Bindin Specificities of Cellular Retinol-binding Protein and Cellular Retinol-binding Protein, Type II*

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Cellular retinol-binding protein (CRBP) and cellular retinol-binding protein, type II (CRBP(II)) are cytoplasmic proteins that bind trans-retinol as an endogenous ligand. These proteins are structurally similar having greater than 50% sequence homology. Employing fluorometric, absorption, and competition studies, the ability of pure preparations of CRBP(II) and CRBP to bind various members of the vitamin A family has been examined. In addition to trans-retinol, CRBP(II) was able to form high affinity complexes (Kd < 5 x 10^-8 M) with 13-cis-retinol, 3-dehydroretinol, and all-trans-retinaldehyde. CRBP bound those retinol isomers with similar affinities, but did not bind trans-retinaldehyde. Neither protein bound retinoic acid nor 9-cis- and 11-cis-retinol. The spectra of 13-cis-retinol and 3-dehydroretinol, when bound, were shifted and displayed fine structure compared to their spectra in organic solution. However, the Amax and fluorescent yield of a particular ligand were different when bound to CRBP(II) versus CRBP. It appears that CRBP(II) and CRBP bind trans-retinol, 13-cis-retinol, and 3-dehydroretinol in a planar configuration. However, the binding sites of CRBP(II) and CRBP are clearly distinct based on the observed spectral differences of the bound ligands and the observation that only CRBP(II) could bind trans-retinaldehyde. The ability of CRBP(II) to bind trans-retinaldehyde suggests a physiological role for the protein in accepting retinaldehyde generated from the cleavage of β-carotene in the absorptive cell.

Vitamin A is an essential nutrient necessary for vision, growth, reproduction, and maintaining the normal differentiated state of epithelial cells (reviewed in Ref. 1). The mechanisms of action of vitamin A in vision and in differentiation remain topics of considerable research interest. Efforts of the past decade have revealed several distinct cytoplasmic proteins that bind various members of the vitamin A family (reviewed in Ref. 2). These proteins solubilize and protect their ligands within the aqueous environment of the cytoplasm and may be involved in the transport of various retinoids to sites of action and/or metabolism.

Cellular retinol-binding protein, type II (CRBP(II)) and cellular retinol-binding protein (CRBP) are two members of this class of vitamin A-binding proteins. They are structurally similar having greater than 50% sequence homology (3). The endogenous ligand of both CRBP(II) and CRBP has been identified as all-trans-retinol (4–6). CRBP is present in a wide variety of tissues (7) but only the small intestine contains significant levels of CRBP(II) in the adult rat (4). Immunohistochemical studies have localized CRBP(II) to the mature absorptive cell of the small intestine (8). CRBP was not detected in absorptive cells but was present in minor amounts in other cells of the small intestine. The localization studies suggested that CRBP(II), but not CRBP, is involved in the absorption of vitamin A. The observation that CRBP(II)-retinol is a better substrate than CRBP-retinol for an intestinal retinol esterifying enzyme provides additional support for this putative role (9).

CRBP has both high affinity and high specificity for retinol. Partially purified preparations of CRBP were unable to bind retinaldehyde, retinoic acid, or retinyl esters based on in vitro competition studies, but did have affinity for some geometric isomers of retinol (10). The binding specificity of CRBP(II) has not yet been examined. In the present study we compared the binding specificity of pure preparations of CRBP(II) and CRBP for various compounds in the vitamin A family. Of particular interest was the discovery that CRBP(II), but not CRBP, binds all-trans-retinaldehyde. This suggests an additional role for CRBP(II) during absorption: CRBP(II) may serve as the acceptor for the retinaldehyde generated by oxidative cleavage of β-carotene during the necessary gut metabolism that produces vitamin A.

**EXPERIMENTAL PROCEDURES**

Materials—All-trans-retinol, all-trans-retinaldehyde, and all-trans-retinoic acid were from Sigma. 9-cis-Retinaldehyde, 11-cis-retinaldehyde, and 3-dehydroretinol were generous gifts from Hoffman-La Roche. 13-cis-Retinaldehyde was isolated after intense illumination of a 1.0 mg/ml solution of all-trans-retinaldehyde in hexane (11). 9-cis-, 11-cis-, and 13-cis-Retinol were prepared by reduction of the corresponding aldehydes with sodium borohydride (12). Dimethyl sulfoxide (spectrophotometric grade) was from Aldrich. Hexane and dioxane (both HPLC grade) were from Burdick and Jackson.

