Differential Interaction of Lecithin–Retinol Acyltransferase with Cellular Retinol Binding Proteins†

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ABSTRACT: Esterification of retinol (vitamin A alcohol) with long-chain fatty acids by lecithin–retinol acyltransferase (LRAT) is an important step in both the absorption and storage of vitamin A. Retinol in cells is bound by either cellular retinol binding protein (CRBP), present in most tissues including liver, or cellular retinol binding protein type II [CRBP(II)], present in the absorptive cell of the small intestine. Here we investigated whether retinol must dissociate from these carrier proteins in order to serve as a substrate for LRAT by comparing Michaelis constants for esterification of retinol presented either free or bound. Esterification of free retinol by both liver and intestinal LRAT resulted in K_m values (0.63 and 0.44 μM, respectively) similar to those obtained for esterification of retinol–CRBP (0.20 and 0.78 μM, respectively) and esterification of retinol–CRBP(II) (0.24 and 0.32 μM, respectively). Because K_d values for retinol–CRBP and retinol–CRBP(II) are 10^{-8}–10^{-10} M, these similar K_m values indicated prior dissociation is not required and that direct binding protein–enzyme interaction must occur. Evidence for such interaction was obtained when apo-CRBP proved to be a potent competitive inhibitor of LRAT, with a K_I (0.21 μM) lower than the K_m for CRBP–retinol (0.78 μM). Apo-CRBP(II), in contrast, was a poor competitor for esterification of retinol bound to CRBP(II). Apo-CRBP reacted with 4 mM *p*-(chloromercuri)benzenesulfonic acid lost retinol binding ability but retained the ability to inhibit LRAT, confirming that the inhibition could not be explained by a reduction in the concentration of free retinol. Thus, LRAT was able to discriminate between the apo and holo forms of the two binding proteins. Ligand binding altered the physical properties of both CRBP and CRBP(II), as demonstrated by changes in elution position during size-exclusion and ion-exchange chromatography, perhaps providing the basis for differential recognition by LRAT. Previous work has shown retinoid binding proteins can restrict the metabolism of the bound retinoid to certain enzymes; here, specific interaction of a particular enzyme with apo and holo binding proteins modulated the extent of that metabolism.

Vitamin A is a fat-soluble micronutrient which is essential for vision, growth, differentiation, and morphogenesis. Retinol, the alcohol form of vitamin A, is extremely lipophilic and is delivered to target tissues bound to the plasma-specific retinol binding protein (RBP).[1] Intracellularly, the retinol is complexed with cellular retinol binding protein (CRBP) in most vitamin A sensitive tissues, including liver, and with CRBP(II) in the absorptive cells of the small intestine [for a review, see Blomhoff (1991)]. Esterification of retinol with long-chain fatty acids in vitro can be carried out by an acyl-CoA-dependent activity, acyl-CoA:retinol acyltransferase (ARAT), present in preparations of microsomes from liver (Ross, 1982), intestine (Helgerud et al., 1982; Ong et al., 1987), and other tissues (Randolph et al., 1991; Chaudhary & Nelson, 1987), or by another activity, lecithin–retinol acyltransferase (LRAT), also present in such microsomes (Ong et al., 1987, 1988; Yost et al., 1988; MacDonald & Ong, 1988a,b; Saari & Bredberg, 1989; Shingleton et al., 1989; Randolph et al., 1991). LRAT utilizes the fatty acid in the *sn*-1 position of phosphatidylcholine as the source of fatty acid for esterification (MacDonald & Ong, 1988a,b; Barry et al., 1989; Saari & Bredberg, 1989). LRAT can esterify retinol in vitro when it is presented bound to either of the two cellular retinol binding proteins, but ARAT requires that the retinol be provided free, under the conditions examined so far (Ong et al., 1987, 1988; Randolph et al., 1991).

The cellular binding proteins, CRBP and CRBP(II), have considerable sequence identity (57% for the rat proteins) (Li et al., 1986). Each is also highly conserved between species, with 97% identity for CRBP from rat and human (Colantuoni et al., 1985) and only 1 amino acid difference between human and rat CRBP(II) in the first 36 residues (Inagami & Ong, 1992). This high degree of sequence conservation between species suggests that recognition of these proteins by other cellular components may play an important role in their function. Recent work by Napoli and others has demonstrated an apparent recognition of CRBP and cellular retinoic acid binding protein, also highly conserved between species (Astrom et al., 1991), by enzymes involved in retinal synthesis in rat liver (Posch et al., 1991), retinyl ester hydrolysis in rat liver (Boerman & Napoli, 1991), and retinoic acid catabolism by rat testes (Fiorelli & Napoli, 1991). Here, we examined possible interactions between LRAT and CRBP and CRBP(II). LRAT differentially recognized the apo and holo forms of the two retinol binding proteins. Apo-CRBP was a potent inhibitor of LRAT while apo-CRBP(II) was not. This differential recognition of apo- and holoproteins by LRAT provides a mechanism to modulate the extent of esterification of vitamin A.

