Retinol-Binding Site in Interphotoreceptor Retinoid-Binding Protein (IRBP): A Novel Hydrophobic Cavity

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PURPOSE. Interphotoreceptor retinoid-binding protein (IRBP) appears to target and protect retinoids during the visual cycle. X-ray crystallographic studies had noted a ββα-spiral fold shared with crotonases and C-terminal protein transferases. The shallow cleft formed by the fold was assumed to represent the retinol-binding site. However, a second hydrophobic site consisting of a highly restricted cavity was more recently appreciated during in silico ligand-docking studies. In this study, the ligand-binding environment within the second module of Xenopus IRBP (X2IRBP) is defined.

METHODS. Pristine recombinant polypeptide corresponding to X2IRBP was expressed in a soluble form and purified to homogeneity for its ligand-attachment capacity. Rhodopsine was subjected to 11-cis and all-trans fluorescence enhancement, quenching of tryptophan fluorescence, and energy transfer. The effect of oleic acid on retinol binding was also examined.

RESULTS. A ligand-binding stoichiometry of ~1:1 was observed for 11-cis and all-trans with Kd in the tens of nanomolar range. The substitution mutants showed little effect on retinol binding in titrations after fluorescence enhancement. However, the W450F and not the W587F mutant showed a markedly reduced capacity for fluorescence quenching for both 11-cis and all-trans retinol. Oleic acid inhibited the binding of 11-cis and all-trans retinol in an apparent noncompetitive manner.

CONCLUSIONS. The binding site for 11-cis and all-trans retinol is a novel hydrophobic cavity that is highly restrictive and probably distinct from the long chain fatty acid-binding site. (Invest Ophtalmol Vis Sci. 2009;50:5577–5586) DOI:10.1167/iovs.08-1857

Vision begins with the photoisomerization of 11-cis to all-trans retinal bound to rhodopsin.1,2 The 11-cis isomer is returned to the outer segments through the "visual cycle."3–5 The cycle utilizes the retinal pigmented epithelium (RPE) and Müller cell glia to enzymatically reconvert all-trans retinol to an 11-cis isomer. The RPE returns 11-cis retinol to the rod outer segments, although the Müller cells, at least in some species, return 11-cis retinol to the cones, which are able to oxidize it to 11-cis retinal.6–10 Interphotoreceptor retinoid-binding protein (IRBP), which is the major soluble protein component of the interphotoreceptor matrix, facilitates the exchange of these retinoids between the photoreceptors, RPE, and Müller cells (reviewed in Refs. 1–3,11–15). Disruption of a phylogenetically conserved salt bridge within IRBP has been associated with a form of recessive retinitis pigmentosa.16 IRBP carries visual-cycle retinoids in a light-dependent manner, functions to remove all-trans retinol from bleached rod outer segments,17–20 delivers all-trans retinol to the RPE,21 releases 11-cis retinal from the RPE,22–24 and delivers 11-cis retinol to the rod outer segments.24 Recent studies of irbp−/− mice support a role for IRBP in the translocation of visual retinoids and cone function.25 Finally, IRBP carries endogenous 11-cis retinol, suggesting that it has a role in the cone visual cycle.6,26–27 These processes may be regulated by various fatty acids bound endogenously to IRBP.28 For example, docosahexaenoic acid (DHA) is thought to trigger the release of 11-cis retinal from IRBP to the outer segments.29,30 More recently, oleic acid was found to bind more tightly to IRBP than DHA.31 IRBP therefore functions in a complex process regulated by fatty acids, and possibly receptors, to accomplish a bidirectional transport of visual-cycle retinoids between diverse cell types while protecting their isomeric and oxidative state.32 IRBP, the largest known retinoid-binding protein, consists of two or four homologous "modules" each approximately 300 amino acids in length (four in tetrapods; two in teleosts33–36). The functional significance of this structure, unique among retinoid-binding proteins, is largely unknown. As the individual modules of IRBP appear to represent functional units of the protein, we and others have attempted to understand the structure and function of IRBP by examining the biochemical and functional properties of its individual modules.37–44 X-ray crystallographic analysis of the second module of IRBP suggests a homology with two enzyme families, the C-terminal transferases (CPTases) and crotonases.45 Photosystem II D1 CPTase (D1P), a prototype CPTase with known structure,46 plays a role in the repair of the oxidative damage to the reaction center by renewing the photosystem II D1...
protein. The repair involves the disassembly of the photosynthesis complex with replacement of damaged D1 protein with new protein. In a final step in this new process, D1P catalyzes the C-terminal cleavage of the new D1 protein. Catalysis requires binding of DIP to the hydrophobic C-terminal of the D1 protein. Each module of IRBP is structurally similar to the D1P domains A and C which correspond to the N- and C-terminal X2IRBP domains, respectively. However, the PDZ domain, located between domains A and C conferring this enzymatic activity in DIP, is not present in IRBP. IRBP therefore retains the hydrophobic ligand-binding ability but not the proteolytic activity.