*High Performance Liquid Chromatography (HPLC)—All HPLC procedures were performed with a solvent delivery system consisting of an LKB 2152 controller and an LKB 2150 pump equipped with a Valco injection valve (200-μl loop). Elution was routinely monitored with a V, variable wavelength detector (ISCO) connected to a Spectra Physics SP4720 integrator. The stationary phase was a Whatman Partisil 5 (4.6 mm × 25 cm) 5-μm silica column coupled to a 5-μm Supelcosil (2 cm) silica guard column.*

**Purification of Retinoids—**The isomers of retinol, retinaldehyde, and 3-dehydroretinol were routinely purified by HPLC immediately prior to binding analysis. Retinol isomers were purified using a mobile phase of hexane and dioxane (95:5) at a flow rate of 2.0 ml/min (13). All-trans-3-dehydroretinol could be separated from all-trans-retinol using identical conditions. Isomers of retinaldehyde were purified using a mobile phase of hexane containing 2.5% dioxane at a flow rate of 2.0 ml/min.
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rate of 1.0 ml/min (13). Retinoid acid was used without further purification.

Generally between 0.3 and 1 μmol of each isomer were injected onto the column for purification. To minimize light catalyzed isomerization of the retinoids, all purification procedures were monitored in the dark. HPLC purifications were monitored by UV absorbance until the isomer of interest began eluting. The detector was then turned off and the peak was collected in the dark. The isomer was immediately dried under a stream of N₂, redissolved in Me₂SO, stoppered under N₂, and protected from light. Concentrations were determined off and the peak was collected in the isomer of interest began eluting. The detector was then turned onto the column for purification. To minimize light catalyzed isomerization, the detector was then turned off and the column was open only long enough to obtain constant readings (approximately 2 s). Immediately following these measurements, the final tube containing 700 pmol of the retinol isomer was extracted and analyzed by HPLC to determine the degree of isomeric purity (see methods below). Control titrations used ovalbumin at 500 pmol/ml in PBS in place of CRBP(II) and CRBP. All procedures were in the dark.

All-trans-3-dehydroretinol, all-trans-retinoic acid, and the cis/trans isomers of retinyldehyde are essentially nonfluorescent under the conditions employed here. Specific binding of these vitamin A compounds was examined by determining their ability to compete with all-trans-retinol for binding to CRBP(II) and CRBP. Ten separate Me₂SO solutions were prepared containing a constant amount of all-trans-retinol at 275 nmol/ml and increasing amounts of competitor at 0-2750 nmol/ml. Two milliliters of each of these prepared competitor solutions were transferred to separate plastic tubes. One microliter of apoprotein solution was added to each of these tubes and incubated for 30 min at room temperature. The solutions were transferred to a spectrofluorometric cuvette and the amount of all-trans-retinol bound to the protein was determined in each incubation by measuring the relative fluorescence. Excitation was at 348 and 350 nm (bandpass 2 nm) for CRBP(II) and CRBP, respectively. Emission was monitored at 480 nm. In some cases, the final tube was extracted and analyzed by HPLC using methods described below. All procedures were carried out in the dark.

Determination of Isomer Purity following Binding Analysis—The cis isomers of retinol and retinyldehyde are unstable and can readily isomerize to the all-trans configuration. The purity of each isomer following binding analysis was assessed by extracting the final incubation into hexane and analyzing the extract by HPLC. Briefly, 0.5 ml of an incubation was transferred to a 15-ml glass conical extraction tube. Two milliliters of ethanol were added and vortexed briefly. Following the addition of 8.0 ml of hexane and 2.0 ml of distilled water the solution was vortexed for 30 s. The upper phase was removed, dried under a stream of N₂, and redissolved in 100 μl of HPLC mobile phase. Ninety microliter were injected onto the Partisil 5 column and the isomers were separated using methods described above. The relative contribution of each cis/trans isomer to the total was determined using the integrated peak area.

Determination of Apparent Dissociation Constants—The apparent dissociation constant for the all-trans-retinyldehyde-CRBP(II) complex was determined by titrating the apoprotein with all-trans-retinyldehyde and monitoring the saturable quench of protein fluorescence (16). A 1.0-mI dilution of apoprotein in PBS was prepared in a 2.0-ml spectrophotometric cuvette. All-trans-retinyldehyde in Me₂SO was added in 0.2-0.4-μl aliquots using a 10-μl Hamilton syringe. The increase in emission of bound retinyldehyde was monitored using excitation wavelengths of 348 nm for CRBP(II) and 350 nm for CRBP. The maximum fluorescence at saturation was corrected for free retinoid contributions and compared to standard curves of [CRBP retinol] versus [CRBP retinol] or [CRBP(II) retinol] versus F to determine concentrations of active protein in the protein preparations.

