of 11-cis-retinal from the two IRBP-binding sites differ by about an order of magnitude (15). The data in the present work demonstrate that upon removal of FAs from IRBP, the rates of dissociation of this ligand from the two IRBP sites are similar and that the addition of DHA dramatically facilitates dissociation from one of the sites. These observations, taken together, verify that facilitation of the dissociation of 11-cis-retinal from the hydrophilic binding site of IRBP originates from the presence of IRBP-bound DHA.

The effect of DHA on binding of 11-cis-retinal to IRBP was remarkably specific. DHA weakened binding of this ligand but did not significantly interfere with the interactions of all-trans-retinol with the protein. The effect was also specific in that DHA only inhibited the interactions of 11-cis-retinal with the hydrophilic site of IRBP but did not alter the characteristics of the interactions of this ligand with the hydrophobic site. Finally, while DHA was found to be a potent inhibitor, palmitic acid had very little effect on binding of 11-cis-retinal to IRBP.

Based on these data and considering the concentration gradient of DHA that exists between photoreceptors and RPE cells (Table II), we propose a mechanism by which IRBP targets 11-cis-retinal to photoreceptor cells. More specifically, we suggest that the interactions of IRBP with DHA and the outcomes of these interactions for binding of 11-cis-retinal in the hydrophilic binding site of the protein serve to target this ligand to its site of action in the eye. According to this model, when IRBP is localized near RPE, it is associated with FAs prevalent in these cells, which are predominantly saturated FAs. Under these conditions, the hydrophilic retinoid-binding site of IRBP possesses a high affinity for 11-cis-retinal. Since 11-cis-retinal is readily available in the vicinity of the RPE cells, the site becomes occupied with this ligand. Movement of IRBP to the vicinity of photoreceptor outer segments results in reequilibration of the FAs associated with the protein. Since photoreceptors are enriched with DHA, the FA pool bound to IRBP will be readjusted to contain a large fraction of DHA. Binding of DHA to IRBP leads to rapid release of 11-cis-retinal from the hydrophilic site. Since the affinity of this site for all-trans-retinol remains high in the presence of the DHA, it will become occupied with the later ligand. The process is reversed when IRBP is again localized near the RPE; DHA is exchanged with saturated FAs, and the retinoid-binding site regains its high affinity for 11-cis-retinal. The outcome of the overall process is that 11-cis-retinal is transferred from RPE to photoreceptor cells where it is required for rhodopsin regeneration, while all-trans-retinol is transferred from photoreceptor to pigment epithelium cells where it can be either resorcinized to the 11-cis form or esterified and stored. An additional outcome is that FAs are transferred down their concentration gradients. DHA moves from photoreceptors to RPE, while palmitic acid is transported to the photoreceptors.

This model thus provides a basis for understanding how direction of fluxes of specific retinoids may be regulated. IRBP can mediate retinoid targeting due to its ability to adjust binding affinity of one ligand in response to its interactions with another. The ultimate source for the ability of the protein to target retinoids to their sites of action is the differential concentration of DHA in photoreceptors versus in RPE cells.

Several questions remain open. For example, the effect of DHA on binding of 11-cis-retinal to IRBP is confined to the interactions within the second/hydrophilic binding site. A complete understanding of the mechanism of action of IRBP would require clarification of how binding of retinoids within the hydrophobic IRBP sites is regulated. In addition, several FAs other than palmitic acid and DHA are associated with bovine IRBP in vivo. The effects of these FAs on binding of retinoids to IRBP are being studied and will be reported separately, but it is of interest to mention here that preliminary studies have demonstrated that arachidonic acid, which like DHA is a polyunsaturated FA, does not affect the interactions of 11-cis-retinal with IRBP. Another issue that remains open relates to the identification and characterization of the FA-binding site(s) of IRBP. The data reported here are consistent with two alternative possibilities. It was found that neither palmitic acid nor DHA significantly affected retinoid binding in the hydrophobic site and that the DHA-induced inhibition of binding of 11-cis-retinal in the hydrophilic site was limited to this ligand. These data seem to indicate that FAs do not associate directly with either of the retinoid binding pockets. Instead, the observations imply that while DHA might induce a change in the configuration of the hydrophilic site, it does not directly compete with retinoids on binding to it. Alternatively, it is possible that, unlike the hydrophobic retinoid-binding site of IRBP, which was shown to accommodate both all-trans-retinol and 11-cis-retinal (Fig. 2), two additional discrete sites might exist for the ligands. In this case, DHA might specifically compete with retinal for binding in its second site, but it does not interfere with binding of retinol, which occurs at a distinct pocket.

In summary, the results of the present work demonstrate that DHA composes a significant fraction of the FAs endogenously associated with bovine IRBP and that this FA specifically modulates the interactions of 11-cis-retinal with one of the retinoid-binding sites of the protein. Based on these observations and on the existence of steep concentration gradients for DHA between ROS and RPE, a mechanism by which IRBP may target specific retinoids to particular cells in the eye is proposed. Although several questions remain open, the suggested model should prove useful in providing a fresh point of view on a long standing question.