The C-terminal domain B of X2IRBP and domain C of D1P exhibit a structural homology with crotonases (enoyl-CoA hydratase/isomerases), as represented by the known structures of dienoyl-CoA isomerase and chlorobenzoyl-CoA dehydrogenase and enoyl-CoA hydratase. Crotonases have a low sequence identity, but adopt the same overall fold. X2IRBP shares with these enzymes a structural core composed of three helices and the large five-stranded β sheet in domain B forming a βββ-spiral fold. These elements are arranged in a topologically identical order despite peripheral insertions/deletions. However, none of the catalytic residues of the crotonase family are conserved in IRBP.

If we are to understand the mechanism underlying the interaction between IRBP’s retinol- and fatty acid-binding sites and the molecular basis of retinitis pigmentosa caused by a recently described mutation in IRBP, it is critical to define the binding domain(s) for these physiological ligands. The structural homology among X2IRBP, crotonases, and CT-Pases suggested that at least one site corresponds to a shallow cavity, and not the shallow cleft, represents the binding site for retinol in X2IRBP. The rationale for the experimental design takes advantage of the fact that X2IRBP has only two tryptophans each of which is located in one of these two putative ligand-binding domains. Tryptophan, with a fluorescence emission close to the excitation of retinol, provides a sensitive probe of the two sites. This type of tryptophan scanning has been useful in defining the ligand-binding site in cellular retinol-binding protein II and uncovering structure-function relationships in various protein systems (reviewed in Ref. 5). For our studies, recombinant X2IRBP were prepared in a manner that has recently allowed preparation of high-quality crystals of full length Xenopus IRBP. This approach, which extends earlier strategies for preparing X2IRBP, includes providing a reducing environment and removing the fusion tag from the final protein. We provide evidence that the hydrophobic cavity, and not the shallow cleft, represents the binding site for 11-cis and all-trans retinol in X2IRBP.

METHODS

The second module of X2IRBP IRBP (X2IRBP), corresponds to amino-acid residues 313 to 612 in the full-length sequence where the first residue corresponds to the internal methionine of the signaling peptide. The expression construct was prepared by PCR amplifying module 2 from X10a, a full-length Xenopus IRBP cDNA that has been isolated in our laboratory. Ligation-independent cloning (LIC) was used to insert the amplified fragment into pET-30 Xa/LIC (Novagen, San Diego, CA). This vector provides polyhistidine and S-Tag domains, and a factor Xa cleavage site 5′ to the cloning site. During the PCR, we added a tobacco-etched virus (TEV) protease cleavage site (GluAsnLeuTrpPheGln ↓ Gly; bold in the forward primer sequence below) between the Xa cleavage and cloning sites (see Fig. 2A). Cleavage with TEV was efficient and added a single Gly residue to the X2IRBP N terminus. The oligonucleotide primers, synthesized by Integrated DNA Technologies (Coralville, IA), were as follows. Forward primer, GGTATTGAGGTGCCAAAATCTGTTATTTCCAGGCgtgcgt- tacatgtacc (italic, primer extension sequence for vector compatibility; bold, TEV protease recognition site; lowercase, Xenopus-IRBP coding sequence beginning at residue 313); Reverse primer, AGAG- GAGAGTTAGAGCCTTAACgatgttctcctggtcgc (italic, primer extension sequence; bold, stop codon; lowercase, Xenopus-IRBP coding sequence ending with residue 612).