Fluorescence Binding Assays—All fluorescence measurements were on an SLM-Aminco SPF-500C spectrophotometer. The instrument was standardized in the ratio mode with a 1.0 μg/ml solution of quinine sulfate dihydrate in 0.1 M H₂SO₄ (Regis). Excitation was at 350 nm (bandpass 2 nm) and emission was monitored at 450 nm (bandpass 20 nm).

The saturable binding of fluorescent retinoids (the cis/trans isomers of retinol) to pure preparations of apo-CRBP(II) and apo-CRBP was examined using a modification of the fluorescence titration method of Cogan et al. (16). These modifications were designed to minimize isomerization of the cis-retinol isomer toward all-trans-retinol during the experiment. In this procedure, 1.0 ml of either CRBP(II) or CRBP at approximately 250 pmol in PBS was added to seven individual plastic tubes. An increasing quantity (0-700 pmol) of the pure retinol isomer solution in Me₂SO was added to each tube using a 10-μl Hamilton syringe. The volume of Me₂SO was always less than 0.8% of the total volume of each incubation. The solutions were vortexed briefly and incubated for 10 min at room temperature. Each solution was transferred to a 2.0-ml spectrophotometric cuvette and the relative fluorescence of the bound retinol was determined. Emission was monitored at 480 nm (bandpass 20 nm) using an excitation wavelength (bandpass 0.5 nm) of 348 and 350 nm for CRBP(II) and CRBP, respectively. The cuvette was inverted several times then incubated for 30 min at room temperature. The solutions were transferred to a spectrofluorometric cuvette and the amount of all-trans-retinol bound to the protein was determined in each incubation by measuring the relative fluorescence. Excitation was at 348 and 350 nm (bandpass 2 nm) for CRBP(II) and CRBP, respectively. Emission was monitored at 480 nm. In some cases, the final tube was extracted and analyzed by HPLC using methods described below. All procedures were carried out in the dark.

The absorption and fluorescence of bound all-trans-retinyldehyde was determined using methods described above.
of added ligand. At least 2 determinations were made on each protein-ligand complex and reported values are averages.

RESULTS

Ability of Various Retinoids to Compete with All-trans-retinol for Binding to CRBP(II) and CRBP—We first examined the ability of several nonfluorescent vitamin A compounds (retinaldehyde, retinoic acid, and 3-dehydroretinol) to compete with all-trans-retinol for binding to CRBP(II) and CRBP. Increasing quantities of all-trans-retinaldehyde or all-trans-3-dehydroretinol were mixed with a constant, saturating level of all-trans-retinol and incubated with apo-CRBP(II) (Fig. 1, left panel). A concentration-dependent decrease in fluorescence intensity was observed with increasing amounts of all-trans-retinaldehyde or all-trans-3-dehydroretinol. A 2.0-2.5-fold molar excess of these compounds was required to compete for 50% of the retinol binding sites indicating that these retinoids may have a slightly lower affinity for CRBP(II) than all-trans-retinol. Alternatively, the greater excess required may reflect different rates of association of the protein with the several retinoids examined. It is possible that true equilibrium was not reached in these experiments.

All-trans-retinoic acid had little if any ability to compete. Competition with a 2-3-fold molar excess of retinoic acid decreased observed retinol fluorescence by only 5%. At high concentrations of retinoic acid (5.5 nmol/ml), up to 20% decreases in retinol fluorescence were occasionally observed. However, this compared to decreases of over 80% observed with similar concentrations of all-trans-retinaldehyde or 3-dehydroretinol.

When these same compounds were examined for their ability to compete with all-trans-retinol for binding to apo-CRBP (Fig. 1, right panel), only all-trans-3-dehydroretinol was able to produce a similar concentration-dependent decrease in bound retinol fluorescence. A 1-fold molar excess of 3-dehydroretinol produced a 50% reduction in fluorescence indicating that all-trans-retinol and 3-dehydroretinol may have similar affinities for CRBP. Consistent with previous reports with partially purified CRBP (10), all-trans-retinaldehyde and all-trans-retinoic acid showed no ability to compete with all-trans-retinol for binding to CRBP.

The observed loss of retinol fluorescence with increasing concentrations of retinaldehyde and/or 3-dehydroretinol was not due to a nonspecific quench of bound retinol fluorescence.

This statement is made on the basis of several observations. Retinoic acid, retinaldehyde, and 3-dehydroretinol have similar spectral properties, yet concentration-dependent decreases were not observed when retinoic acid was utilized in this competition assay. Retinaldehyde produced a reduction in retinol fluorescence when added to CRBP(II), but did not produce a similar effect with CRBP. Retinaldehyde, retinoic acid, and 3-dehydroretinol did not quench retinol fluorescence in organic solution (data not shown).