EXPERIMENTAL PROCEDURES

Materials. CRBP(II) was purified from rat intestine as previously described (Schaefer et al., 1989). CRBP was purified from an *Escherichia coli* strain (GW 5837) trans-

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[1] Abbreviations: LRAT, lecithin–retinol acyltransferase; ARAT, acyl-CoA:retinol acyltransferase; CRBP, cellular retinol binding protein; CRBP(II), cellular retinol binding protein type II; DTT, dithiothreitol; BHT, butylated hydroxytoluene; PMSF, phenylmethanesulfonyl fluoride; HPLC, high-performance liquid chromatography; pCMS, *p*-(chloromercuri)benzenesulfonic acid; DFP, diisopropyl fluorophosphate.

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formed with the pGX2608-CRBP plasmid, graciously provided by Donald Stump, Ph.D., and Frank Chytil, Ph.D. (Stump et al., 1991). Grown in LB media at 37 °C, the *E. coli* cells were harvested by centrifugation at 3300*g* for 30 min and then resuspended in 10 mM Tris, 1 mM EDTA, and 100 mM KCl, pH 7.5. The suspension was sonicated to lyse all cells and then centrifuged at 30000*g* for 30 min. The resulting supernatant liquid was concentrated by ultrafiltration with an Amicon YM 3 membrane to approximately 40 mL. The concentrated material was applied to a 5 cm × 78 cm Sephadex G-75 column equilibrated in 0.02 M Tris/acetate, pH 8.3, at a flow rate of 1.5 mL/min. The fractions were monitored for the ability to bind retinol. Fractions containing CRBP were pooled and concentrated to less than 5 mL. An LKB HPLC system equipped with a Tosohaas DEAE 5 PW column (15 cm × 20 cm) with a guard column was equilibrated in 0.05 M Tris/acetate, pH 8.3. After the concentrated material was loaded, a gradient from 0.05 to 0.33 M Tris/acetate, pH 8.3, was applied at a flow rate of 3 mL/min for 80 min. The fractions with the ability to bind added retinol were in a protein peak centered at 60 min. The purity of the protein was assessed both by SDS gradient gels (8–25%) and by sequence determination of the N terminus to 34 residues. Both procedures demonstrated pure protein corresponding to CRBP. This *E. coli*-expressed CRBP has been partially characterized previously (Stump et al., 1991).

Retinol was labeled at the C-15 position by reduction with NaB^3H_4 as previously described (Liau et al., 1981). Typical specific activity of the [^{3}H]retinol was 13–15 Ci/mmol. [^{3}H]-Retinol–CRBP and [^{3}H]retinol–CRBP(II) were prepared as described previously (Ong et al., 1987).

Me_2SO (spectrophotometric grade) and aluminum oxide (activated, neutral, Brockmann I) were from Aldrich. β-Mercaptoethanol was from Fluka. Dithiothreitol, *p*-(chloromercuri)benzenesulfonic acid, diisopropyl fluorophosphate, β-hydroxytoluene, and phenylmethanesulfonyl fluoride were from Sigma. Lipidex-1000 was from United Technologies Packard. All solvents used in the extraction procedure were HPLC-grade and were obtained from Sigma. The Statworks program is distributed by Cricket Software Inc.

Preparation of Microsomes. Rat liver microsomes were prepared as before with the resulting microsomal pellet resuspended in 0.2 M KH_2PO_4, pH 7.2, containing 1 mM DTT (Ong et al., 1988). Rat intestinal microsomes were prepared essentially as before (Ong et al., 1987). The intestines were removed from the rat, flushed with cold saline, opened with a longitudinal cut, and rinsed with three washes of cold saline. These rinsed intestines were then subjected to homogenization and centrifugation as before and the resulting microsomal pellets resuspended in 0.2 M KH_2PO_4, pH 7.2, containing 1 mM DTT.

