Binding of Retinol in Both Retinoid-binding Sites of Interphotoreceptor Retinoid-binding Protein (IRBP) Is Stabilized Mainly by Hydrophobic Interactions*

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Interphotoreceptor retinoid-binding protein (IRBP) is an ocular protein which is believed to participate in the visual cycle by mediating transport of retinoids between pigment epithelium and photoreceptor cells. The molecular mechanism underlying the ability of IRBP to target particular retinoids to the specific cells that are their sites of action and metabolism is not completely clear, and little information is available regarding the structure of the protein's multiple ligand-binding sites. IRBP possesses two retinoid-binding sites, and it was reported that binding of the visual chromophore, 11-cis-retinal, in one of these sites, but not in the other, is tightly regulated by another IRBP ligand, docosahexaenoic acid (Chen, Y., Houghton, L. A., Brenna, J. T., and Noy, N. (1996) J. Biol. Chem. 271, 20507). The two sites are thus functionally distinct. Here, the thermodynamic parameters governing the interactions of retinol with the IRBP retinoid-binding sites were measured. The data demonstrate that the interactions of retinol with both sites are stabilized mainly by hydrophobic interactions, and that the hydroxyl head group of retinol is not involved in formation of protein-ligand complexes. Nevertheless, the data indicate that the two sites are structurally distinct, and that binding of retinol in them occurs by remarkably different modes of interactions.

Shuttling of retinoids across the interphotoreceptor matrix (IPM), the extracellular space separating between retinal pigment epithelium (RPE) and photoreceptor cells in the eye, is essential for the function of the visual cycle. Rhodopsin regeneration requires transport of the visual chromophore 11-cis-retinal from its site of synthesis in the RPE to photoreceptor cells. On exposure to light, rhodopsin-bound 11-cis-retinal is isomerized to all-trans-retinal, hydrolyzed from the protein, and is enzymatically reduced to all-trans-retinol. Regeneration of the visual chromophore requires movement of all-trans-retinol from photoreceptors back to the RPE for re-oxidation and isomerization (reviewed in Ref. 1). Thus, the visual cycle critically includes continuous transfer of all-trans-retinol from photoreceptors to the RPE and of 11-cis-retinol from RPE to the neural retina. The mechanisms by which retinoids, which are poorly soluble in water, are transported through the IPM at rates that are sufficient to support visual function are not completely clear at present. It has been repeatedly suggested, however, that the interphotoreceptor retinoid-binding protein (IRBP), plays an important role in this process (reviewed in Ref. 2).

IRBP is a 140-kDa glycoprotein that is the major soluble protein in the IPM. It binds various isomeric and chemical forms of retinoids as well as long chain fatty acids (3–6), and is believed to mediate transfer of retinoids through the IPM (3, 7–16). IRBP possesses two retinoid-binding sites (3, 8, 17) that seem to be functionally distinct. The specific function of one of these sites (termed “site 1” in this article) is not clear at present but it has been shown to possess a retinoid selectivity in the order of 11-cis-retinal > all-trans-retinol > all-trans-retinal > 11-cis-retinol. The higher binding affinities for 11-cis-retinal and all-trans-retinol correspond to the physiological need to shuttle these particular retinoids across the IPM (18). It is thus conceivable that site 1 serves mainly to allow IRBP to maintain a large storage pool of these retinoid forms in the extracellular space of the IPM.

The recent observations that the interactions of 11-cis-retinal with the other retinoid-binding site of IRBP (“site 2”) are tightly regulated by the polyunsaturated long chain fatty acid docosahexaenoic acid (DHA), provided clues regarding the possible function of site 2. It was shown that binding of DHA to IRBP dramatically reduces the affinity of site 2 for this ligand, and facilitates the rate by which 11-cis-retinal is released from this site by about an order of magnitude (6). In contrast, DHA has very little effect on the interactions of 11-cis-retinal with site 1, or on the affinities of either site 1 or site 2 for all-trans-retinol (6). Further evidence indicated that DHA comprises a significant fraction of fatty acids associated with IRBP in the IPM, and that a steep concentration gradient of free DHA exists across the IPM such that the level of this fatty acid is about 8-fold higher in photoreceptor versus pigment epithelium cells (6).