The possibility existed that CRBP(II) might have two separate binding sites, one specific for retinol, the other for retinaldehyde. The above results might then be explained if binding of both retinaldehyde and retinol by the same protein molecule would quench retinol fluorescence. However, the full fluorescence characteristic of bound retinol could be recovered in the above experiments by adding additional retinol to the protein solution containing a 10:1 molar ratio of retinaldehyde:retinol. This strongly suggested the retinol was displacing the retinaldehyde from a single binding site and was not consistent with two separate, specific sites.

Demonstration of Retinaldehyde Binding to CRBP(II) by Monitoring Quench of Protein Fluorescence—Decreases in the intrinsic protein fluorescence of CRBP(II) and CRBP were observed upon binding of all-trans-retinol. Binding constants can be determined by monitoring the quench of protein fluorescence during titration as has been done for retinol-binding protein and retinoic acid (16). This technique was used to determine an apparent dissociation constant for the CRBP(II)-all-trans-retinaldehyde complex and to confirm the binding specificity of CRBP(II). Fig. 2 shows the quench of protein fluorescence (corrected for the Me2SO background) when increasing amounts of either all-trans-retinol, all-trans-retinaldehyde, or all-trans-retinoic acid are added to a preparation of apo-CRBP(II). A concentration-dependent, saturable decrease in the protein fluorescence of apo-CRBP(II) was observed upon addition of increasing amounts of either all-trans-retinol or all-trans-retinaldehyde. No effects beyond that of Me2SO alone was observed when CRBP(II) was titrated with retinoic acid. These observations confirm the results of the competition studies. When the data was analyzed by the method of Cogan et al. (16), apparent dissociation constants of approximately 10 nm were obtained for both the all-trans-retinol-CRBP(II) and all-trans-retinaldehyde-CRBP(II) complexes (Fig. 2, inset). Thus, the competition experiments described in the previous section may not have provided equilibrium values.

Determination of the Specificity of CRBP(II) for cis Isomers of Retinaldehyde—The competition studies with retinaldehyde and CRBP(II) were extended to include several monocis isomers of retinaldehyde including 9-cis-, 11-cis-, and 13-cis-retinaldehyde. The cis isomers of retinaldehyde were examined for their ability to compete with all-trans-retinol for binding to apo-CRBP(II) by monitoring the decrease in fluorescence of protein-bound retinol (Fig. 3). All-trans-retinaldehyde was the most effective competitor producing a curve similar to the initial experiments of Fig. 1. The cis isomers of retinaldehyde appeared to be less effective with 11-cis-, 13-cis-, and 9-cis-retinaldehyde demonstrating decreasing abilities to reduce the observed fluorescence. However, the cis isomers of retinaldehyde are relatively unstable under these conditions and can readily isomerize to the all-trans configuration. To determine if the apparent competition observed using the cis isomers could be explained by cis/trans isomerization, the final tube of each assay was extracted into hexane and analyzed by HPLC. Over 10% of the 11-cis-retinaldehyde in the final tube had isomerized to all-trans-retinaldehyde,
Protein fluorescence was determined by excitation at 280 nm and determined by quench concentration of 0.16.

Enhancement of fluorescence is indicative of protein-bound all-trans-retinol and 13-cis-retinol for both proteins. This is while only 3.0-3.5% of the 9-cis and 13-cis pools were isomerized. This suggests that the cis isomers of retinaldehyde are not bound significantly by CRBP(II).

Demonstration of the Specificity of CRBP(II) and CRBP for the cis Isomers of Retinol—The fluorescence intensity of all-trans-retinol when bound to CRBP(II) or CRBP is over 50 times higher than when added to solutions of a nonbinding protein such as ovalbumin. Saturable binding of all-trans-retinol to retinol-binding protein (16), CRBP (15), and CRBP(II) (9) has been characterized using a fluorescent titration technique that determines bound retinol. We have used modifications of this technique to investigate the ability of CRBP(II) and CRBP to bind 9-cis-, 11-cis-, and 13-cis-retinol. Increasing amounts of all-trans-retinol or 13-cis-retinol were added to CRBP(II) (Fig. 4, top panel) and CRBP (Fig. 4, bottom panel). A saturable enhancement of retinol fluorescence was observed with increasing concentrations of all-trans-retinol and 13-cis-retinol for both proteins. This enhancement of fluorescence is indicative of protein-bound retinol. Control titrations of ovalbumin solutions were essentially nonfluorescent (data not shown). Extraction and HPLC analysis of the final titration points of the 13-cis-retinol curves revealed less than 15% of the 13-cis-retinol had isomerized to all-trans. Thus, the fluorescence observed at each point was primarily due to protein-bound 13-cis-retinol. Although the number of data collection points had to be limited in order to reduce light catalyzed isomerization to all-trans-retinol, an apparent dissociation constant of 40 nM was estimated for both the CRBP(II)-13-cis-retinol and CRBP-13-cis-retinol.

