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Differential Binding of Retinol Analogs to Two Homologous Cellular Retinol-binding Proteins*

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A comparative study of the interactions of rat cellular retinol-binding protein (CRBP) and cellular retinol-binding protein II (CRBP II) with a number of synthetic phenyl-substituted analogs of all-*trans*-retinol was performed using fluorescence and nuclear magnetic resonance analysis. These studies indicate that CRBP II is more sensitive to modifications of the ring moiety than CRBP. Removal of the two methyl substituents on the ring which are ortho to the polyene chain abolishes binding to CRBP II. Conformational analysis of the ligands indicates that these two methyl groups influence the planarity of the ligand. The identification of monospecific ligands may prove useful for studying the physiological roles of these two proteins.

Cellular retinol-binding protein (CRBP)[1] and cellular retinol-binding protein II (CRBP II) are homologous cytoplasmic proteins that bind all-*trans*-retinol and all-*trans*-retinaldehyde. Although these two proteins share 56% amino acid sequence identity (Li *et al.*, 1986), they exhibit very different tissue distributions. CRBP II is confined to the columnar absorptive cells (enterocytes) on small intestinal villi in adult animals, where it represents ~1% of the cytosolic proteins and is 1000-fold more abundant than CRBP (Ong, 1984; Crow and Ong, 1985). In contrast CRBP is present in a wide variety of tissues, including liver, kidney, and testis (Chytil and Ong, 1984; Levin *et al.*, 1987). These differences in CRBP and CRBP II gene expression strongly suggest that these two proteins serve different physiological functions and that CRBP II is specifically adapted for intestinal absorption and metabolism of retinol.

MacDonald and Ong (1987) reported that retinol is a better substrate for esterification by intestinal microsomes *in vitro* when it is presented bound to CRBP II rather than to CRBP. Napoli *et al.* (1991) have reported *in vitro* studies suggesting that CRBP is involved in mediating the conversion of retinol to retinoic acid and in the hydrolysis of retinyl esters. *In vitro* studies on retinoid metabolism rely on the addition of specific cofactors and inhibitors to monitor a particular metabolic pathway. *In vivo* studies are hampered by problems in tracking the specific type of binding protein that the retinol molecule is associated with. Studies on the molecular details of the binding interactions of the two CRBPs provide a basis for identifying or designing ligands that bind only to a specific retinoid binding protein. Such monospecific ligands may prove useful in delineating the specific metabolic pathways that these two proteins mediate.

We have previously carried out comparative ^{19}F nuclear magnetic resonance (NMR) studies of *Escherichia coli*-derived CRBP and CRBP II highly substituted with the nuclear spin label ^{19}F attached to the 6 position of the tryptophan residue (Li *et al.*, 1991). The incorporated ^{19}F nuclei provide sensitive residue-specific probes for monitoring retinoid-protein interactions in solution. Ligand-induced perturbations were observed for 2 of the 4 tryptophan residues (Trp^9 and Trp^{107}) in both 6-FTrp-CRBP and 6-FTrp-CRBP II. However, in contrast to 6-FTrp-CRBP II where binding of all-*trans*-retinol results only in downfield shifts of its Trp^9 and Trp^{107} resonances, binding of all-*trans*-retinol to 6-FTrp-CRBP results in an upfield shift in the Trp^{107} resonance. These observations suggest that there are differences in how these two proteins interact with all-*trans*-retinol. To further map differences in the ligand-binding interactions of these two proteins, ^{19}F NMR analyses of a number of synthetic fluororetinol analogs complexed with CRBP and CRBP II were performed. We report here the identification of two ring-modified synthetic retinols that bind only CRBP and fail to bind to CRBP II.

EXPERIMENTAL PROCEDURES

Materials—6-Fluorotryptophan, deuterium oxide (D_2O), and all-*trans*-retinol were purchased from Sigma. Plasmids and bacterial strains used for expression of rat CRBP II and rat CRBP in *E. coli* have been described in earlier publications (Li *et al.* 1987, 1989; Levin *et al.* 1988).

Preparation of Retinol Analogs—(*E,E,Z,E*)-6-Fluoro-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid methyl ester (acid ester form of **1**), (all-*E*)-9-[4-(trifluoromethoxyl)phenyl]-3,7-dimethyl-2,4,6,8-nonatetraenoic acid ethyl ester (acid ester form of **2**), (all-*E*)-9-(4-trifluoromethoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (**3**), (all-*E*)-3,7-dimethyl-9-phenyl-2,4,6,8-nonatetraen-1-ol (**4**), (all-*E*)-9-(2,4,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (**5**) were obtained from Hoffmann-La Roche (see Fig. 1). Retinoid analogs which were received in the acid ester form were reduced to the corresponding alcohol by diisobutylaluminum hydride in hexane at 0 °C under nitrogen. The crude product was then further purified by preparative thin layer chromatography on glass-backed silica 60 plates (Merck), hexane/ethyl acetate (60:40, v/v). All manipulations were carried out in the dark. The product was stored at −70 °C.

The products were characterized by ^1H and ^{19}F NMR spectroscopy. The ^1H and ^{19}F NMR spectra of the retinol analogs are as follows

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[1] The abbreviations used are: CRBP, cellular retinol binding protein; $FABP_c$, fatty acid binding protein (cytoplasmic); 6-FTrp, 6-fluorotryptophan; TFA, trifluoroacetic acid.