These observations led us to propose that DHA serves as a switch that allows IRBP to specifically target 11-cis-retinol to photoreceptor cells. We thus suggested that when IRBP is in the vicinity of pigment epithelium cells, where DHA concentrations are relatively low, site 2 possesses a high affinity for 11-cis-retinal which consequently binds to it. Movement of IRBP to the vicinity of photoreceptor cells exposes the protein to high DHA levels which then induces rapid release of 11-cis-retinal from site 2 at this location (6). The functional interrelationships between the association of 11-cis-retinal and DHA with IRBP, which manifests itself in the regulatory features of site 2, may thus comprise the underlying molecular mechanism responsible for the directional transfer of retinoids across the IPM.

In addition to functional differences reflected by differential

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1 The abbreviations used are: IPM, interphotoreceptor matrix; IRBP, interphotoreceptor retinoid-binding protein; RPE, retinal pigment epithelium; DOPC, dioleoylphosphatidylcholine; DHA, docosahexaenoic acid.

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regulatory features and distinct retinoid-binding affinities (6, 18), the two IRBP retinoid-binding sites also display structural dissimilarities. For example, binding of retinol in site 1 results in a marked enhancement in the fluorescence of this ligand (10, 19), reflecting its movement from the aqueous phase to a more restrictive and hydrophobic environment within the binding site (20). In contrast, association of retinol with site 2 does not affect the fluorescence of the ligand (17). We previously suggested that these observations might indicate that while site 1 comprises a hydrophobic pocket, site 2 may be a more shallow site located at the surface of the protein, and that it may interact with retinol via its polar head group rather than by providing a non-polar environment which accommodates the β-ionone and the isoprenoid moiety of this ligand (18).

The present work was undertaken to further characterize the interactions of retinol with the two IRBP retinoid-binding sites. The data support the suggestion that the two sites are structurally distinct. The results indicate, however, that despite the dissimilarities of the two sites, the interactions of retinol with both of them are stabilized mainly by hydrophobic interactions, and that the hydroxyl head group of retinol is not involved in interactions of this ligand with either.

**EXPERIMENTAL PROCEDURES**

**Bovine Retina**—Fresh bovine eyes were obtained from a local slaught- terhouse, stored in Hank’s balanced salt solution containing 1.3 mM CaCl₂ and 20 mM HEPES, pH 7.0, and processed within 6 h after removal. The anterior half of the eye was removed, the eye cup washed with Hank’s balanced salt solution containing 20 mM HEPES and the retina was removed. Retinas were stored in the same buffer at −80 °C.

**Bovine IRBP**—This was purified from bovine retina essentially as described (21), except that fast-flow DEAE-Sepharose was used in place of DEAE-cellulose. Purified protein was dialyzed against a buffer containing 20 mM HEPES, pH 7.0, 150 mM NaCl, and 0.1 mM dithiothreitol (buffer A), concentrated, and stored at −20 °C in buffer A containing 50% glycerol. Protein concentration was determined using ε₂₈₀ nm = 134,124 (21).

**Unilamellar Vesicles of Dioleoylphosphatidylcholine (DOPC)**—Vesi- cles were prepared in buffer A as described previously (18). Phospholipid concentration was determined according to Dittmer and Wells (22).

**Ligands**—Retinol and retinyl methyl ether (generously provided by Drs. Hill and Shealy, Southern Research Institute, Birmingham, AL) were dissolved in ethanol and their concentrations determined using ε₂₈₀ nm = 52,770 and 52,200, respectively.

**Fluorescence Measurements**—Fluorescence was measured using a Fluorolog 2 spectrophotofluorometer (SPEx Instruments, Edison, NJ). Kinetic measurements were carried out using a High-Tech (Salisbury, United Kingdom) rapid mixing apparatus in conjunction with the spectrophotofluorometer.