Esterification Assay. The esterification assay for microsomal LRAT activity was as previously described (MacDonald & Ong, 1988). Either 4 μg of liver microsomal protein or 13 μg of intestinal microsomal protein was assayed in a final volume of 100 μL. The incubation mixture contained 0.2 M KH_2PO_4 in all cases and included 1 mM DTT unless otherwise noted. All assays were carried out under dim yellow light. For the kinetic analyses, [^{3}H]retinol was added dissolved in Me_2SO. A final concentration of 1% Me_2SO was maintained for all assays, a level that does not inhibit LRAT activity (Herr et al., 1991). Reactions were initiated by the addition of [^{3}H]retinol, [^{3}H]retinol–CRBP, or [^{3}H]retinol–CRBP(II) and incubated with shaking at 37 °C for 10 min. Reactions were stopped by the addition of 400 μL of cold EtOH containing 1 mg/mL BHT. The retinyl esters were extracted into 1.6 mL of hexane and isolated by aluminum oxide chromatography as described (Ong et al., 1988). The results are expressed as picomoles of [^{3}H]retinyl ester formed per minute per milligram of microsomal protein. The competition studies were performed as above but without Me_2SO in the reaction mixture. Increasing concentrations of retinol–CRBP, retinol–CRBP(II), apo-CRBP, apo-CRBP(II), or pCMS-treated-protein were added to the assay mixture, and the reaction was initiated by addition of 400 pmol of protein of either [^{3}H]-retinol–CRBP, for liver LRAT, or [^{3}H]retinol–CRBP(II), for intestinal LRAT. The reaction proceeded at 37 °C for 10 min, and the total [^{3}H]retinyl esters were determined as above. Radioactive ester production in the competition experiments is expressed as the percentage of the production observed in the absence of competitor.

Stability of the Retinol–CRBP Complex. Liver microsomes (2 mg/mL) were incubated at 37 °C with 5 mM PMSF (added in Me_2SO) for 10 min to inhibit LRAT activity. The fluorescence excitation spectra of retinol–CRBP were determined between 300 to 400 nm (band-pass 2 nm) with the emission set at 500 nm (band-pass 20 nm). Fluorescence measurements were taken on an SLM-Aminco SPF-500C spectrofluorometer. Fluorescence excitation spectra were compared for retinol–CRBP immediately after addition of 1 nmol to 500 μL of 0.2 M KH_2PO_4 containing 20 μg of the treated microsomal protein, after incubation of 1 nmol of retinol–CRBP at 37 °C for 10 min in 500 μL of 0.2 M KH_2PO_4, and after incubation of 1 nmol of retinol–CRBP at 37 °C for 10 min in 500 μL of 0.2 M KH_2PO_4 containing 20 μg of the treated microsomal protein.

Reaction of Binding Proteins with p-(Chloromercuri)benzenesulfonic Acid. Reaction of CRBP and CRBP(II) with *p*-(chloromercuri)benzenesulfonic acid (pCMS) was monitored by observing the loss of the typical fluorescence spectrum of bound retinol (MacDonald & Ong, 1987). For these studies, 1.8 mg of apo-CRBP or 1.7 mg of apo-CRBP(II) was incubated with 4 μmol of pCMS in 1-mL final volume with shaking in the dark at 25 °C. At various time intervals, 15-μL aliquots of the protein solution were removed (≈1.8 nmol of protein) and added to 285 μL of 0.2 M KH_2PO_4, pH 7.2, in a microcuvette with the further addition of 4.4 nmol of retinol in Me_2SO. The fluorescence excitation spectrum was then monitored between 300 and 400 nm (emmission set at 500 nm). Free retinol has considerably less fluorescence, without fine structure, under these conditions. Apo-CRBP required a 2-h incubation with 4 mM pCMS before complete loss of the ability to bind retinol was noted, while apo-CRBP(II) required 5 h for complete loss. Following treatment, the proteins were applied to a PD-10 Sephadex G-25M column (Pharmacia) equilibrated in 0.2 M KH_2PO_4, pH 7.2, to remove excess reagent. Modified proteins were used immediately.

To test the reversibility of the pCMS treatment, 20 mM β-mercaptoethanol was introduced to a sample containing 33 nmol of pCMS-treated CRBP, unable to bind retinol as monitored above. The sample was incubated with shaking in the dark at 25 °C. At various time intervals, a 15-μL aliquot of the protein sample (≈1.8 nmol) was added to 285 μL of 0.2 M KH_2PO_4 buffer with further addition of retinol as above and the fluorescence spectrum recorded. A time-dependent recovery of the typical retinol–CRBP fluorescence spectrum was observed, with 71% recovery after 90 min.