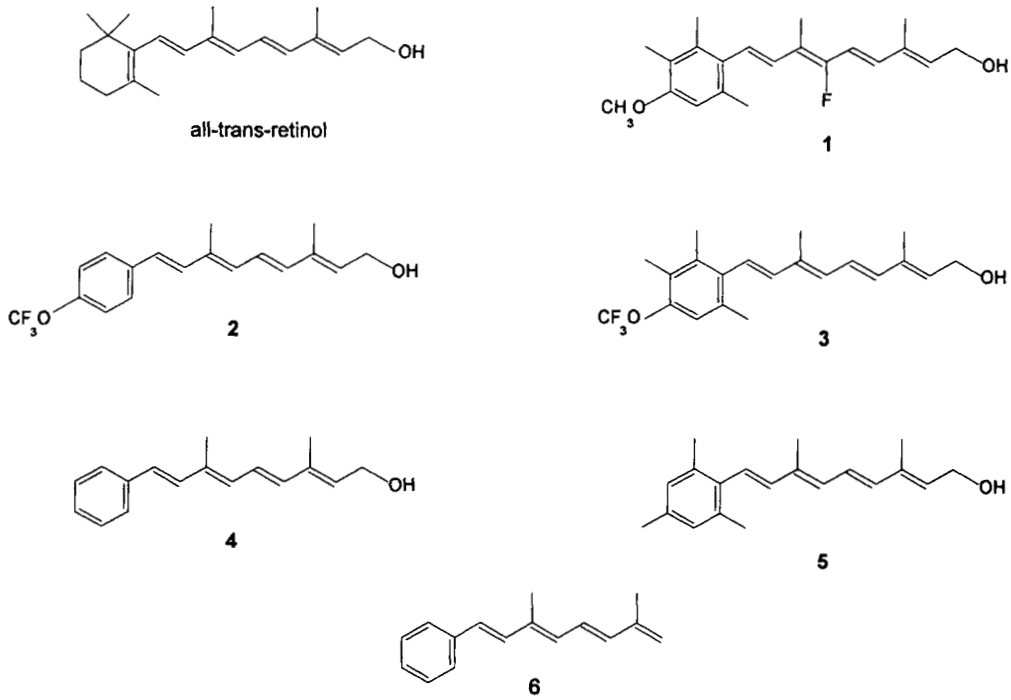

FIG. 1. **Structure of retinol analogs.**

with the ^1H chemical shifts (δ) referenced to trimethylsilane and the ^{19}F chemical shifts (δ) referenced to trifluoroacetic acid (TFA): (*E,E,Z,E*)-6-fluoro-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (**1**). ^1H NMR ($CDCl_3$) δ 6.78 (1H, d, J = 17 Hz), 6.60 (1H, d, J = 17 Hz), 6.58 (1H, s), 6.57 (1H, d, J = 18 Hz), 6.39 (1H, dd, $J_{7,8}$ = 16, $J_{8,F}$ = 27), 5.80 (1H, t, J = 5.5 Hz), 4.32 (2H, d, J = 5.5 Hz), 3.80 (3H, s), 2.29 (3H, s), 2.23 (3H, s), 2.13 (3H, s), 1.99 (3H, d, J = 18 Hz), 1.86 (3H, s). ^{19}F NMR ($CDCl_3$) form TFA: −47.90 (d, J = 27 Hz) ppm.

(All-*E*)-9-[4-(trifluoromethoxyl)phenyl]-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (**2**): ^1H NMR ($CDCl_3$) δ 7.41 (2H, d, J = 8.8 Hz), 7.14 (2H, d, J = 8.3 Hz), 6.82 (1H, d, J = 16 Hz), 6.61 (1H, dd, J = 11 Hz, J = 15 Hz), 6.52 (1H, d, J = 17 Hz), 6.35 (1H, d, J = 16 Hz), 6.28 (1H, d, J = 11 Hz), 5.72 (1H, t, J = 7.0 Hz), 4.310 (2H, m), 2.00 (^3H, s), 1.86 (^3H, s). ^{19}F NMR ($CDCL_3$) 17.83 ppm.

Measurement of ^1H-^1H nuclear Overhauser effect confirmed that these retinol analogs were in the all-trans-configuration. HPLC analysis indicated that the compounds were 99% pure.

Production of Rat CRBP II and CRBP in E. coli—Unlabeled rat apoCRBP and apoCRBP II were expressed in *E. coli* strain JM101 harboring the appropriate recombinant expression vectors as described previously (Li *et al.*, 1987; Levin *et al.*, 1988). 6-Fluorotryptophan was incorporated into the *E. coli* tryptophan auxotroph, W3110 trpA33 (Drapeau *et al.*, 1968) and inducing expression in media containing the analog (Li *et al.*, 1989, 1991). The proteins were purified as described previously (Cheng *et al.*, 1991). The purified proteins were passed through a column of Lipidex 1000 at 37 °C (Lowe *et al.*, 1987) to remove any bound fatty acids derived from *E. coli*.

Fluorescence Measurements—Steady state fluorescence measurements were performed on a Photon Technology International spectrophotometer. Ligand binding studies were performed by fluorometric titration (Cogan *et al.*, 1976; Li *et al.*, 1987) using delipidated apoprotein solutions at 0.8–1.2 μM. The protein was excited at 290 nm, and the (tryptophan) fluorescence at 340 nm was monitored.