**Measurement of Rates of Dissociation of Retinol from IRBP**—To induce dissociation of retinol from IRBP, the holo-protein was mixed with vesicles of DOPC which served as an acceptor phase. An access of lipid over protein (typically, 2–6 mol lipid/mol IRBP) was used to ensure that over 95% of the ligand transferred from the protein to the vesicles at equilibrium.

To monitor dissociation of retinol from site 1, the decrease in the fluorescence of the ligand as it moves out of this site was followed. To monitor dissociation of retinol from both IRBP’s retinoid-binding sites, the intrinsic fluorescence of the protein, which increases as the ligand moved away from both sites, was monitored. In all cases, the dissociation reaction could be well fit for a single first order equation and data analyses were carried out using the software package Origin (Microcal Co.).

**Thermodynamic Parameters of the Kinetics by Which IRBP-retinol Complexes Dissociate**—Rate constants for dissociation of IRBP-retinol complexes were plotted in the form of an Arrhenius plot, in ln kₘₐₓ versus 1/T. Energy of activation (Eₐ) of the reaction was obtained from the slope of the plot (slope = Eₐ/R). Enthalpy of activation (ΔH*) was calculated using ΔH* = Eₐ − RT. Entropy of activation (ΔS*) was calculated using ΔS* = 2.3log(h/kₐT), where h is the Avogadro number, R is the Planck constant, and kₐT = e⁻⁻ΔH*/ΔS*. Free energy of activation (ΔG*) was calculated using ΔG* = ΔH* − TΔS*.

**Fluorescence Titrations of IRBP**—Binding of retinol or retinyl methyl ether to IRBP was studied by fluorescence titrations as described previously (17). The interactions of the ligands with site 1 were followed by monitoring the enhancement of the fluorescence of the ligand upon binding at this site (10, 15, 18, 23–25). Binding in the two retinoid-binding sites was followed by monitoring the decrease in the fluorescence of the protein which occurs concomitantly with ligand binding in both sites (18). Data were analyzed by fitting the experimental points to an equation derived from simple binding theory using the software package Origin (Microcal Co.).

**Thermodynamic Parameters Governing the Formation of IRBP-Retinol Complexes at Equilibrium**—Equilibrium constants (Kᵣ) were plotted as a function of temperature in the form of a van’t Hoff plot, ln Kᵣ versus 1/T. In this plot, the slope of the line corresponds to −ΔH/R, while the y intercept corresponds to ΔS/R. The free energy of the reaction was calculated using ΔG = ΔH − TΔS.

**RESULTS**

To explore possible differences between the modes by which the two IRBP retinoid-binding sites interact with retinol, the thermodynamic parameters governing the rates of dissociation of retinol from the two sites were assessed.

IRBP was precomplexed with retinol at a ligand/protein mole ratio of 2/1, and mixed with unilamellar vesicles of DOPC. Due to the high affinity of lipid bilayers for retinol, following mixing, the ligand moved from the protein to the vesicles (24).

Utilization of a large excess of lipid vesicles over protein ensured that over 95% of the ligand transferred into the vesicles at equilibrium. We and others previously showed that retinol moves from IRBP to lipid vesicles by a process that involves dissociation of the ligand from the protein, diffusion through the aqueous phase, and association with the lipid bilayers (18, 26). The rate-limiting step of the overall process is the dissociation of retinol from the protein. Thus, the observed rate of movement of retinol between IRBP and vesicles in these experiments directly reflects the rate of dissociation of the ligand from the protein (18, 26).

As detailed below, two fluorescent properties within the system proved useful for probing dissociation of retinol from IRBP. One of these was used to monitor the dissociation of the ligand from site 1, while the other was used as a read-out for probing the dissociation of retinol from both site 1 and site 2 simultaneously. To date, no optical method for probing site 2 individually has been developed.