Comparison of Apo and Holo Binding Proteins by HPLC. In all cases, the HPLC studies were performed using protein from the same preparation. Holo-CRBP was obtained by

addition of at least 2-fold molar excess of retinol to purified apo-CRBP protein (1 mg/mL), followed by application to a 1-mL Lipidex-1000 column equilibrated in and eluted with 0.2 M KH_2PO_4, pH 7.2. Apo-CRBP(II) was obtained by irradiation of pure holo-CRBP(II) (1 mg/mL) with ultraviolet light as previously described (MacDonald & Ong, 1987), with subsequent Lipidex-1000 chromatography as above.

An LKB HPLC system was equipped with an LDC Milton Roy SM4000 programmable-wavelength detector. An LKB DEAE-5PW (8 cm × 75 mm) column was used for the ion-exchange studies. The column was equilibrated in 0.05 M Tris/acetate buffer, pH 8.3. All proteins were exchanged into this starting buffer prior to injection onto the column. For CRBP, approximately 1.6 nmol of apoprotein or 1.3 nmol of holoprotein was loaded onto the column and then eluted by application of a gradient from 0.05 to 0.33 M Tris/acetate, pH 8.3, over 20 min at a flow rate of 1 mL/min. For CRBP-(II), approximately 1.3 nmol of apoprotein or 1.6 nmol of holoprotein was loaded onto the column and then eluted with a gradient from 0.02 to 0.15 M Tris/acetate, pH 8.3, over 10 min at a flow rate of 1 mL/min.

Size-exclusion chromatography was carried out on a Toso-Haas TSK-Gel G3000SW_{XL} (30 cm × 7.8 mm) column, equilibrated in 0.1 M Na_2PO_4, pH 6.7, containing 0.1 M Na_2SO_4 and 0.05% NaN_3. Approximately 2.5 nmol of apo-CRBP, 1 nmol of holo-CRBP, 2 nmol of CRBP(II), or 2 nmol of holo-CRBP(II) was injected onto the column and eluted at 1 mL/min. The elution of apoproteins was monitored by their absorbance at 280 nm; holoproteins were monitored by their absorbance at 350 nm. The results for each of the HPLC trials are presented as the mean retention time ± standard deviation. The data were subjected to a two-way analysis of variance using the Statworks statistical program.

RESULTS

Comparison of Protein-Bound and Free Retinol as Substrate for LRAT. Microsomal LRAT produces retinyl esters when provided with retinol bound to either cellular retinol binding protein (CRBP) or CRBP(II) (Ong et al., 1987, 1988; Yost et al., 1988; Shingleton et al., 1989). In contrast, microsomal ARAT is essentially inactive when retinol is provided bound to the cellular proteins but is active if retinol is provided free (Ong et al., 1987, 1988; Helgerud et al., 1982; Ross, 1982). It is possible, however, that the true physiological substrate for LRAT is also free retinol, in equilibrium with the bound. Because the apparent K_m values for LRAT and ARAT differ considerably, the binding proteins could direct esterification primarily to LRAT, which has a considerably lower K_m, by limiting the free retinol concentration. If this were the case, one would then expect the apparent K_m of LRAT for esterification of free retinol to be significantly less than the K_m determined with protein-bound retinol, but this has not yet been examined. Consequently, kinetic analyses of LRAT-catalyzed esterification of retinol, provided both free and bound, were carried out for both liver and intestinal microsomes. Retinol provided free produced typical Michaelis–Menten curves for its esterification by LRAT, as shown in Figure 1. The Eadie–Hofstee plots (Figure 1, insets) resulted in a calculated K_m of 0.68 μM and a V_{max} of 64 pmol/[min·(mg of protein)] for intestinal LRAT and a K_m of 0.67 μM and a V_{max} of 69 pmol/[min·(mg of protein)] for liver LRAT, in the examples shown. Similar studies with retinol–CRBP and retinol–CRBP(II) were then carried out with the same microsomal preparations for comparison, and the averages of a number of determinations are presented in Table I. The

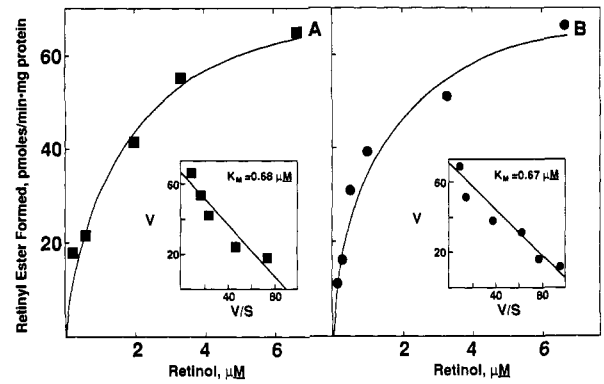

FIGURE 1: Effect of retinol concentration on rate of esterification by microsomal LRAT. Increasing amounts of [^{3}H]retinol (in Me_2SO) were added to reaction mixtures containing either 13 μg of intestinal microsomal protein (panel A) or 4 μg of liver microsomal protein (panel B) in 0.2 M KH_2PO_4 with a final assay volume of 100 μL. Reaction time was 10 min at 37 °C. Radioactive ester production was analyzed by chromatography on alumina columns as described (Ong et al., 1988). The averages of two determinations are shown. The inset is the Eadie–Hofstee transformation of the data used to calculate K_m and V_{max}.