NMR Measurements—Purified proteins were concentrated and equilibrated with D_2O as reported previously (Li *et al.*, 1989). The buffer used for NMR studies contained 10 mM potassium phosphate, pH 7.4, 5 mM 2-mercaptoethanol, and 0.05% sodium azide. The protein concentration was 0.1–1 mM. ^{19}F NMR spectra were recorded at 470.3 MHz without proton decoupling on a Varian VXR-500 spectrometer. The chemical shift was referenced to an internal standard (potassium salt of trifluoroacetic acid in solution). A 1.5-s delay time between pulses (~3 T_1 values for proteins and bound retinoids resonances) was employed in all the experiments. Temperature was controlled to within 0.1 °C with an Oxford temperature controller. An apodization function resulting in 12-Hz line broadening was applied to all the ^{19}F NMR spectra.

^1H NMR spectra of the retinol analogs dissolved in $CDCl_3$ were recorded at 499.6 MHz on a Varian VXR-500 spectrometer at 25 °C. The samples for nuclear Overhauser effect experiments were filtered and degassed by bubbling with nitrogen gas.

Conformational Analysis—Molecular orbital calculations were performed with the AM1 hamiltonian from MOPAC (version 6.0; Stewart, 1990) on a silicon graphics 4D/35 computer workstation. All structures of the retinoids were submitted for MOPAC AM1 geometry optimization. The constructed retinoids were initially minimized with an empirical force field provided with the SPARTAN package followed by a treatment with the AM1 program for a single point energy minimization. The resulting structure was then submitted for geometry optimization. Calculations were considered converged when the energy difference between successive optimization cycles was less than 0.5×10^{-3} Kcal/mol. The torsion angle of the calculated energy-minimized structure of etretinate was 52.9° using this method as compared with the torsion angle measured by x-ray studies of crystalline etretinate (Pfoertner, 1987) of 66.8°. To estimate the energy barrier between stable conformations of phenyl-substituted retinoid analogs (lacking the ortho methyl substituents), molecular mechanics calculations were performed with the MM2 program on **6** (see Fig 1).

RESULTS

^{19}F NMR Spectra of Retinol Analogs Complexed with 6-FTrp-CRBPII and 6-FTrp-CRBP—We examined the interactions of three synthetic fluorinated phenyl-substituted retinol analogs, (*E,E,Z,E*)-6-fluoro-9-(4-methoxy-2, 3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (**1**), (all-*E*)-9-(4-trifluoromethoxyphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (**2**), and (all-*E*)-9-(4-trifluoromethoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (**3**) (see Fig. 1) with 6-FTrp-CRBP and 6-FTrp-CRBP II. We have shown previously (Li *et al.*, 1991) that the addition of an equimolar amount of all-*trans*-retinol to 6-FTrp-CRBP II results in downfield shifts of its Trp^9 and Trp^{107} F-resonances (0.5 and 2.0 ppm, respectively) (see Fig. 2, *A* and *C*). Addition of an equimolar amount of all-*trans*-retinol to 6-FTrp-CRBP results in a 0.4 ppm downfield shift of its Trp^9 F-resonance but in a 0.4 ppm upfield shift of its Trp^{107} F-resonance (see Fig. 2, *B* and *D*). Binding of **1** to 6-FTrp-CRBP II (see Fig. 2*E*) results in similar perturbations in the chemical shifts of the F-resonances corresponding to Trp^{107} (−43.2 ppm relative