**Temperature Dependence of the Rate of Dissociation of Retinol from IRBP’s Retinoid-binding Site I**—Retinol is an efficient fluorophore and the intensity of its fluorescence is markedly enhanced upon its binding in site 1. Consequently, dissociation of retinol from this site can be followed by monitoring the time-dependent decrease in retinol fluorescence following mixing of holo-IRBP with vesicles. Dissociation of retinol from site 1 at various temperatures in the range of 12–40 °C was studied. Representative traces obtained at 12 °C and 40 °C are shown in Fig. 1, A and B, respectively. At all of the temperatures studied, the dissociation reaction followed first order kinetics and the respective rate constants could be obtained by fitting the data to a single exponential. Fig. 2 shows an Arrhenius plot describing the temperature dependence of the rate constants. The energy of activation of the reaction and the calculated values of the free energy (ΔG*), entropy (ΔS*), and enthalpy (ΔH*) of activation characterizing the reaction by which retinol dissociates from site 1 are shown in Table I.

**Temperature Dependence of the Rate of Dissociation of Retinol from Both of IRBP’s Retinoid-binding Sites**—Binding of retinol in both of the retinoid-binding sites of IRBP results in a significant decrease in the intrinsic fluorescence of the protein which can be monitored at excitation and emission wavelengths of 280 and 340 nm, respectively (17). Consequently, movement of retinol away from either binding site results in recovery of the protein’s fluorescence intensity and the time-
To assess the thermodynamic parameters of the dissociation of retinol from the two sites, the temperature dependence of the apparent rate constants of the dissociation reaction was studied. Representative traces at 12 °C and at 40 °C are shown in Fig. 3, A and B, respectively, and an Arrhenius plot of the data is shown in Fig. 4. The thermodynamic parameters characterizing the dissociation reactions are shown in Table I.

The energy of activation of the dissociation of retinol from site 1 was found to be about 7 kcal/mol larger than that of the dissociation of retinol from both of the IRBP’s retinoid-binding sites. As the later value represents a mean between the activation energies for dissociation of the ligand from site 1 and site 2, it is clear that the energy required to convert retinol bound at site 1 to the activated state is remarkably higher than that required to overcome energy barriers for dissociation of the ligand from site 2. Inspection of the thermodynamic parameters characterizing the processes by which retinol dissociates from the two sites reveal additional differences: the entropic contribution to the free energy of activation of retinol dissociation from site 1 is significantly smaller as compared with site 2, while the enthalpic component is several kcal/mol larger. Overall, these observations indicate that the barriers for dissociation of retinol from site 1 versus site 2 are different both in magnitude and nature.

**Thermodynamic Parameters Governing the Interactions of Retinol with Site 1 at Equilibrium**—To obtain better insight into the forces that stabilize the interactions of retinol within the IRBP retinoid-binding sites, the thermodynamic parameters characterizing these interactions were measured. Equilibrium constants at various temperatures were determined by fluorescence titrations of the protein with retinol.

To monitor binding of retinol at site 1, fluorescence titrations were followed by monitoring the enhancement of the fluorescence of the ligand upon binding at this site. A representative titration is shown in Fig. 5A. The data indicated that the number of binding sites obtained for the titration shown in Fig. 5A was 0.86 mol/mol IRBP, verifying that following the enhancement of the fluorescence of retinol reports on binding of the ligand in a single site. A van’t Hoff plot of the temperature dependence of the equilibrium constants is shown in Fig. 5B. The thermodynamic parameters governing the interactions of retinol with site 1 are presented in Table II.

**Thermodynamic Parameters Governing the Interactions of Retinol with Both IRBP’s Retinoid-Binding Sites at Equilibrium**—To probe binding of retinol in both of the IRBP retinoid-binding sites, the decrease of the intrinsic fluorescence of the protein upon titration with retinol was monitored. A representative titration of this type is shown in Fig. 6A. Analysis of these data indicated that the number of retinol-binding sites probed by this method was 1.69 mol/mol IRBP. In 9 independent protein preparations, titration data obtained by monitoring the fluorescence of the protein yielded a number of binding sites that was always about double the number of sites obtained by monitoring the enhancement in the fluorescence of retinol. These observations verify that quenching of the fluorescence of retinol with both IRBP’s retinoid-binding sites at equilibrium is shown in Table I.

| Site | $E_a$ (kcal/mol) | $\Delta G^a$ (kcal/mol) | $\Delta H^a$ (kcal/mol) | $T\Delta S^a$ (kcal/mol K) |
|------|-----------------|------------------------|------------------------|--------------------------|
| 1    | 17.9            | 18.2                   | 17.3                   | -0.9                     |
| 1 + 2| 11.1            | 14.4                   | 10.5                   | -3.9                     |

*At 25 °C.* Activation energies for dissociation of retinol from site 1 and from both sites were obtained from the data in Figs. 2 and 4, respectively. Thermodynamic parameters were calculated as described under “Experimental Procedures.”