Table I: Kinetic Parameters for Esterification of Free vs Bound Retinol by LRAT

	intestinal microsomes		liver microsomes	
	K_m (μM)	V_{max} (pmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (pmol min^{-1} mg^{-1})
retinol	0.63 ± 0.30[a]	54 ± 13	0.44 ± 0.23	80 ± 15
retinol–CRBP	0.20 ± 0.10	33 ± 12	0.78 ± 0.09	76 ± 6
retinol–CRBP(II)	0.24 ± 0.04	47 ± 3	0.32 ± 0.02	61 ± 5

[a] Each value is the average of at least four determinations ± SEM.

only statistically significant difference in K_m between free retinol and protein-bound retinol was seen in the intestinal microsomes where the K_m for free retinol was larger than that for retinol–CRBP ($p < 0.05$).

Although it is clear that free retinol was an effective substrate for LRAT (and the carrier proteins were not *required*), the K_m values determined indicated that the binding proteins could not be simply serving as a reservoir to provide *free* retinol but must be presenting retinol directly to LRAT. This was clear when the degree of dissociation expected in this system was considered. The values reported for the apparent K_d range from 1 × 10^{-8} to 1 × 10^{-10} M for retinol–CRBP (Ong & Chytil, 1978; Ong et al., 1987; Levin et al., 1988; Noy & Blaner, 1991; Li et al., 1991) and (1–3) × 10^{-8} M for retinol–CRBP(II) (Ong et al., 1987; MacDonald & Ong, 1987; Levin et al., 1988). One can calculate from the higher estimated value for K_d of 1.0 × 10^{-8} M for retinol–CRBP that the free retinol concentration present at 0.2 μM retinol–CRBP (the K_m concentration for LRAT from intestinal microsomes) would be only 0.04 μM. This then should be equivalent to the measured K_m for free retinol, if free retinol were the required substrate. However, the measured K_m for free retinol was 0.63 μM, 17-fold higher. Analyses of the other values in Table I by this procedure would result in similar dramatic differences (data not shown).

However, because the reported K_d values had been determined for pure proteins, it was possible that the membranes of the microsomal preparation might be serving as a sink for free retinol, shifting the equilibrium. Loss of retinol from CRBP to plasma membranes had previously been observed (Noy & Blaner, 1991). Consequently, we examined the stability of the retinol–CRBP complex under the conditions of the assay in the presence of the microsomal preparation.

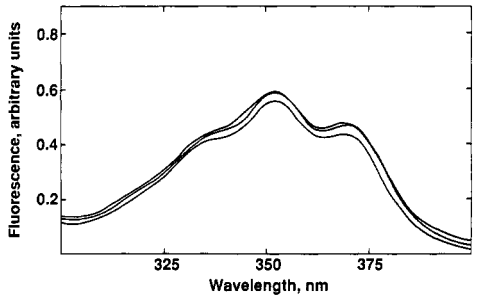

FIGURE 2: Stability of the retinol–CRBP complex in the presence of liver microsomes. Fluorescence excitation spectra were recorded for 1 nmol of retinol–CRBP for the following three conditions: immediately after addition to 20 μg of liver microsomal protein, previously treated with 5 mM PMSF; after 10 min at 37 °C in the presence of 20 μg of treated microsomal protein; and after 10 min at 37 °C in the presence of buffer only. Final volume of 300 μL in all three cases. The tracings shown are representative of two separate trials.