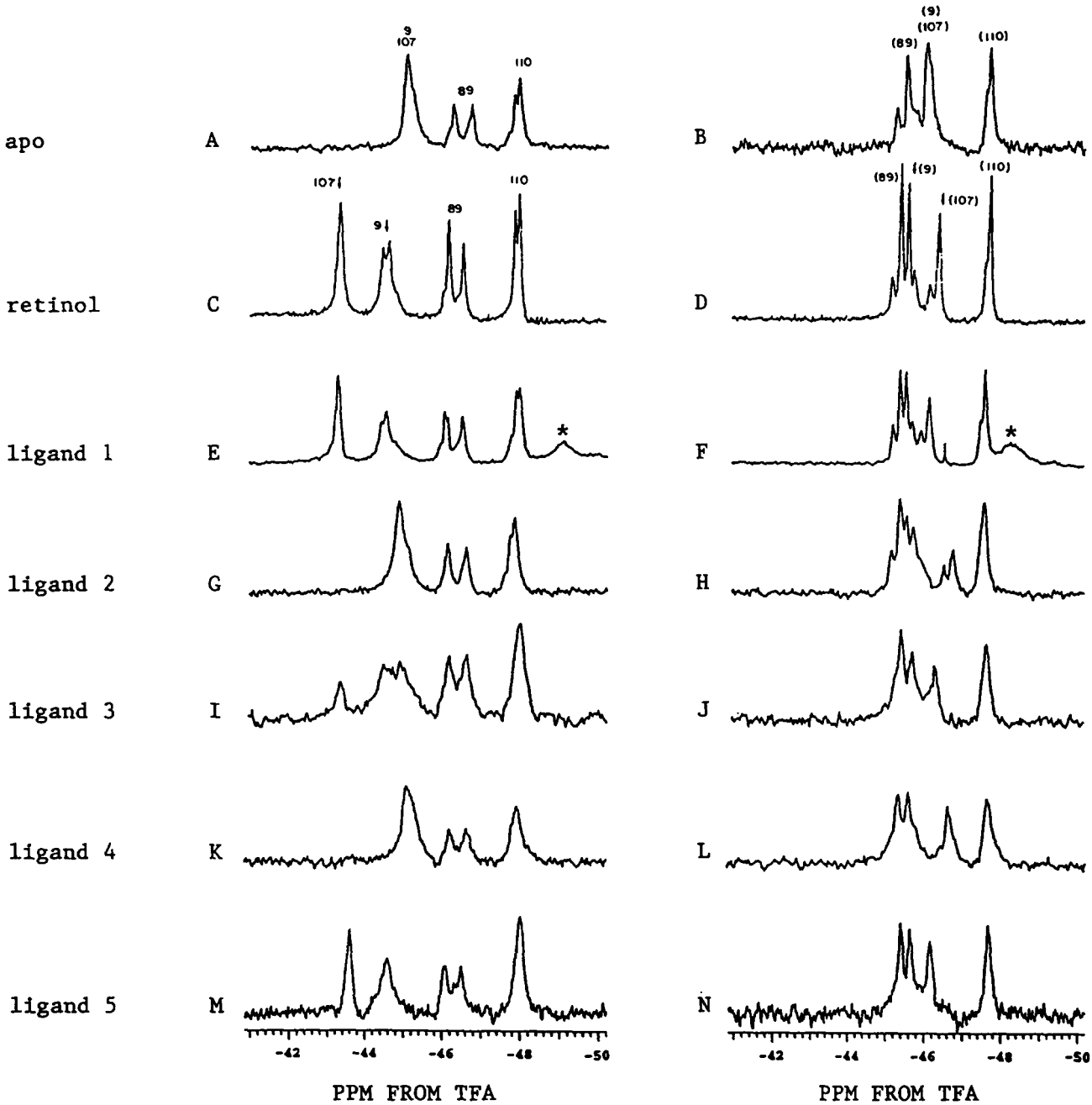

FIG. 2. **^{19}F NMR spectral analysis of retinol analogs binding to 6-F-Trp-CRBP II and 6-F-Trp-CRBP.** The ^{1}H coupled 470.3-MHz ^{19}F NMR spectra were collected as described under "Experimental Procedures" at ambient room temperature 20–25 °C, unless otherwise specified. Chemical shifts are referenced to the ^{19}F signal for trifluoroacetic acid (*TFA*). An apodization function resulting in a line-broadening of 12 Hz was applied for all spectra. 400–6000 free induction decays were obtained for each spectrum. *A*, 0.40 mM apo6-FTrp-CRBP II; *B*, 0.20 mM apo6-FTrp-CRBP; *C*, 0.5 mM 6-FTrp-CRBP II with 0.5 mM all-*trans*-retinol; *D*, 0.5 mM 6-FTrp-CRBP with 0.5 mM all-*trans*-retinol; *E*, 35.0 °C, 0.40 mM 6-FTrp CRBP II with 0.40 mM **1**; *F*, 0.20 mM 6-FTrp-CRBP with 0.2 mM **1**; *G*, 0.2 mM 6-FTrp-CRBP II with 0.2 mM **2**; *H*, 0.20 mM 6-FTrp-CRBP with 0.2 mM **2**; *I*, 0.2 mM 6-FTrp-CRBP II with 0.2 mM **3**; *J*, 0.2 mM 6-FTrp-CRBP with 0.2 mM **3**; *K*, 0.2 mM 6-FTrp-CRBP II with 0.2 mM **4**; *L*, 0.11 mM 6-FTrp-CRBP with 0.11 mM **4**; *M*, 0.2 mM 6-FTrp-CRBP II with 0.2 mM **5**; *N*, 0.2 mM 6-FTrp-CRBP with 0.2 mM **5**. The *numbers* indicate the tryptophan residue to which the F-resonance was assigned. * indicates the F-resonance of the bound ligand.

to TFA) and Trp^{9} (−44.5 ppm relative to TFA), to those observed with the addition of all-*trans*-retinol (see Fig. 2*C*). The broad peak (−48.4 to −49.4 ppm relative to TFA) labeled * corresponds to the fluorine nuclei of bound ligand **1**. The signals corresponding to Trp^{9}, Trp^{89}, Trp^{107}, Trp^{110}, and **1** have an integrated area ratio of 0.7:1:1:1:0.9. The addition of an equimolar amount of all-*trans*-retinol reduced the signal corresponding to bound **1** to 44%, suggesting that the relative affinities of **1** and all-*trans*-retinol are roughly comparable. Addition of two equivalents of all-*trans*-retinol resulted in the disappearance of the signal corresponding to bound **1**. Further analysis of this signal is described elsewhere.[2]

Binding of **1** to 6-FTrpCRBP (see Fig. 2*F*) also results in similar perturbations in the chemical shifts of the F-resonances corresponding to Trp^{107} (−45.5 ppm and −45.63 ppm relative to TFA), and Trp^{9} (−46.1 and −45.9 ppm relative to TFA), to those observed with the addition of all-*trans*-retinol (see Fig. 2*D*). The small sharp peak at −46.55 ppm is assigned to the unbound ligand because free ligand in the same buffer produces the same signal. The broad peak (−47.6 to −49.2

[2] D. Rong and E. Li, manuscript in preparation.