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the protein is indeed a valid readout for probing the association of retinol with both binding sites. Equilibrium constants obtained from these titrations thus reflect a mean between the values of the equilibrium constants characterizing the interactions of retinol with the two IRBP retinoid-binding sites. The temperature dependence of the mean equilibrium constant is shown in Fig. 6

B

in the form of a van’t Hoff plot, and the mean thermodynamic parameters governing the interactions of retinol are presented in Table II.

Inspection of the thermodynamic parameters obtained for the interactions of retinol in site 1 (Table II) shows that only 12% of the energy stabilizing the association of retinol with this site is contributed by enthalpic components. The thermodynamic parameters for the interactions of retinol within site 2 were calculated from the values obtained for site 1 and the values for sites 1 and 2 (Table II). The results indicate that the enthalpic contribution for binding at site 2 comprised about 30% of the overall free energy stabilizing retinol-protein interactions at this site. Thus, the enthalpic contribution for association of retinol in both sites, which could emanate either from van der Waals or from hydrogen bond interactions between the protein and bound retinol, is relatively small. The predominant force that acts to stabilize the interactions of retinol in both sites is entropic. These observations thus indicate that protein-retinol interactions in both of IRBP’s retinoid-binding sites

![Fig. 3. Rate of dissociation of retinol from both retinol-binding sites.](image)

![Fig. 4. Arrhenius plot for dissociation of retinol from both binding sites.](image)

![Fig. 5. Temperature dependence of the equilibrium constant characterizing binding of retinol in site 1.](image)

**TABLE II**

| Site     | ΔG* (kcal/mol) | ΔH (kcal/mol) | TΔS° (kcal/mol) |
|----------|----------------|---------------|----------------|
| 1        | -9.6           | -1.2          | 8.5            |
| 1 + 2    | -9.2           | -2.0          | 7.2            |
| 2 (calculated) | -8.8       | -2.8          | 6.0            |

*At 25 °C. Thermodynamic parameters for the association of retinol with site 1 and with both sites were obtained from the data in Figs. 5 and 6, respectively. Thermodynamic parameters for the association of retinol in site 2 were calculated assuming that the values obtained for site 1 + 2 is a mean of the values characterizing the 2 sites.
molecule, a model that accounts for all of these observations can be put forward. It is thus reasonable to suggest that site 1 is comprised of a deeper pocket which accommodates both the \(\beta\)-ionone ring and the isoprenoid chain of retinol. Such a pocket will provide a hydrophobic environment for the ligand and will lead to a marked increase in its fluorescence. In contrast, site 2 may be a shallower pocket which interacts with the \(\beta\)-ionone moiety of retinol by hydrophobic association, but does not accommodate the fluorescent portion of the molecule.

This model for the structure of site 2 implies that the polar head group of retinol will not be involved in protein-ligand interactions in this location. To further explore this hypothesis, the interactions of retinyl methyl ether, a retinol derivative lacking the hydroxyl head group, with IRBP were studied. Fluorescence titrations of IRBP with retinyl methyl ether are shown in Fig. 7. Similarly to studies with retinol, the interactions of retinyl methyl ether with IRBP were probed both by monitoring the fluorescence of the ligand (Fig. 7A), and by monitoring the decrease in the intrinsic fluorescence of the protein upon binding of this ligand (Fig. 7B). Also similarly to studies with retinol, fluorescence titrations with retinyl methyl ether indicated that the number of binding sites probed by the later method was double the number of binding sites observed by the former. Furthermore, the data yielded equilibrium dissociation constants for the interactions of retinyl methyl ether with site 1 of 95 nM. The apparent mean dissociation constant of the ether derivative with both sites was found to be 142 nM (mean of two measurements). The \(K_d\) values characterizing the interactions of retinyl methyl ether with both of the IRBP retinoid-binding sites are thus essentially identical to those of retinol (see Table III).