Tryptophan was replaced with phenylalanine or valine at positions 450 and 587. The mutations were introduced directly into the X2IRBP-pET30 construct by using site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA). Briefly, hot-start PCR was performed on a thermocycler (model 2210; Applied Biosystems, Foster City, CA), using high-fidelity DNA polymerase (PfuUltra; Stratagene) for 18 cycles (95°C, 30 seconds; 58°C, 1 minute; 68°C, 7.5 minutes) with HPLC-purified primers. W450F: forward caccttttagattgaatacgctttg- acccataaattgaaaccacakgttg- tgaatctagaaaggtg; W587F: forward catagatagaaatgatcctttggt from the column at 300 to 320 mM NaCl with a 1.0 M NaCl linear gradient over 12 column volumes. The freed X2IRBP, which had little affinity for the immobilized nickel domain cleaved from the X2IRBP with TEV for 16 hours at 4°C. The populated fraction after centrifugation at 43,000g for 30 minutes was subjected to affinity chromatography on a 5-mL nickel column at 6°C (HisTrap; GE Healthcare, Piscataway, NJ). These columns use the tetradeutate chelator Ni-NTA, which has superior Ni2+ affinity to tridentates, and is stable in DTT up to 5 mM. X2IRBP-polyhistidine fusion proteins eluted from the column at ~100 mM imidazole with a 0- to 300-mM imidazole linear gradient over 15 column volumes (pH 7.4). The imidazole was removed by ultrafiltration, and the fusion protein domain cleaved from the X2IRBP with TEV for 16 hours at 4°C. The freed X2IRBP, which had little affinity for the immobilized nickel resin, was separated from residual uncut fusion protein, by reapplication to the column. The protein was further purified by ion-exchange chromatography (resin, 20 mM bed volume; Q Sepharose HP, XK16/20 column; GE Healthcare). X2IRBP eluted from the column at 500 to 320 mM NaCl with a 1.0 M NaCl linear gradient over 12 column volumes. Protein aliquots were held at ~80°C and never refrozen. The concentration of the purified protein was determined by absorbance spectroscopy (extinction 27,590 M−1 cm−1, at 280 nm), and amino acid analysis (AАА). For AAA, the samples were hydrolyzed in vacuo for 16 hours at 115°C with 6 N HCl/0.2% phenol. The acid contains 2 nanomoles norvaline per 100 μL as an internal standard. Analyses were performed with an amino acid analyzer (L-8900PH; Hitachi, Tokyo, Japan) that uses an ion-exchange column to separate the amino acids and a postcolumn reaction with ninhydrin for detection at 570 and 440 nm. AAA also allowed quantification of the number of phenylalanine residues present in the recombinant protein. Although the analysis did not support direct quantification of tryptophan, the ability to measure the increased number of phenylalanines per mole of polypeptide provides support for the presence of the intended amino acid substitutions.

Ligand-Binding Assays by Fluorescence Spectroscopy

All-trans retinol, at a purity of 99% by HPLC, was obtained from Fluka (Buchs, Switzerland). Working stocks were prepared fresh in ethanol and held under argon at ~80°C for no more than 10 days. The
extinction coefficient of all-trans retinol in ethanol was taken as 38,300 M⁻¹ cm⁻¹, 325 nM. 11-cis Retinol was prepared from 11-cis retinal by chemical reduction. In brief, sodium borohydride (4.5 mg, 0.119 mmol) was added to 11-cis retinal (11.1 mg, 39.1 mmol) dissolved in 550 µL of molecular sieve-dried 2-propanol and flushed with argon. After the reaction was stirred at 21°C for 1 hour, most of the solvent was removed in a stream of argon. Water (300 µL) was added dropwise. The product was extracted three times with benzene (1 mL each time). The three benzene phases, which contained the product, were combined. The product in the benzene was dried over MgSO₄ and the solvent removed with reduced pressure under 30°C to yield 7.3 mg (65.2%) of pure 11-cis retinol. The purity of the product was confirmed by HPLC and absorption maximum (318 nm, hexane).