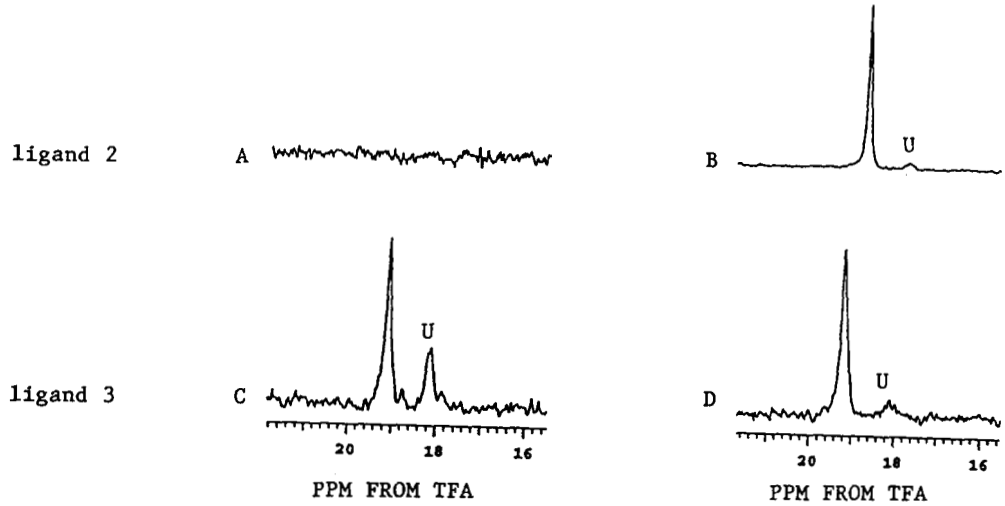

FIG. 3. **^{19}F NMR spectral analysis of retinol analogs binding to 6-F-Trp-CRBP II and 6-F-Trp-CRBP.** The ^{1}H-coupled 470.3-MHz ^{19}F NMR spectra were collected as described under "Experimental Procedures" at ambient room temperature 20–25 °C. Partial spectra showing only the signal corresponding to the bound and unbound (labeled *U*) are shown. Chemical shifts are referenced to the ^{19}F signal for trifluoroacetic acid (*TFA*). An apodization function resulting a line broadening of 12 Hz was applied for all spectra. 400–6000 free induction decays were obtained for each spectrum. *A*, 0.40 mM 6-FTrp CRBP II with 0.40 mM **2**; *B*, 0.20 mM 6-FTrp-CRBP with 0.2 mM **2**; *C*, 0.2 mM 6-FTrp-CRBP II with 0.2 mM **3**; *D*, 0.20 mM 6-FTrp-CRBP with 0.2 mM **3**. *U* indicates the F-resonance of the unbound ligand.

ppm relative to TFA) labeled * corresponds to the fluorine nuclei of bound ligand **1**. The signals corresponding to Trp^{110} and **1** have an integrated area ratio of 1:1. The addition of an equimolar amount of all-*trans*-retinol reduced the signal corresponding to bound **1** to 53%, suggesting that the relative affinities of **1** and all-*trans*-retinol were comparable. Addition of two equivalents of all-*trans*-retinol resulted in the disappearance of the signal corresponding to bound **1**. Further analysis of this signal is described elsewhere.[2] These results indicate that substitution of the cyclohexenyl ring by a trimethylmethoxyphenyl group does not substantially affect binding to either of the CRBPs.

In contrast, addition of **2** to 6-FTrp-CRBP II (see Fig. 2*G*) results in a ^{19}F NMR spectrum that is identical to the spectrum obtained for the apoprotein (see Fig. 2*A*). These results indicate that **2** fails to bind CRBP II. Addition of **2** to 6-FTrp-CRBP (see Fig. 2*H*) results in similar perturbations in the chemical shifts of the F-resonances corresponding to Trp^{9} (−45.50 and −45.63 ppm relative to TFA) and to Trp^{107} (−46.6 and −46.8 ppm relative to TFA) to those observed with the addition of all-*trans*-retinol. As shown in Fig. 3*B*, the F-resonance corresponding to the bound ligand **2** was detected at 18.8 ppm relative to TFA. The signals corresponding to **2** and Trp^{110} have an integrated area ratio of 1:2.4. This signal was reduced to 29% by the addition of an equimolar amount of all-*trans*-retinol, suggesting that the relative affinities of **2** and all-*trans*-retinol are comparable. Addition of two equivalents of all-*trans*-retinol resulted in the disappearance of the signal corresponding to bound **2**.

To determine whether the presence of a trifluoromethoxy group, or the absence of the methyl substituents, is responsible for differential ligand binding, ligand **3**, which is the trifluoromethoxytrimethylphenyl analog of all-*trans*-retinol was obtained from Hoffmann-La Roche. As shown in Fig. 2*I*, addition of an equimolar amount of **3** to 6-FTrp-CRBP II resulted in a roughly 50% decrease in the signal at −44.8 ppm relative to TFA, corresponding to Trp^{9} and Trp^{107} in 6-FTrp-apo-CRBP II, and the appearance of two new downfield signals at −43.3 ppm and −44.4 ppm relative to TFA. This suggests that approximately 50% of the added ligand complexed with 6-FTrp-CRBP II. The signal corresponding to the bound ligand (see Fig. 3*C*) is observed as a sharp peak at 19.1 ppm relative to TFA. The signals corresponding to Trp^{110} and bound **3** have an integrated area ratio of 1:2. Addition of an equimolar amount of all-*trans*-retinol results in complete disappearance of the signal. The small signal at 18.08 ppm relative to TFA corresponds to unbound ligand. These observations suggest that addition of methyl substituents back on the phenyl ring partially restores ligand binding to CRBP II. However the decreased binding of **3** compared with **1** to CRBP II, also suggests that substitution of the methoxy group with a trifluoromethoxy group at position 4 of the phenyl ring reduces the affinity of the ligand to CRBP II. As shown in Fig. 2*J*, the ^{19}F NMR spectrum of 6-FTrp-CRBP with an equimolar amount of **3** is similar to the ^{19}F NMR spectrum of 6-FTrp-CRBP complexed with all-*trans*-retinol (see Fig. 2*D*). The signal corresponding to bound **3** was observed at 19.2 ppm relative to TFA. The signals corresponding to Trp^{110} and **3** have an integrated area ratio of 1:2.7. Addition of an equimolar amount of all-*trans*-retinol reduced the signal to 45%, suggesting that the relative affinities of CRBP for **3** and all-*trans*-retinol are comparable. Addition of two equivalents of all-*trans*-retinol resulted in the disappearance of the signal corresponding to bound **3**.