**Fig. 2.** Binding of retinol and retinaldehyde to CRBP(II) determined by quench of protein fluorescence. Apo-CRBP(II) was prepared by acetone precipitation and diluted to a final concentration of 0.16 μM in 0.14 M NaCl, 0.01 M potassium phosphate, pH 7.4. Increasing quantities of all-trans-retinol (O), all-trans-retinaldehyde (.), or all-trans-retinol acid (■) were added in 0.2-0.4-μl aliquots of Me2SO to 1.0 ml of apo-CRBP(II). After each addition, the titration solution was gently stirred, incubated for 3 min, and protein fluorescence was determined by excitation at 280 nm and monitoring emission at 350 nm. All procedures were in the dark at room temperature. All points were corrected for Me2SO solvent effects. The protein quench saturation curve for all-trans-retinaldehyde was linearly transformed by the method of Cogan (16) (inset).

**Fig. 3.** Ability of the geometrical isomers of retinaldehyde to compete with retinol for binding to CRBP(II). All-trans-retinaldehyde (C), 13-cis-retinaldehyde (□), 11-cis-retinaldehyde (■), and 9-cis-retinaldehyde (△) were examined for their ability to compete with all-trans-retinol for binding to CRBP(II) by methods identical as described in the legend of Fig. 1. The final point of the cis isomer curves were corrected for the potential competition caused by contaminating all-trans-retinaldehyde (represented by open symbols with cross). The broken line illustrates cis isomer competition curves corrected for all-trans-retinaldehyde contamination.

**Fig. 4.** Binding of retinol isomers to CRBP(II) or CRBP. Pure preparations of CRBP(II) or CRBP were diluted to 0.31 or 0.29 μM, respectively, in 0.14 M NaCl, 0.01 M potassium phosphate, pH 7.4. The dilutions were irradiated with UV light to destroy endogenous retinol. HPLC pure retinol isomers were dissolved in Me2SO at 80-100 nmol/ml. All-trans-retinol (O), 13-cis-retinol (□), 11-cis-retinol (■), or 9-cis-retinol (△) were added to increasing quantities to separate 1.0-ml aliquots of UV-treated CRBP(II) (top panel) or CRBP (bottom panel). The solutions were incubated at room temperature for 5 min. Bound retinol was determined by monitoring emission at 480 nm (bandpass 20 nm) with excitation (band pass 0.5 nm) at 348 and 350 nm for CRBP(II) and CRBP, respectively.
cis-retinol complexes. Comparable values for the all-trans-retinol-CRBP and CRBP(II) complexes were obtained by applying this technique. These determinations should be considered less precise than when more data points could be collected, as shown in Fig. 2, for example. However, these observations suggest that 13-cis-retinol and all-trans-retinol have similar affinities for these proteins.

The 9-cis and 11-cis isomers of retinol showed little fluorescence enhancement in the presence of CRBP(II) and CRBP (Fig. 4) compared to ovalbumin control solutions. The minor amount of fluorescence observed above ovalbumin control for these isomers was fully accounted for by isomerization to all-trans-retinol, determined by extraction of the final titration point into hexane and analysis of the extract by HPLC. It seems unlikely that 9-cis-retinol and 11-cis-retinol bind to CRBP(II) or CRBP but do not experience enhanced fluorescence. However, if that were the case, binding would be revealed by using these isomers as competitors for all-trans-retinol binding and observing the 13-cis-retinol fluorescence in fluorescence. The presence of 10-fold greater amounts of 9-cis-retinol and 11-cis-retinol did not reduce fluorescence when all-trans-retinol was provided for binding, confirming that the isomers do not bind to CRBP(II) and CRBP (data not shown).

Comparison of the Spectral Properties of 13-cis-Retinol and All-trans-retinol in Ethanol and Bound to Protein—The spectral properties of all-trans-retinol in organic solvents are markedly different from its properties when complexed to CRBP(II) and CRBP. To confirm that 13-cis-retinol was specifically bound by CRBP(II) and CRBP, we examined the fluorescence excitation spectra of 13-cis-retinol in ethanol, complexed to CRBP(II), and complexed to CRBP (Fig. 5). As a free ligand in ethanol, 13-cis-retinol has a rather broad, featureless excitation spectrum with a $\lambda_{\text{max}}$ of 327 nm (Fig. 5, bold line in left panel). The excitation spectrum of 13-cis-retinol in the presence of CRBP(II) (Fig. 5, bold line in middle panel) or CRBP (Fig. 5, bold line in right panel) was red shifted and displayed vibronic fine structure. In addition, a 280-nm peak appeared that apparently represents energy transfer from tryptophan residues to the cis-retinol in fluorescence.