The binding of all-trans and 11-cis retinol to X2IRBP was characterized in titrations using a DM 45 scanning spectrofluorimeter (On-Line Instrument Systems, Inc., Bogart, GA). Titrations monitoring enhancement of retinol fluorescence, quenching of intrinsic protein fluorescence, and energy transfer were performed as previously described. Enhancement

**Figure 1.** Two putative retinol-binding sites within the second module of Xenopus IRBP (X2IRBP). (A) Hydrophobicity surface representation, where white represents high hydrophobicity, and blue represents high hydrophilicity. Within the bracketed area, the arrowhead identifies a shallow hydrophobic cleft. Arrow: an opening located in a hydrophobic patch that leads to a hydrophobic cavity. Modified with permission from Loew A, Gonzalez-Fernandez F. Crystal structure of the functional unit of interphotoreceptor retinoid binding protein. *Structure.* 2002;10(1):43–49. © Cell Press. (B) Stereoimage of computer docking of all-trans retinol in each of the two possible hydrophobic ligand-binding domains. We have previously shown that retinol-binding quenches tryptophan fluorescence in X2IRBP (Gonzalez-Fernandez et al. 2006). There are two tryptophan residues within X2IRBP that could support this quenching. Each of these tryptophans (red) is located in one of the putative ligand-binding domains. W450 is located in the hydrophobic cavity containing a molecule of retinol (yellow); W587 is located in the more shallow hydrophobic cleft containing a molecule of retinol (blue). Image modified with permission from Gonzalez-Fernandez F, Ghosh D. Focus on molecules: interphotoreceptor retinoid-binding protein (IRBP). *Exp Eye Res.* 2006;86:169–170. © Elsevier. The image may be viewed in 3-D without specialized stereo glasses. Suggestions for viewing molecular stereo images are available at http://spdbv.vital-it.ch/TheMolecularLevel/0Help/StereoView.html.
of retinol fluorescence was determined by monitoring the increase in retinol fluorescence (all-trans retinol: excitation, 330 nm; emission, 480 nm; 11-cis retinol: excitation, 324 nm; emission, 475 nm). A fluorescence-matched solution of Nacetyltryptophanamide, which possesses a typical protein fluorescence, but is unable to interact with retinol, was used as a blank. Assays after the quenching of protein fluorescence used excitation and emission wavelengths of 280 and 340 nm, respectively. In these experiments, the inner filter effect was accounted for by graphic correction. Energy transfer assays monitored retinol fluorescence at 480 nm for all-trans retinol and 475 nm for 11-cis retinol (excitation, 280 nm). The dissociation constant ($K_d$) and number of binding sites ($N$) was determined by nonlinear least-squares fit to a binding equation that assumes a single type of noninteracting site(s). The error associated with the determination of binding parameters is given as the mean ± SE. The standard errors are estimates of the uncertainties in the estimates of the regression coefficients (analogous to the SEM). Nonlinear least-squares analysis, determination of the binding parameter SE, determination of $R^2$ (coefficient of determination), and plotting of the fitted curves were performed (SigmaPlot Version 7; SPSS, Chicago, IL). In some experiments (see Fig. 5), each titration run was alternated. That is, during the same experiment, a titration was run with one mutant, followed by the second mutant. This was repeated a total of three times alternating between the two proteins. Collecting the data in this manner eliminates time-dependent systematic error. The data from each mutant was then combined and analyzed, as just described.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933449/) **Figure 2.** X2IRBP and the W450T and W587T mutants were expressed in a soluble form as polyhistidine-fusion proteins. The fusion domain was removed by cleavage with tobacco-etch virus (TEV) protease. (A) Map of the plasmid construct used to express the wild-type and mutant forms of X2IRBP. Arrow: cleavage site in the TEV protease recognition site (GluAsnLeuTyrPheGln Gly). Amino-acid residues are numbered according to the sequence of the full-length Xenopus IRBP. (B) Purification of X2IRBP and removal of its polyhistidine fusion tag. **Lane a:** total soluble E. coli lysate. **Lane b:** proteins binding to the HisTrap-nickel column. **Lane c:** Proteins not binding to a second HisTrap column after TEV cleavage. **Lane d:** Cleaved X2IRBP following Q-Sepharose HP ion-exchange chromatography. **Arrows:** positions of the fusion protein before and after TEV cleavage.**