To further delineate the molecular basis for differential binding of ligands to CRBP and CRBP II, two additional nonfluorinated phenyl-substituted retinol analogs were studied (see Fig. 1): (all *E*)-9-phenyl-3,7-dimethyl 2,4,6,8- nonatetraene-1-ol (ligand **4**) and (all-*E*)-9-(2,4,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraene-1-ol (ligand **5**). As shown in Fig. 2*K*, addition of an equimolar amount of **4** to 6-FTrp-CRBP II results in a ^{19}F NMR spectrum that is identical to that of 6-FTrp-apoCRBP II, indicating that **4** does not bind to CRBP II. In contrast, the ^{19}F NMR spectrum of 6-FTrp-CRBP complexed with an equimolar amount of **4** (see Fig. 2*L*) is similar to the spectrum observed for 6-FTrp-CRBP complexed with all-*trans*-retinol (see Fig. 2*D*).

As shown in Fig. 2*M*, addition of an equimolar amount of **5** to 6-FTrp-CRBP II creates a similar perturbation to the chemical shifts of the F-resonances corresponding to Trp^{107} (−43.5 ppm) and Trp^{9} (−44.8 ppm) to those observed with addition of all-*trans*-retinol (Fig. 2*C*). The addition of one equimolar amounts of **5** to 6-FTrp-CRBP also induces similar chemical shift changes for Trp^{9} (to −45.3 ppm) and Trp^{107} (to −46.1 ppm) (Fig. 2*N*) to those observed with addition of all-*trans*-retinol.

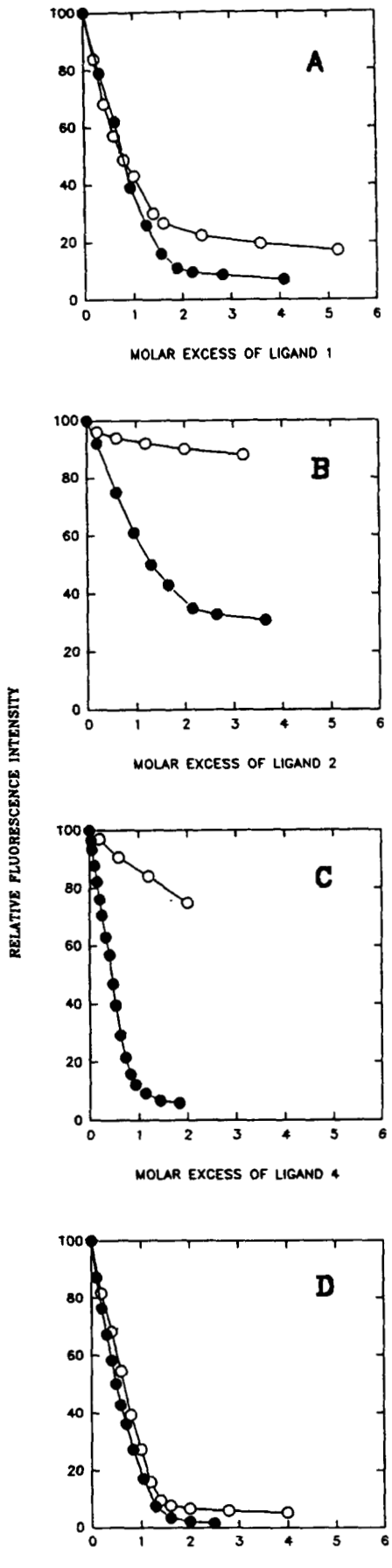

FIG. 4. **Titration of apoCRBP II (○) and apoCRBP (●) with retinol analogs.** Protein fluorescence was monitored at 340 nm with excitation at 290 nm with the addition of increasing amounts of ligand (see "Experimental Procedures"). The protein concentration was 1 μM. *A*, CRBP II (○) and CRBP (●) were titrated with **1**. *B*, CRBP II (○) and CRBP (●) were titrated with **2**. *C*, CRBP II (○) and CRBP (●) were titrated with **4**. *D*, CRBP II (○) and CRBP (●) were titrated with **5**.

TABLE I

Dissociation constant of CRBP II and CRBP with retinol analogs ***1–5***

The apparent dissociation constants, K_d, was determined using fluorometric titration as described under "Experimental Procedures."

Retinoid	K_d	
	CRBP II	CRBP
	nM	
All-*trans*-retinol	10[a]	10[a]
1	225 ± 50	38 ± 13
2	No binding	30 ± 10
3[b]		
4	No binding	146 ± 54
5	24 ± 11	79 ± 50

[a] Levin *et al.* (1987).
[b] Ligand **3** is too unstable to perform fluorescence studies.