As previously discussed for trans-retinol-CRBP (15), the fluorescence intensity of 13-cis-retinol in complex with CRBP(II) and CRBP is enhanced 20-fold over its fluorescence in ethanol. These observations are similar to the spectral changes observed when all-trans-retinol binds specifically to CRBP(II) (4) or CRBP (15) and illustrated in Fig. 5 (thin line spectra).

Interesting spectral differences between the CRBP(II) and CRBP complexes are apparent in Fig. 5. A reproducible 2-nm difference in the $\lambda_{\text{max}}$ of all-trans-retinol complexed to CRBP(II) versus CRBP (348 versus 350 nm) has been previously reported (4) and is repeated here. This characteristic difference was also observed when 13-cis-retinol bound to CRBP(II) and CRBP. The CRBP(II)-13-cis-retinol complex had a $\lambda_{\text{max}}$ of 350 nm while the CRBP-13-cis-retinol complex displayed maximal fluorescence when excited at 352 nm. Thus, the spectra of both 13-cis-retinol and all-trans-retinol were red shifted 23 nm when bound to CRBP(II) and 25 nm when bound to CRBP. Finally, the fluorescence intensity of all-trans-retinol was 2-fold greater in the CRBP(II) complex than in the CRBP complex. The different intensities were also apparent in the titration curves of Fig. 4 and in standard curves of retinol-protein concentration versus fluorescence (data not shown).

Spectral Properties of 3-Dehydroretinol Complexed to CRBP(II) and CRBP—3-Dehydroretinol was an effective competitor of all-trans-retinol for binding to CRBP(II) or CRBP (Fig. 1). The spectral properties of 3-dehydroretinol were examined in the presence of CRBP(II) or CRBP to confirm binding. The absorbance spectrum of 3-dehydroretinol was red shifted 26 or 28 nm, respectively, compared to the spectrum of the free ligand in ethanol (Fig. 6B). The characteristic 2-nm difference between the $\lambda_{\text{max}}$ of a ligand bound to CRBP(II) and CRBP was preserved. Both spectra of bound 3-dehydroretinol displayed vibronic fine structure. The estimated extinction coefficients of 3-dehydroretinol complexed to CRBP(II) or CRBP were 45,000 $M^{-1} \text{cm}^{-1}$ and 48,000 $M^{-1} \text{cm}^{-1}$, respectively. These values are 10–15% higher than published values of 41,000 $M^{-1} \text{cm}^{-1}$ for the free ligand in ethanol (14). The absorbance spectrum of all-trans-retinol complexed to either CRBP(II) or CRBP is also red shifted, displays vibronic fine structure.

![Fig. 5. Fluorescence excitation spectra of all-trans-retinol and 13-cis-retinol in ethanol, complexed with CRBP(II) or CRBP. All excitation spectra were examined by monitoring retinol fluorescence at 480 nm. Left panel, excitation spectra of HPLC pure all-trans-retinol (thin line) and 13-cis-retinol (bold line), each at 2.0 $\mu$M in ethanol, were corrected for the ethanol blank spectrum. Middle panel, approximately 420 pmol of all-trans-retinol (thin line) or 13-cis-retinol (bold line) were added in 2.0 $\mu$l of Me$_2$SO to 1.0 ml of UV-irradiated CRBP(II) (0.20 $\mu$M). The solution was incubated in the dark for 5 min before spectra were run. Right panel, approximately 420 pmol of all-trans-retinol (thin line) or 13-cis-retinol (bold line) were added in 2.0 $\mu$l of Me$_2$SO to 1.0 ml of UV-irradiated CRBP, 0.18 $\mu$M. The solution was incubated in the dark for 5 min before spectra were run.](image1)

![Fig. 6. Absorption spectra of 3-dehydroretinol in ethanol, complexed to CRBP(II) or to CRBP. The absorption spectra of the CRBP(II)-3-dehydroretinol complex (14 $\mu$M) (panel A, bold line) and the CRBP-3-dehydroretinol complex (5.8 $\mu$M) (panel A, thin line) were examined. Also shown is the absorption spectrum of HPLC pure 3-dehydroretinol (19 $\mu$M) in ethanol (panel B, thin line). A difference spectrum representing 3-dehydroretinol bound to CRBP(II) (panel B, bold line) was obtained by adding 8.6 nmol of pure 3-dehydroretinol to a 1.0-ml preparation of UV-irradiated CRBP(II) (27 $\mu$M). The reference cuvette contained the identical UV-irradiated CRBP(II) preparation.](image2)
structure, and exhibits a higher extinction coefficient compared to its spectrum in organic solutions (4, 15). On the basis of these spectral changes, it has been proposed that all-trans-retinol is held in a fully planar configuration in the CRBP(II)-retinol and CRBP-retinol complexes (4, 15). It appeared that 3-dehydroretinol also was binding in a rigid planar configuration to CRBP(II) and CRBP.