**RESULTS**

Crotonases and CTPases use the same fold to stabilize unique ligands. In the case of CTPases, the fold stabilizes the hydrophobic C-terminal end of a protein substrate. For dienoyl-CoA isomerase, the fold confers an ability to isomerize fatty acids, allowing their entry into the β-oxidation pathway. Superposition of X2IRBP with one of the crotonase superfamily members suggests a similar ligand-binding site. Of note, such comparisons place the ligand-binding site between the two domains of X2IRBP in the βα-spiral fold. Furthermore, an equivalent superposition of 2-enoyl-CoA hydratase with D1P protease also places the ligand into the known active site of D1P. Although the catalytic residues, and the function between the crotonase superfamily and CTPases are not conserved, the location of the substrate-binding domain is. The structural homology between X2IRBP, crotonases and CTPases suggests that at least one binding site in X2IRBP is located in the βα-spiral corresponding to the shallow cleft in Figure 1.

Although computer modeling studies are not inconsistent with this prediction, they also identified a second putative binding site for all-trans retinol. In addition to the βα-spiral fold, retinol could also be docked within a separate “hydrophobic cavity.” Based on their size and hydrophobicity, both of these domains represent candidate retinol-binding sites. The arrow in Figure 1A identifies the entrance to the hydrophobic cavity, and the arrowhead identifies a more open cleft-like domain. The hydrophobic cavity, is more restricted, and lined by a higher percentage of hydrophobic residues compared with the larger, less restrictive cleft-like fold. The “shallow cleft” is large enough to provide a binding site for a long chain fatty-acid molecule like DHA. In contrast, the hydrophobic cavity is not large enough to accommodate these fatty acids, but easily docks all-trans retinol in silico. (Fig. 1B).

**Figure 3.** In silico energy minimization for the tryptophan-to-phenylalanine substitution in each of the two putative retinol-binding sites. The binding pockets were energy minimized in a 4.5-A sphere surrounding the phenylalanine. (A) Hydrophobic cavity showing the W450F substitution docked with all-trans retinol (yellow). (B) Shallow cleft showing the W587F substitution docked with all-trans retinol (purple). In each panel, the native structure is shown in green with the tryptophan in red. The energy-minimized structure is shown in blue, with the orientation of the phenylalanine in blue.
proteins was determined by absorbance spectroscopy and AAA. From the translated amino acid sequence, 12 phenylalanines are predicted to be present in X2IRBP of wild-type sequence. Of those, 11.9 were actually detected by AAA. For both W450F and W587F, the number of phenylalanines would be expected to increase to 13.0. Consistent with this prediction, the number of phenylalanines detected by AAA was 13.2 and 13.4, respectively (±0.15). These studies support the presence of a tryptophan-to-phenylalanine substitution in each of the mutants.

To address whether the purified X2IRBP of wild-type sequence, and the W450F and W587F mutants were in a correctly folded state, we took advantage of the fact that misfolded polypeptides often exist in solutions as protein aggregates.64 Each of the purified recombinant proteins freed of its polyhistidine fusion domain were subjected to analytical size-exclusion chromatography on a calibrated S-200 column. SDS-PAGE was used to analyze the composition of the peaks obtained. Each of the recombinant proteins migrated as a sharp peak at an elution volume consistent with the expected size of the monomer (data not illustrated). Evidence of dimers or significant multimer formation was not found. Since metastable, partially folded states generally lead to protein aggregation, the finding that the protein exists as a monomer in solution suggests that each of the proteins are present in a correctly folded state.65,66

In silico energy minimization was used to anticipate potential local effects of substituting phenylalanine for tryptophan at each site. In Figure 3, the binding pockets were energy minimized in a 4.5-Å sphere surrounding the phenylalanine. Superimposition of the X2IRBP structure with the energy-minimized mutants showed little alteration of the environment of the ligand-binding pocket. No increased steric hindrance was appreciated, suggesting that the substitution would not affect the ability of the sites to accommodate a molecule of all-trans retinal. This finding does not rule out an effect on protein folding. However, as discussed earlier, misfolding is unlikely, given the monodispersity of the mutants in solution.