These observations indicate, in agreement with the above proposed model, that the -OH head group of retinol is not involved in the interactions of this ligand with either of the IRBP-binding sites.

**DISCUSSION**

IRBP, a retinoid- and fatty acid-binding protein of the IPM, is believed to participate in the visual cycle by mediating rapid shuttling of retinoids between photoreceptors and RPE cells (1, 2). The molecular mechanism underlying the ability of IRBP to target specific retinoids to particular locations in the eye is not completely understood at present. One approach for obtaining better insights into the mechanism of action of IRBP is to investigate the interactions of this protein with its ligands and the factors that regulate these interactions. As discussed in the Introduction, available data on the interactions of retinoids with the two IRBP retinoid-binding sites suggest that while site 1 might serve as a passive reservoir that allows for elevated concentrations of particular retinoids within the IPM, the regulatory features of site 2 might comprise the mechanism by which IRBP specifically targets \(11\text{-cis}\)-retinal to photoreceptor cells, while delivering all-trans-retinol from the photoreceptors to the RPE (6).

In the effort to understand the functional roles of the two IRBP retinoid-binding sites, it is interesting to consider the
structurally distinct.

In an additional set of experiments, the thermodynamic parameters of the interactions of retinol with the two IRBP sites at equilibrium were measured (Table II). The data clearly demonstrated that the main contribution to the forces that stabilize the interactions of retinol in both sites originates from entropic components. These observations reveal that the interaction energy is derived mainly from hydrophobic associations between the protein and the ligand bound in both sites (27). This conclusion is in agreement with the additional observations that retinyl methyl ether, a retinol derivative lacking the hydroxyl head group of the parent compound, binds to both of the IRBP retinoid-binding sites with affinities that are essentially identical to those of retinol. The head group of retinol is thus not required, and is unlikely to be involved in the association of retinoids in either binding site.

The results of the present study indicate further that while both of the IRBP retinoid-binding sites interact with retinoids mainly by hydrophobic association, the structures of the two sites and their mode of interactions with retinoids are substantially different. As discussed above, binding of retinol in site 1 results in a significant enhancement in the fluorescence of the ligand, indicating that the isoprenoid chain of retinol is removed from the aqueous phase to a hydrophobic environment upon binding in this site. In contrast, association of retinol with site 2 does not affect the fluorescence of the ligand, suggesting that the isoprenoid moiety of retinol is not transferred to a hydrophobic environment upon binding in this site. Comparison between the kinetic parameters governing the interactions of retinol with the two sites (Table III) shows that site 2 binds as well as releases this ligand at significantly faster rates as compared with site 1, and thus that the interactions of retinol with site 2 are looser as compared with site 1. It is reasonable to suggest, in view of these observations, that site 1 might be a deep hydrophobic pocket which engulfs both the β-ionone ring and the isoprenoid chain of retinoids, while the hydrophobic region in site 2 is smaller and while it binds the β-ionone ring by hydrophobic interactions, it does not associate with the isoprenoid chain or the head group of the molecule. The observations that the enthalpic component contributing to the interactions of retinol in site 2 is somewhat larger as compared with site 1 (30 versus 12% of total binding energy), suggest further that the isoprenoid chain may be bound in an additional, more polar, region within site 2, perhaps by van der Waals interactions.

An important question that remains open regarding structure-function relationship in IRBP is how the two dissimilar retinoid-binding sites of IRBP are constructed from the four highly homologous amino acid stretches within the protein sequence. It is also not clear at present how binding of DHA to the protein regulates the interactions of site 2 with 11-cis-retinol without affecting the interactions of all-trans-retinol in the same location. These questions are currently under investigation.