REFERENCES

1. Saari, J. C. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sorn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 351–386, Raven Press, New York
2. Pepperberg, D. R., Okajima, T.-I. L., Wiggert, B., Rippes, H., Crouch, R. K., and Chader, G. J. (1993) Mol. Neurobiol. 7, 61–85
3. Fors G.-S., Lång, G. I., Landers, R. A., Alvarez, R. A., and Bridges, C. D. (1984) J. Biol. Chem. 259, 6534–6542
4. Bazan, N. G., Reddy, T. S., Redmond, T. M., Wiggert, B., and Chader, G. J. (1985) J. Biol. Chem. 260, 13677–13680
5. Adler, A. J., and Spencer, S. A. (1991) Exp. Eye Res. 53, 337–346
6. Ho, M.-T. P., Massey, J. B., Pownall, H. J., Anderson, R. E., and Hollyfield, J. G. (1989) Exp. Eye Res. 51, 717–728
7. Flannery, J. G., O’Day, W., Pfeffer, S. A., Horwitz, J., and Bok, D. (1990) Exp. Eye Res. 51, 251–255
8. O’Day, W., Flannery, J. G., and Bok, D. (1992) Photochem. Photobiol. 56, 928–935
9. Crouch, R. K., Hazard, E. S., Lind, T., Wiggert, B., Chader, G., and Corson, W. (1992) J. Biol. Chem. 267, 21983–21989
10. Okajima, T.-I. L., Wiggert, B., Chader, G. J., and Pepperberg, D. R. (1994) J. Biol. Chem. 269, 21983–21989
11. O’Day, W., Flannery, J. G., Pfeffer, S. A., Horwitz, J., and Bok, D. (1990) Exp. Eye Res. 53, 717–728
12. Carlson, A., and Bok, D. (1992) Biochemistry 31, 9056–9062
13. Okajima, T.-I. L., Wiggert, B., Chader, G. J., and Pepperberg, D. R. (1994) J. Biol. Chem. 269, 21983–21989
14. Jones, G. J., Crouch, R. K., Wiggert, B., Cornell, M. C., and Chader, G. J. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 9066–9070
15. Okajima, T.-I. L., Pepperberg, D. R., Rippes, H., Wiggert, B., and Chader, G. J. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 6907–6911
16. Y. Chen and N. Noy, unpublished results.
Modulation of IRBP-Retinoid Interactions by DHA

13. Saar, J. C., Teller, D. C., Crab, J. W., and Bredberg, L. (1985) J. Biol. Chem. 260, 195–201
14. Chen, Y., Saari, J. C., and Noy, N. (1993) Biochemistry 32, 11311–11318
15. Chen, Y., and Noy, N. (1994) Biochemistry 33, 10658–10665
16. Fleisler, S. J., and Anderson, R. E. (1983) Prog. Lipid Research 22, 79–131
17. Chen, H., Wiegand, R. E., Koutz, C. A., and Anderson, R. E. (1992) Exp. Eye Res. 55, 93–100
18. Anderson, R. E., Lissandrello, R. E., Maude, M. B., and Matthes, M. T. (1976) Exp. Eye Res. 23, 9–131
19. Saari, J. C., and Bredberg, L. (1988) Exp. Eye Res. 44, 569–578
20. Dittmer, J. C., and Wells, M. A. (1969) Methods Enzymol. 14, 482–530
21. Cogan, U., Kopelman, M., Makady, S., and Shintzky, M. (1976) Eur. J. Biochem. 65, 71–78
22. Norris, A. W., Cheng, L., Giguere, V., Rosenberger, M., and Lee, E. (1994) Biochim. Biophys. Acta 1209, 10–18
23. Ong, D., and Chytil, F. (1980) Methods Enzymol. 67, 288–296
24. Noy, N., and Blaner, W. S. (1991) Biochemistry 30, 6380–6386
25. Okajima, T-I. L., Pepperberg, D. R., Ripps, H., Wiggert, B., and Chader, G. J. (1989) Exp. Eye Res. 49, 629–644
26. Adler, A. J., and Evans, C. D. (1985) Invest. Ophthalmol. Vis. Sci. 26, 273–282
27. Connors, K. A. (1987) Binding Constants: The Measurement of Molecular Complex Stability, pp. 175–183, John Wiley and Sons, Inc., New York
28. Royer, C. A., and Beechem, J. M. (1992) Methods Enzymol. 210, 481–504
29. Noy, N., and Xu, Z-J. (1990) Biochemistry 29, 3878–3883
30. Noy, N., and Xu, Z-J. (1990) Biochemistry 29, 3883–3888
31. Noy, N., Kelsoe, D. J., and Scoot, A. W. (1995) J. Lipid Res. 36, 375–382
32. Kamp, F., Zakim, D., Zhang, F., Noy, N., and Hamilton, J. A. (1995) Biochemistry 34, 11928–11937
33. Daniels, C., Noy, N., and Zakim, D. (1985) Biochemistry 24, 3286–3292
34. Lee, R. H., Farber, D. B., and Lolley, R. N. (1982) Methods Enzymol. 81, 496–499
35. Ottonello, S., and Maraini, G. (1984) Curr. Eye Res. 3, 1085–1096