Fluorescence spectroscopy allows noninvasive monitoring of ligand binding by monitoring changes in quantum yield.61 Observing the binding of all-trans or 11-cis retinal took advantage of such changes concomitant with ligand entry into the binding pocket. The amino-acid residues lining the two pockets have been tabulated.58 The hydrophobic environment of the sites support retinal fluorescence enhancement. The quenching of endogenous protein fluorescence on ligand binding is supported by the presence of tryptophan. As shown in Figure 1, the entire X2IRBP contains only two tryptophan residues. Each is located in the vicinity of one of the ligand-binding pockets. The average ring center-to-center distances between tryptophan and the docked retinol are 5.2 and 11.9 Å for the hydrophobic cavity and the shallow cleft, respectively. In contrast, W450 is 32 Å from the retinol in the shallow cleft; W587 is 20 Å from the retinol in the hydrophobic cavity.

To determine whether the hydrophobic cavity, or the shallow cleft represents the principal ligand-binding site, we took advantage of the modeling indicating that the bound retinol would be near a tryptophan residue in either site. W450 is located in the hydrophobic cavity and W587 is located in the shallow cleft (Fig. 1B). In addition to the titrations with X2IRBP of wild-type sequence, Figure 4 superimposes data for the binding of all-trans retinol to the tryptophan-to-phenylalanine substitution mutants. All-trans retinal binding was observed by monitoring the enhancement of retinol fluorescence (excitation 330 nm, emission 480 nm) and quenching of tryptophan fluorescence (excitation 280 nm, emission 340 nm). The amount of enhancement was determined by subtracting the

### Table 1. Summary of All-trans Retinal Binding to X2IRBP and Its Tryptophan to Phenylnalanine Substitution Mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sites (N)</th>
<th>K&lt;sub&gt;e&lt;/sub&gt; (μM)</th>
<th>Enhancement</th>
<th>Quenching</th>
<th>Energy Transfer</th>
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<td>X2IRBP</td>
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<tr>
<td>W450F</td>
<td>N</td>
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<td>0.033 ± 0.008</td>
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</tr>
<tr>
<td>W587F</td>
<td>N</td>
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</table>

Calculated parameters were derived from titrations illustrated in Figure 4, except for those indicated by †, which correspond to titrations in Figure 5 where the protein used came from a separate expression and purification. ND, not done.

† Not calculated, insufficient quenching.
nonspecific fluorescence changes measured by titration of all-trans retinol into a fluorescence-matched NAT solution (data not shown). NAT, which has protein-like fluorescence from its indole ring, does not significantly interact with all-trans retinol, making it an ideal control solution.62 The number of binding sites per molecule of X2IRBP of wild-type (WT) sequence was calculated (see the Methods section) to be \(N = 1.27 \pm 0.03\), with \(K_d = 0.07 \pm 0.02\) \(\mu\)M. Figure 4B shows tryptophan fluorescence quenching, giving \(N = 1.26 \pm 0.02\) with \(K_d = 0.008 \pm 0.003\) \(\mu\)M (WT). Inner filter effect was accounted for as described by Mertens and Kagi.63 The plot in Figure 4C shows the energy transfer, giving \(N = 1.26 \pm 0.02\) with \(K_d = 0.033 \pm 0.008\) \(\mu\)M. These titrations suggest that there is at least one binding site for all-trans retinol in X2IRBP.

Titrations monitoring all-trans retinol fluorescence enhancement showed significant binding for both mutants (Table 1). In contrast, titrations monitoring tryptophan quenching detected a site only in W587F (0.97 \(\pm\) 0.02). No significant quenching was observed with the W450F mutant (Fig. 4B). Measurements of energy transfer detected approximately one site in both mutants.

Figure 5 shows the titrations, which were performed in a side-by-side manner to directly compare the mutants during the same experiment. In the experiments depicted, each of the titration runs for W450F and W587F were alternated in the same experiment, with a total of three titrations performed for each mutant. The mutants showed a marked difference in

**FIGURE 6.** Binding of 11-cis retinol to X2IRBP of wild-type sequence. The titrations monitored the enhancement of retinol fluorescence (excitation 330 nm, emission 480 nm) and quenching of endogenous protein fluorescence (excitation 280 nm, emission 340 nm). Enhancement: \(N = 0.95 \pm 0.03\) and \(K_d = 0.077 \pm 0.015\) \(\mu\)M. Quenching: \(N = 0.93 \pm 0.02\) and \(K_d = 0.042 \pm 0.009\). The calculated number of binding sites and dissociation constants are summarized in Table 2.