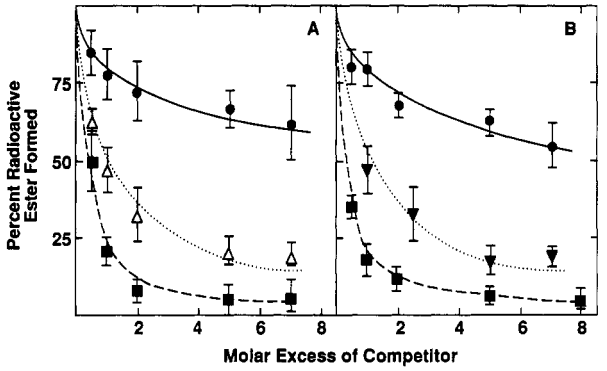

FIGURE 3: Reduction in radioactive retinyl ester formation by the addition of apo and holo binding protein. Either 13 μg of intestinal microsomal protein (panel A) or 4 μg of liver microsomal protein (panel B) was assayed by initiating the reactions with 400 pmol of [^{3}H]retinol–CRBP(II) for intestinal LRAT (panel A) or 400 pmol of [^{3}H]retinol–CRBP for liver LRAT (panel B). The assays contained increasing concentrations of apo-CRBP (■), apo-CRBP(II) (●), retinol–CRBP (▼), or retinol–CRBP(II) (△) and were analyzed as described under Experimental Procedures. The results are given as the percentage of radioactive ester formed in the absence of any additional binding protein and are the average of at least four determinations (±standard deviation).

As shown in Figure 2, no detectable loss of the characteristic retinol–CRBP fluorescence was observed after a 10-min incubation at 37 °C in the presence of a microsomal preparation, indicating no significant perturbation due to the presence of microsomes. LRAT in these preparations had been previously inactivated by PMSF to avoid loss of retinol from CRBP by esterification. Consequently, these results indicate that dissociation of retinol from the binding protein was not increased by the presence of the microsomes in this system, in contrast to the observations of others noted above. Therefore, the above comparisons of K_m values versus K_d values remain valid. Either retinol must be transferred directly from the binding proteins to the active sites of LRAT or the bound retinol itself served as substrate without being removed from the carrier protein.

Differential Recognition of Binding Proteins by Microsomal LRAT. The ability of LRAT to interact directly with the retinol binding protein complexes raised the question of whether the apoproteins might also be recognized by the enzyme. To test for this possibility, increasing amounts of apo binding protein were added to the standard incubation mixture containing either [^{3}H]retinol–CRBP or [^{3}H]retinol–CRBP(II) to see if the amount of radioactive retinyl ester formed would be reduced, indicating competition between the apo and holo forms for LRAT. To provide a basis for comparison, the effect of the addition of increasing amounts of binding protein complexes with unlabeled retinol, which would, of course, compete with [^{3}H]retinol binding protein complex for LRAT, was also determined. Addition of increasing amounts of retinol–CRBP(II) to [^{3}H]retinol–CRBP(II) and of retinol–CRBP to [^{3}H]retinol–CRBP indeed reduced the recovery of radioactive ester produced by LRAT, with the expected 50% reduction observed at approximately equal molar ratios (Figure 3). In contrast, apo-CRBP(II) did not appear to be recognized well by LRAT because it was not an effective competitor of ester production. A 7-fold molar excess of apo-CRBP(II) only reduced ester formation by 35% (Figure 3, left panel), indicating that LRAT was able to discriminate between retinol–CRBP(II) and the apoprotein. It should be noted that, at the concentration of [^{3}H]retinol–CRBP(II) employed in the assay, the addition of a 7-fold molar excess of apo-CRBP(II) would reduce the free retinol concentration about 100-fold, on the basis of a K_d of 3×10^{-8} μM for the complex. This again indicates that free retinol cannot be the required substrate for LRAT.

Results obtained with CRBP proved to be surprisingly different. Apo-CRBP was a potent competitor of retinol esterification. A 2-fold molar excess of apo-CRBP reduced esterfication by 87% (Figure 3, right panel), indicating that LRAT had a stronger interaction with the apoprotein than with the holoprotein. These results were independent of the source of LRAT as apo-CRBP was also an effective competitor for esterfication by intestinal LRAT and apo-CRBP(II) had little effect on esterfication by liver LRAT (Figure 3). The heterologous transfer of retinol from one binding protein to the other can occur though it does not appear to be a problem in this system due to the similarity in reaction between retinol–CRBP and retinol–CRBP(II) (Table I, Figure 3).