The bottom panel of Fig. 6 compared the spectral properties of 3-dehydroretinol as a free ligand in ethanol to its properties as a bound moiety. The bold line is a difference spectrum in which the reference cuvette contains apo-CRBP(II) and the sample cuvette contained the identical protein solution, but with a subsaturating amount of 3-dehydroretinol. A significant decrease in absorbance was observed in the 280-290-nm region of the spectrum comparing bound to free ligand. Only a minor shift of 3-4 nm to the red was observed in this region of the bound spectrum. The absorbance at 290-299 has been attributed to the partial chromophore of the ring when it is out of plane with the double-bonded side chain (14).

Absorbance Spectrum of the CRBP(II)-All-trans-retinaldehyde Complex — The absorbance spectrum of the CRBP(II)-all-trans-retinaldehyde complex was also examined for spectral changes (Fig. 7, bold line). The spectrum of all-trans-retinaldehyde complexed to CRBP(II) was shifted approximately 25 nm to the red and was noticeably broader than its spectrum in hexane (Fig. 7, thin line). Viscous fine structure was not observed in the CRBP(II)-retinaldehyde spectrum, suggesting that retinaldehyde does not fit into the binding site of CRBP(II) in the same manner as retinol. The extinction coefficient of all-trans-retinaldehyde in the complex was estimated by adding subsaturating amounts of retinaldehyde dissolved in Me2SO to CRBP(II) and measuring retinaldehyde absorbance at 395 nm. The concentration of protein and ligand were chosen such that greater than 99% of added ligand would bound to the protein, even if the true \( K_d \) were as high as 100 nm. Retinaldehyde was added to 20 \( \mu \)M CRBP(II) in two additions to achieve a final concentration of 9.4 \( \mu \)M. The absorbance at 395 nm was determined after each addition. At equilibrium, the concentration of free ligand after the final addition would be only 87 nm or 0.9% of the total retinaldehyde added. An average of two such determinations yielded an extinction coefficient for retinaldehyde complexed to CRBP(II) of 38,000 M\(^{-1}\). This value is significantly lower than the extinction coefficient of retinaldehyde in hexane of 48,000 M\(^{-1}\) (17).

**DISCUSSION**

CRBP(II) and CRBP are structurally related proteins having greater than 50% sequence homology (3). These proteins have been shown to bind all-trans-retinol as a high affinity ligand in vivo (4-6). Here we have compared the abilities of CRBP(II) and CRBP to bind several biologically active vitamin A compounds in vitro. The results of this study demonstrate that CRBP(II) and CRBP have quite similar binding properties and specificities for several isomers of retinol, suggesting that these proteins have very similar binding sites. However, an important difference between these proteins was the ability of CRBP(II), and not CRBP, to bind all-trans-retinaldehyde.

The ability of CRBP(II) to bind retinaldehyde may have considerable significance. The primary sources of vitamin A in the diet are retinyl esters and plant pigments such as \( \beta \)-carotene. Retinyl esters are hydrolyzed in the lumen of the intestine and the resulting retinol enters the enterocyte (18). \( \beta \)-Carotene is absorbed as such and oxidatively cleaved to all-trans-retinaldehyde within the enterocyte (19, 20). Retinaldehyde is then reduced to retinol (21, 22). Retinol from either source is esterified, and the resulting retinyl esters are incorporated into chylomicrons to exit in the lymph (21, 22). The high levels of CRBP(II) in the enterocyte combined with its ability to bind all-trans-retinaldehyde suggests that CRBP(II) may serve as a cytosolic acceptor for retinaldehyde generated from cleavage of carotenes. This dual capacity to bind trans-retinol and trans-retinaldehyde may circumvent the need for two distinct proteins to handle vitamin A absorption. Similarly, cellular retinaldehyde-binding protein, a cytoplasmic protein of the retinal pigment epithelium and Mueller cell, binds both 11-cis-retinol and 11-cis-retinaldehyde in vivo (6) and permits a facile reduction/oxidation of the bound retinoid by the specific 11-cis-retinol dehydrogenase in the pigment epithelium (24). CRBP(II) may have a similar role in the enterocyte.