**FIGURE 7.** 11-cis Retinol binding to mutants W450F (○) and W587F (●). (A) Binding determined by monitoring the enhancement of 11-cis fluorescence (excitation 324 nm, emission 475 nm). (B) Binding determined by monitoring quenching of tryptophan fluorescence (excitation 280 nm, emission 340 nm). Fluorescence intensity is normalized relative to the maximum amount of quenching. Calculated number of binding sites and dissociation constants are summarized in Table 2.
fluorescence quenching compared to enhancement. Although the binding affinity of the two mutants was subtly altered, the enhancement titration did not detect a significant difference in the ligand-binding stoichiometry. In contrast, the W587F mutant showed a reduction in fluorescence quenching compared with W450F. As in Figure 4, the level of fluorescence quenching observed did not permit meaningful fitting of the binding equation. The calculated number of binding sites and dissociation constants are summarized in Table 1. The data suggest that all-trans retinol fluorescence quenching is markedly reduced when the tryptophan residue (W450) located in the hydrophobic cavity, but not in the shallow cleft (W587), is replaced with phenylalanine. The fact that all-trans retinol binding can still be measured by fluorescence enhancement suggests that the binding site remains intact in both mutants.

To address the possibility that one of the sites might be specific for the 11-cis and the other for the all-trans isomer of retinol, we carried out a second set of titrations shown in Figures 6 and 7. Figure 6 shows the binding of 11-cis retinol to X2IRBP of wild-type sequence. The superimposed titrations appear symmetrical in shape, and the calculated parameters were similar (by enhancement $N = 0.95 \pm 0.03$, and $K_d = 0.077 \pm 0.015 \mu M$; by quenching $N = 0.93 \pm 0.02$, and $K_d = 0.042 \pm 0.009 \mu M$). Titrations attempting to monitor energy transfer were not sensitive enough to obtain reliable data, because 11-cis retinol is not as fluorescent as all-trans retinol.

The results were similar for the two mutants when fluorescence enhancement was used to follow 11-cis retinol binding (Fig. 7A). One binding site with similar dissociation constants was detected in each mutant (Table 2). In contrast, when tryptophan quenching was used to monitor 11-cis retinol binding, the results were different (Fig. 7B). For W587F, values of $N = 0.77 \pm 0.02$, with $K_d = 0.024 \pm 0.010$ were obtained. However, for W450F, the quenching was not sufficient to obtain a meaningful fit of the ligand-binding equation. In silico docking shows that the $\beta$-ionone rings of both 11-cis and all-trans retinol are centered within the hydrophobic cavity (Fig. 8). Their side chains extend from the cavity into a less hydrophobic environment. Although the local environments of the side chain show some differences, the binding site can accommodate both retinol isomers. Taken together, the results suggest that 11-cis and all-trans retinol bind in the hydrophobic cavity.

The known ability of X2IRBP to bind fatty acids is not easily explained by an interaction with the hydrophobic cavity, because the cavity is not large enough to accommodate a molecule the size of DHA or oleic acid. To investigate, we performed 11-cis and all-trans retinol titrations with X2IRBP in the presence of oleic acid ranging in concentration from 0.67 to 2.66 $\mu M$. The titrations, which monitored retinol fluorescence enhancement, are shown in Figure 9. For both 11-cis and all-trans retinol, the presence of oleic acid depressed the degree of fluorescence enhancement for retinol without altering significantly the inflection point of the binding–titration curve.

**TABLE 2. Summary of 11-cis Retinol Binding to X2IRBP and Its Tryptophan to Phenylalanine Substitution Mutants**

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<tr>
<td></td>
<td>$K_d$</td>
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<td>0.042 ± 0.009</td>
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<tr>
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<td></td>
<td>$K_d$</td>
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<tr>
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* No significant quenching was observed.