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REFERENCES

1. Saari, J. C. (1994) in The Retinoids, Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) pp. 351–386, Raven Press, New York
2. Pepperberg, D. R., Okajima, T.-I., Wiggert, B., Ripps, H., Crouch, R. K., and Chader, G. J. (1993) J. Biol. Chem. 268, 13677–13680
3. Fong S.-L., Liou, G. I., Landers, R. A., Alvarez, R. A., and Bridges, C. D. (1984) J. Biol. Chem. 259, 6534–6542
4. Baran, N. G., Reddy, T. S., Redmond, T. M., Wiggert, B., and Chader, G. J. (1985) J. Biol. Chem. 260, 13677–13680
5. Putilina, T., Sittenfeld, D., Chader, G. J., and Wiggert, B. (1993) Biochemistry 32, 3797–3803
6. Chen, Y., Houghton, L. A., Brenna, J. T., and Noy, N. (1996) J. Biol. Chem.
7. Lai, Y. L., Wiggert, B., Liu, Y. P., and Chader, G. J. (1982) Nature **298**, 848–849
8. Saari, J. C., Teller, D. C., Crabb, J. W., and Bredberg, L. (1985) *J. Biol. Chem.* **260**, 195–201
9. Jones, G. J., Crouch, R. K., Wiggert, B., Cornwall, M. C., and Chader, G. J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 9606–9610
10. Okajima, T.-I., Pepperberg, D. R., Ripps, H., Wiggert, B., and Chader, G. J. (1989) *Exp. Eye Res.* **49**, 629–644
11. Okajima, T.-I. L., Pepperberg, D. R., Ripps, H., Wiggert, B., and Chader, G. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6907–6911
12. Adler, A. J., and Spencer, S. A. (1991) *Exp. Eye Res.* **53**, 337–346
13. Flannery, J. G., O'Day, W., Pfeffer, B. A., Horwitz, J., and Bok, D. (1990) *Exp. Eye Res.* **51**, 717–728
14. Carlson, A., and Bok, D. (1992) *Biochemistry* **31**, 9056–9062
15. Crouch, R. K., Hazard, E. S., Land, T., Wiggert, B., Chader, G., and Corson, W. (1992) *Photochem. Photobiol.* **56**, 251–255
16. Okajima, T.-I. L., Wiggert, B., Chader, G. J., and Pepperberg, D. R. (1994) *J. Biol. Chem.* **269**, 21983–21989
17. Chen, Y., Saari, J. C., and Noy, N. (1993) *Biochemistry* **32**, 11311–11318
18. Chen, Y., and Noy, N. (1994) *Biochemistry* **33**, 10658–10665
19. Adler, A. J., Evans, C. D., and Stafford, W. F., III (1985) *J. Biol. Chem.* **260**, 4850–4855
20. Cogan, U., Kopelman, M., Mokady, S., and Shinitzky, M. (1976) *Eur. J. Biochem.* **65**, 71–78
21. Saari, J. C., and Bredberg, L. (1988) *Exp. Eye Res.* **44**, 569–578
22. Dittmer, J. C., and Wells, M. A. (1989) *Methods Enzymol.* **14**, 482–530
23. Noy, N., and Xu, Z.-J. (1990) *Biochemistry* **29**, 3878–3883
24. Noy, N., and Xu, Z.-J. (1990) *Biochemistry* **29**, 3883–3888
25. Noy, N., and Blaner, W. S. (1991) *Biochemistry* **30**, 6380–6386
26. Ho, M.-T. P., Massey, J. B., Pownall, H. J., Anderson, R. E., and Hollyfield, J. G. (1989) *J. Biol. Chem.* **264**, 928–935
27. Ross, P. D., and Subramanian, S. (1981) *Biochemistry* **20**, 3096–3102
28. Borst, D. E., Redmond, T. M., Elser, J. E., Gonda, M. A., Wiggert, B., Chader, G. J., and Nickerson, J. M. (1989) *J. Biol. Chem.* **264**, 1115–1123
29. Feng S.-L., and Bridges, C. D. B. (1988) *J. Biol. Chem.* **263**, 15330–15334