The potent inhibition by apo-CRBP, in contrast to the results with apo-CRBP(II), might suggest that these effects could still be due to a reduction in the free retinol concentration, particularly because CRBP has been estimated to have a considerably greater affinity for retinol than does CRBP(II) (Li et al., 1991). To eliminate this possibility, apo-CRBP and apo-CRBP(II) were reacted with *p*-(chloromercuri)benzenesulfonic acid (pCMS), which is known to block the retinol binding ability of the two proteins (Li et al., 1986), producing modified proteins which could then be tested as competitors in the LRAT esterfication assay. The loss of retinol binding ability was carefully monitored by two different methods. First, retinol was added to aliquots of the proteins, taken during the course of reaction with pCMS, and the amount of fluorescence enhancement and the characteristic fine structure of the fluorescence excitation spectrum that is indicative of retinol binding were determined. Two hours of incubation with 4 mM pCMS resulted in loss of the ability of apo-CRBP to bind retinol when compared to untreated protein, as assessed by this measure (Figure 4). Addition of retinol to the treated protein gave the same retinol fluorescence spectrum as seen for addition of retinol to buffer alone. Similar results were obtained when apo-CRBP(II) was incubated with 4 mM pCMS (data not shown).

Because it was possible that the modified proteins were still binding retinol but had simply lost the ability to produce the fluorescence enhancement normally seen, a second test of binding ability was conducted. An aliquot of pCMS-reacted protein was combined with [^{3}H]retinol and subjected to chromatography on Lipidex-1000, which removes unbound retinol (Timmers et al., 1986). Radioactivity in the eluate from the

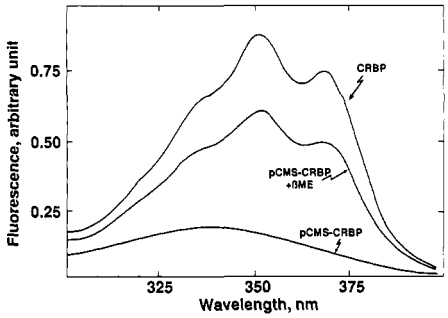

FIGURE 4: Reversible blockage of CRBP retinol binding ability by *p*-(chloromercuri)benzenesulfonic acid. Fluorescence excitation spectra are shown for 1.8 nmol of CRBP in 0.2 M KH_2PO_4 after mixture with 4.4 nmol of retinol ("CRBP"), for 1.8 nmol of CRBP, preincubated with 4 μmol of pCMS, prior to addition of retinol ("pCMS–CRBP"), and for pCMS-treated CRBP after incubation with 20 mM β-mercaptoethanol prior to the addition of retinol ("pCMS–CRBP + βME"). Spectra were obtained as described under Experimental Procedures.

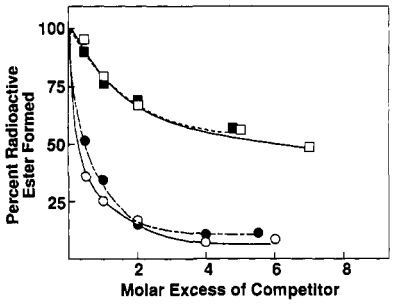

FIGURE 5: Comparison of LRAT inhibition by native and treated apo binding proteins. Increasing concentrations of apo-CRBP (○), pCMS-treated CRBP (●), apo-CRBP(II) (□), or pCMS-treated CRBP(II) (■) were added to 4 μg of liver microsomal protein, and the assay for LRAT activity was initiated by addition of 400 pmol of [^{3}H]retinol–CRBP. Assays were analyzed as described under Experimental Procedures. The results are expressed as the percentage of radioactive ester produced in the absence of competitor and are the average of two determinations.

Lipidex-1000 column was determined to measure the retinol binding ability of the reacted protein. Ovalbumin, which does not bind retinol, was added to all samples (1 mg/mL) immediately prior to Lipidex-1000 chromatography to prevent nonspecific adsorption of the low levels of binding protein being tested. With this procedure, the radioactivity recovered with the pCMS-reacted proteins was less than 5% of the radioactivity recovered with unreacted binding proteins, indicating loss of at least 95% of retinol binding ability for the modified proteins. This confirmed the results from the fluorescence studies and demonstrated that the modified proteins had lost most if not all ability to bind retinol. The treatment did not permanently alter the protein as addition of β-mercaptoethanol to the treated protein produced a time-dependent recovery of retinol binding ability (71% recovered after 90 min; longer times were not examined) (Figure 4).

The modified proteins were then compared to the apoproteins as competitors for [^{3}H]retinol–CRBP esterfication by liver LRAT. Each proved to be as potent as the corresponding native protein (Figure 5). These assays contained no DTT to avoid reversal of pCMS treatment of the binding protein. This similarity in competition between treated and native protein clearly indicated that retinol binding was not involved in the potent inhibition of LRAT activity by apo-CRBP but that LRAT was able to specifically recognize the apoprotein. Interestingly, modification of the proteins did not appreciably alter this recognition.