**DISCUSSION**

Our reductionist approach of examining an individual module has the disadvantage of not taking into account the complete quaternary structure of the protein. However, focusing on the individual module reduces the complexity of the system, allowing insight into structure–function relationships that would be difficult to obtain in the full-length protein. Recently, we established conditions to produce pristine full-length Xenopus IRBP that supports the formation of diffraction-quality crystals. We extend those studies to X2IRBP, by including DTT during the purification and concentration processes and introducing a TEV protease cleavage site allowing efficient removal of the fusion tag. These improvements afforded routine preparation of soluble X2IRBP free of fusion tags. Taking advantage of the fact that there is one tryptophan within each of the two putative retinol-binding pockets, we used site-directed mutagenesis and fluorescence spectroscopy to provide evidence that the hydrophobic cavity, and not the $\beta$-barrel fold, as previously thought, represents the binding site for both 11-cis and all-trans retinol.

X-ray crystallographic studies of X2IRBP have pointed to two hydrophobic domains representing candidate ligand-binding sites. In the original report of the X2IRBP structure, particular attention was given to a shallow cleft (see Fig. 1). Of interest, this cleft, which is formed by the $\beta$-barrel fold and also used by IRBP’s relatives, the crotonases and CPTases, to bind diverse hydrophobic ligands, was considered to be the most promising candidate for the retinol-binding site. However, in silico docking studies called attention to a second more hydrophobic and restricted hydrophobic cavity. There appears to be only one entrance into the cavity as illustrated (Fig. 1). This cavity, which may have structural differences between the modules, can accommodate a molecule of all-trans retinol, but is too small to accommodate a long chain fatty acid such as DHA. In the present study, we took advantage of the fact that, according to the crystal structure, both the hydrophobic cavity and shallow cleft contain a single tryptophan.
residue, W450 and W587, respectively. To probe these sites we substituted phenylalanine for tryptophan at each of these position. Phenylalanine, provides a highly conservative substitution. In contrast, the less conservative valine substitutions appear to destabilize the protein promoting insoluble expression.

Substituting phenylalanine for W450 and W587 does not significantly perturb 11-cis or all-trans retinol binding when monitoring retinol-fluorescence enhancement. However, the ability to support quenching was markedly reduced in the W450F mutant. In contrast, the W587F mutant showed little change, even when binding was determined by monitoring fluorescence quenching. These findings suggest that the hydrophobic cavity, not the shallow cleft, is the primary binding site for both 11-cis and all-trans retinol. That substitution of phenylalanine for W450 and W587 does not significantly perturb the overall structure of the ligand-binding site is supported by the energy-minimization studies and the fact that X2IRBP and the mutants were expressed and purified in a soluble monodispersed form. The effect of the substitution could be further addressed in future x-ray crystallographic studies.

The shallow cleft is a good candidate for a fatty acid-binding site. Previous studies have shown that X2IRBP binds the fluorescent fatty-acid analogue 9-(9-anthroyloxy) stearic acid with stoichiometry of N = 1.49 ± 0.15. Caution is warranted in interpreting these data, because the effect of the fluorescent tag is unknown. A recent study has measured the binding of fatty acids particularly to purified bovine IRBP by observing protein fluorescence quenching. However, we were not successful in using quenching to monitor DHA or oleic acid binding to X2IRBP. This is probably related to sensitivity, as these fatty acids do not absorb well at 350 nm making quenching measurements difficult. To examine the interaction of oleic acid with X2IRBP, we monitored the binding of 11-cis and all-trans retinol in the presence of various concentrations of oleic acid. We found that oleic acid inhibits the binding of both retinols. The inability of higher retinol concentrations to overcome the inhibitory effect suggests that oleic acid inhibits retinol binding through a noncompetitive mechanism. An allosteric mechanism is consistent with the likelihood that the hydrophobic cavity is not large enough to accommodate a molecule of oleic acid. We anticipate that the hydrophobic cavity is the site of 11-cis and all-trans retinol binding and that oleic acid binds within the shallow cleft. This model of separate retinol and fatty acid-binding sites in the same module is similar to that proposed for human IRBP. Ongoing x-ray crystallographic studies of the holoprotein will address this question.

Acknowledgments

The authors thank Andreas Loew for help during the early phases of the work, Tom Loelch for preparing the expression construct, Joseph Armstrong for assistance in the initial ligand-binding studies, and Myron Crawford and Fernando Pineda (Keck Center, Yale University, New Haven, CT) for performing the amino acid analyses.

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