TABLE II

The C5(C6,C7)C8 torsion angles of the calculated energy-minimized structures of retinol analogs ***1–5***

The calculations were made by the AM1 program in MOPAC (version 6.0) as described under "Experimental Procedures."

Retinoids	Torsion angles
1	52.0 °
2	19.7 °
3	54.5 °
4	21 °
5	54 °

Based on an analysis of the interactions of **1-5**, CRBP II appears to be more sensitive to modifications of the ring than CRBP. Removal of the ortho dimethyl substituents on the phenyl ring abolishes binding selectively to CRBP II.

Fluorescence Studies of the Binding of Retinol Analogs to CRBP and CRBP II—Binding of **1**, **2**, **4**, and **5** to CRBP and CRBP II was also studied by fluorometric titration, by monitoring quenching of tryptophan fluorescence with the addition of increasing amounts of ligand. As shown in Fig. 4, addition of **2** or **4** failed to quench CRBP II fluorescence, confirming the NMR studies indicating that **2** and **4** both fail to bind CRBP II. Addition of **1** and **5** quenched both CRBP and CRBP II fluorescence, confirming the NMR studies showing that these two ligands bound to both proteins. The apparent dissociation constants of **1**, **2**, **4**, and **5** complexed with CRBP and CRBP II calculated as described previously (Li *et al.*, 1987) are shown in Table I. These studies confirm that although **2** and **4** fail to bind CRBP II, both of these ligands bind CRBP tightly.

Conformational Analysis of Retinol Analogs—Calculations of the energy minimized structures of **1-5** performed using semiempirical quantum mechanics (AM1, see "Experimental Procedures") indicated that the absence of the ortho dimethyl substituents of **2** and **4** favors a more planar orientation of the ring and side chain. The torsion angle between the ring and the chain of the predicted energy-minimized structures of **1-5** is listed in Table II. The torsion angles of **1**, **3**, and **5** are 52, 54.5, and 54° respectively. The torsion angles of **2** and **4**, which both lack the ortho dimethyl substituents are 19.7 and 21°.

To estimate the energy barrier between stable conformations of phenyl-substituted retinoid analogs (lacking the ortho dimethyl substituents), molecular mechanics calculations

(MM2) were performed on **6** (see Fig. 1). The conjugated *pi*-system is modelled in this calculations by scaling the 2-fold torsional barriers and the bond parameters via a modified p-p-p MO method. The calculation estimated that the energy barrier between the two stable conformations (at torsion angles of 11 and 169°) is 2.97 kcal/mol.

DISCUSSION

Comparative binding studies performed on rat CRBP and CRBP II with various ring-modified retinol analogs have led to the identification of two retinol analogs that bind only to CRBP and not to CRBP II. These studies utilized two different methods for measuring ligand binding, fluorometric titration and ^{19}F NMR. Both methods demonstrated unequivocally that retinol analogues missing the ortho dimethyl groups failed to bind CRBP II while retaining their ability to bind to CRBP. These studies suggest that *removal* of the two ortho dimethyl substituents on the phenyl ring is associated with loss of binding to CRBP II. In contrast, CRBP appears to be much less sensitive to modifications of the ring.

Both CRBP and CRBP II belong to a family of cytoplasmic proteins which bind hydrophobic ligands (Gordon *et al.*, 1991), including intestinal fatty acid binding protein (I-FABP$_c$), liver FABP$_c$ (L-FABP$_c$), heart FABP$_c$ (H-FABP$_c$), ileal lipid-binding protein (ILBP), cellular retinoic acid binding protein (CRABP), the P2 protein of peripheral nerve myelin mammary-derived growth inhibitor (MDGI), and adipocyte lipid-binding protein (ALBP) (aP2). The crystal structures of three members of this family, the P2 myelin protein (Jones *et al.*, 1988), I-FABP$_c$ (Sacchettini *et al.*, 1988), and ALBP (Xu *et al.*, 1992) have been reported. These three proteins contain two orthogonal β-sheets, each consisting of five anti-parallel β-strands all with +1 connections. Since this structure resembles a clam shell, it has been named a β-clam (Sacchettini *et al.*, 1989). Recently the crystal structures of CRBP[3] and CRBP II[4] have been solved. Both proteins share the β-clam structure. The conformation of bound all-*trans*-retinol in the crystalline CRBP-retinol complex is close to the s-*trans* conformation with an estimated torsion angle of −160°.[3] The conformation of bound all-*trans*-retinol in the crystalline CRBP II-retinol complex derived from the four different molecules present in the triclinic form appears to be more twisted from planarity, with torsion angles of −78°, −117°, −127°, −150°.[4]

Two major factors play in determining the minimum energy conformations of the retinol analogs. Conjugation between the phenyl ring and the polyene chain favors an essentially planar structure. However, steric hindrance between the methyl substituents on the ring ortho to the polyene chain and the 8-olefinic proton would make the planar s-*cis* conformation unfavorable. The calculated potential of the cyclohexenyl ring of all-*trans*-retinaldehyde for rotation about the 6–7 bond showed a broad energy minimum at 50° for the s-*cis* conformation, and a second higher energy minimum close to the s-*trans* geometry. NMR measurements (Honig *et al.*, 1971) indicated s-*cis* geometry between the ring and the polyene side chain with a range of rotation of 25–90° at ambient temperatures. All-*trans*-retinoic acid has been crystallized in two forms. The more stable triclinic crystal (Stam, 1972) contained the 6-s-*cis* conformer and the less stable monoclinic crystal (Oberhäensli *et al.*, 1974) contained an almost planar 6-s-*trans* conformer. Modeling of the energy-minimized conformations of the ligands indicates that the presence or absence of the two ortho dimethyl substituents influences the torsion angle between the ring and the polyene chain. However based on the small energy barrier (<3 kcal/mmol) estimated for twisting **4** from its energy minimized form to a torsion angle of 54°, it is difficult to attribute the effect of the ortho dimethyl substituents on differential binding solely to their effect on the torsion angle.