The ability to bind retinaldehyde did not seem to indicate a general loss of binding specificity as CRBP(II) was unable to associate with retinoic acid under the conditions employed in this study. Because only minor amounts of retinoic acid are present in most diets, retinoic acid is not a significant source of vitamin A for the animal. If any mediated absorption of retinoic acid does occur, it must be by some other route than one that would utilize CRBP(II).

Liver storage and growth bioassays have demonstrated that all-trans-retinol and 13-cis-retinol are the most biologically potent isomers of retinol (25). The 9-cis, 11-cis-, and various di-cis-retinol isomers show considerably less vitamin A activity in vivo. Previous studies demonstrated that the binding specificity of partially pure preparations of CRBP agreed well with the biological potency of the isomer (10). We have confirmed these observations on pure preparations of CRBP. In addition, CRBP(II) was observed to have binding specificity for the isomers of retinol identical to that of CRBP. CRBP(II) and CRBP were able to form high affinity complexes with all-trans- and 13-cis-retinol. These proteins did not bind the less active 9-cis and 11-cis isomers under conditions employed here. The low biological potency of 9-cis and 11-cis-retinol may arise from their inability to associate with CRBP(II) and/or CRBP. Moreover, the lower storage of vitamin A in the liver of deficient animals fed the 9-cis and 11-cis isomers may reflect impaired intestinal absorption of these isomers. The tendency of the cis-retinols to readily isomerize...
suggests that the small amount of vitamin A present in the liver of these animals may be the all-trans form. Further studies are required to address these possibilities.

The spectral properties of all-trans-retinol, 13-cis-retinol, and 3-dehydroretinol complexed to CRBP(II) and CRBP were very similar. The bound ligands of both proteins had red shifted spectra that displayed vibronic fine structure compared to their spectra as free ligands in organic solutions. In addition, the bound retinol isomers had higher extinction coefficients than in organic solvents. Thus, CRBP(II) and CRBP appear to bind the retinol isomers in a similar fashion. Specifically, 3-dehydroretinol and 13-cis-retinol were bound in a more rigid planar configuration as previously suggested for all-trans-retinol bound to CRBP (15), CRBP(II) (4), and β-lactoglobulin (26). The difference spectrum illustrating bound 3-dehydroretinol (Fig. 6B) provided additional evidence for the planarity of the bound ligand. Bound 3-dehydroretinol had significantly lower absorbance in the 280–290-nm region of the spectrum compared to 3-dehydroretinol in organic solution. This region of absorbance has been attributed to a partial chromophore arising when the diene ring is out of plane with the hydrocarbon side chain (14). The loss in absorbance in the 280–290-nm region on binding of 3-dehydroretinol to CRBP(II) may be interpreted as a loss of the diene ring partial chromophore as it is brought into conjugation with the side chain in a planar configuration. The corresponding increase in ε280 would be due to an increased population of fully planar molecules when 3-dehydroretinol is bound to CRBP(II) compared to its orientation in organic solutions. Since the contribution of free ligand to the spectrum was negligible, the residual absorbance at 280–290 nm may represent a population of bound ligand that is out of plane with the side chain. Thus, binding to CRBP(II) did not confer full rigidity on 3-dehydroretinol in the binding site.

The fact that CRBP(II) and CRBP bind identical retinol isomers in a similar manner implies considerable similarities in the binding sites for retinol. However, an obvious difference in binding sites was revealed by the ability of CRBP(II) and CRBP with affinities comparable to all-trans-retinol. Differences in the retinol binding sites of CRBP(II) and CRBP illustrates differences in the binding sites as well. The differences in the retinol binding sites of CRBP(II) and CRBP appear to have functional significance. Retinol is bound to CRBP(II) in a manner that renders its hydroxyl group more accessible to enzymatic reaction. We have recently shown that retinol complexed to CRBP(II) is a better substrate for esterification by intestinal microsomes than is CRBP-retinol (9). Thus, retinol absorption may be better facilitated when bound to CRBP(II) versus CRBP.

This study examined vitamin A compounds that bind to CRBP(II) and CRBP with affinities comparable to all-trans-retinol. Competitive binding studies utilized a 1 log range of competitor concentrations. The tendency of these hydrophobic compounds to form micelles precluded the use of higher competitor concentrations. Thus, we cannot exclude binding of some of these compounds at much higher concentrations. Such low affinity binding would have little physiological significance since the predominant forms of vitamin A in the intestine are all-trans-retinol and the trans-retinaldehyde generated from carotene cleavage. Based on these conditions, we have observed remarkably similar binding properties in these two distinct proteins. A novel difference with potential physiological significance was the ability of CRBP(II), and not CRBP, to bind all-trans-retinaldehyde.

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