Fluorescence and NMR measurements yielded somewhat different results with regard to the estimated relative affinities of some of the analogues and all-*trans*-retinol. We previously observed discrepancies in results obtained using these two techniques when comparing the relative affinities of all-*trans*-retinol from CRBP and CRBP II (Li *et al.*, 1991). The reasons for the discrepancies were probably multifactorial and probably related to the hydrophobic properties of the ligands, difficulties in estimating the true concentration of free ligand, as well as the instability of the ligands when exposed to light.

The biological activity of phenyl-substituted retinoic acid derivatives, lacking the methyl substituents, appears to be diminished (Sporn and Roberts, 1984). The biological activity of the corresponding alcohols has thus far not been tested.

The identification of monospecific ligands which bind only to a single CRBP may prove useful in testing the hypothesis that association with a particular CRBP preferentially channels the ligand into certain metabolic pathways.

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[3] T. A. Jones, S. Cowan, and M. Newcomer, personal communication.

[4] N. S. Winter, J. M. Bratt, and L. J. Banaszak, personal communication.

REFERENCES

Cheng, L., Qian, S., Rothschild, C., d'Avignon, A., Lefkowith, J. B., Gordon, J. I., and Li, E. (1991) *J. Biol. Chem.* **266,** 24404–24412
Chytil, F., and Ong, D. E. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) Vol. 2, pp. 89–122, Academic Press, Orlando, FL
Cogan, U., Kopelman, M., Mokady, S., and Schinitsky, M. (1976) *Eur. J. Biochem.* **65,** 71–78
Crow, J. A., and Ong, D. E. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 4707–4711
Drapeau, G. R., Brammer, W. J., and Yanofsky, C. (1968) *J. Mol. Biol.* **35,** 357–367
Gordon, J. I., Sacchettini, J. C., Ropson, I., Frieden, C., Li, E., Rubin, D. C., Roth, K. A., and Cistola, D. P. (1991) *Curr. Top. Lipidology* **2,** 125–137
Honig, B., Hudson, B., Sykes, B. D., and Karplus, M. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 1289–1293
Jones, T. A., Bergfors, T., Sedzik, J., and Unge, T. (1988) *EMBO J.* **7,** 1597–1604
Levin, M. S., Li, E., Ong, D. E., and Gordon, J. I. (1987) *J. Biol. Chem.* **262,** 7118–7124
Levin, M. S., Locke, B., Yang, N. C., Li, E., and Gordon, J. I. (1988) *J. Biol. Chem.* **262,** 17715–17723
Li, E., Demmer, L. A., Sweetser, D. A., Ong, D. E., and Gordon, J. I. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8102–8106
Li, E., Locke, B., Yang, N. C., Ong, D. E., and Gordon, J. I. (1987) *J. Biol. Chem.* **262,** 13773–13779
Li, E., Qian, S., Nader, L., Yang, N. C., d'Avignon, A., Sacchettini, J. C., and Gordon, J. I. (1989) *J. Biol. Chem.* **264,** 17041–17048
Li, E., Qian, S., Winter, N. S., d'Avignon, A., Levin, M. S., and Gordon, J. I. (1991) *J. Biol. Chem.* **266,** 3622–3629
Lowe, J. B., Sacchettini, J. C., Laposata, M., McQuillan, J. J., and Gordon, J. I. (1987) *J. Biol. Chem.* **262,** 5931–5937
MacDonald, P. N., and Ong, D. E. (1987) *J. Biol. Chem.* **262,** 10550–10556
Napoli, J. L., Posch, K. P., Fiorella, P. D., and Boerman, M. H. E. M. (1991) *Biomed. & Pharmacother.* **45,** 131–143
Oberhänsli, W. E., Wagner, H. P., and Isler, O. (1974) *Acta Crystallogr. Sect. B* **30,** 161–166
Ong, D. E. (1984) *J. Biol. Chem.* **259,** 1476–1482
Pfoertner, K. H., Englert, G., and Schoenholzer, P. (1987) *Tetrahedron* **43,** 1321–1334
Sacchettini, J. C., Gordon, J. I., and Banaszak, L. J. (1988) *J. Biol. Chem.* **263,** 5815–5819
Sacchettini, J. C., Gordon, J. I., and Banaszak, L. J. (1989) *J. Mol. Biol.* **208,** 327–340
Sporn, M. B., and Roberts, A. B. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) Vol. 1, pp. 235–279, Academic Press, Orlando, FL
Stam, C. H. (1972) *Acta Crystallogr. Sect. B* **28,** 2936–2945
Stewart, J. T. (1990) *J. Comput. Aided Mol. Des.* **4,** 1–105
Xu, Z., Bernlohr, D. A., and Banaszak, L. J. (1992) *Biochemistry* **31,** 3484–3492