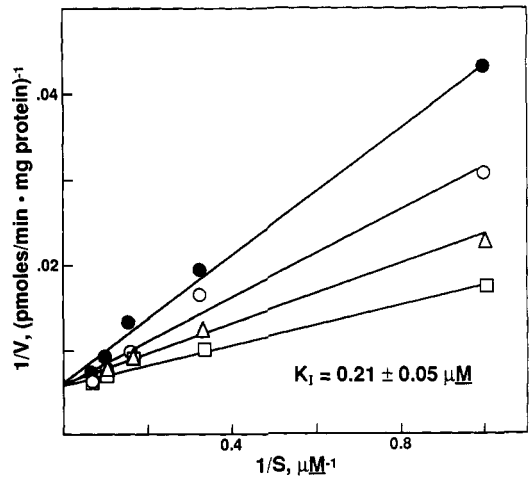

FIGURE 6: Competitive inhibition of LRAT by apo-CRBP. Three concentrations of apo-CRBP were added to an assay mixture containing 4 μg of liver microsomal protein and assays initiated by addition of increasing amounts of [^{3}H]retinol–CRBP. The production of radioactive retinyl esters was analyzed as described under Experimental Procedures. The Lineweaver–Burk transformations are shown for the absence of competitor (□) and for the presence of 0.25 μM apo-CRBP (△), 0.5 μM apo-CRBP (○), and 0.75 μM apo-CRBP (●). The K_I was calculated for each data set by using the slope of the reciprocal plot in the following equation: $\text{slope}_{1/S} = (K_m/V_{max})\{1 + [I]/K_I\}$. The average of the three calculated K_I values is shown ± the standard deviation.

Characterization of Apo-CRBP Inhibition of LRAT. To determine if the inhibition of LRAT by apo-CRBP was indeed competitive, and to measure the K_I of inhibition, the concentration dependence for ester formation by LRAT with [^{3}H]-retinol–CRBP as substrate was determined in the presence of three different concentrations of apo-CRBP (0.25, 0.50, and 0.80 μM). The Lineweaver–Burk transformation of these data is shown in Figure 6, demonstrating competitive inhibition of LRAT by apo-CRBP, with a calculated K_I of 0.21 ± 0.05 μM. This K_I value is significantly less than the K_m of 0.78 μM determined for retinol–CRBP ($p < 0.01$) and is therefore consistent with the differential recognition of apo- and holo-CRBP by LRAT (Figure 3). These studies were not extended to apo-CRBP(II), but clearly the K_I for apo-CRBP(II) would be appreciably greater than the K_m for retinol–CRBP(II) given the modest reduction in ester formation observed in the presence of a 7-fold molar excess of apoprotein. Therefore, the strengths of the interactions between LRAT and the two apo binding proteins differed, with apo-CRBP interacting more strongly with LRAT than did retinol–CRBP while apo-CRBP-(II) interacted less strongly with LRAT than did retinol–CRBP(II).

Effects of Retinol Binding on the Physical Properties of CRBP and CRBP(II). The demonstration that LRAT was able to discriminate between the apo and holo forms of the same protein as well as between the two apoproteins (Figure 3) suggested that conformational change might occur upon binding of ligand. To look for evidence of such change, we examined certain physical properties of the apo- and holoproteins.

First, binding of the uncharged retinol ligand was found to change the charge properties of both proteins. High-performance anion-exchange chromatography resulted in significantly different retention times for the apo and holo forms of the two proteins (Table II). Apo-CRBP was retained 0.44 min longer on the column then holo-CRBP under identical conditions and thus was more anionic, while apo-CRBP(II) had a shorter retention time than holo-CRBP(II) by 0.14 min, and was therefore less anionic in character, at pH 8.3.

Table II: Effect of Retinol Binding on Physical Properties of CRBP and CRBP(II)[a]

		CRBP retention time (min)		CRBP(II) retention time (min)	
	trial	apo[b,d]	holo	apo[c,e]	holo
ion exchange	1	17.45 ± 0.01 (3)[f]	17.01 ± 0.01 (3)	8.56 ± 0.02 (4)	8.70 ± 0.02 (4)
size exclusion	1	13.40 ± 0.03 (7)	13.34 ± 0.01 (6)	ND[g]	ND
	2	13.32 ± 0.02 (3)	13.28 (1)	13.24 ± 0.02 (3)	13.28 ± 0.01 (2)
	3	13.29 ± 0.01 (8)	13.29 ± 0.01 (6)	13.23 ± 0.01 (8)	13.28 ± 0.02 (7)
	4	13.37 ± 0.01 (6)	13.35 ± 0.01 (6)	13.32 ± 0.01 (6)	13.35 ± 0.02 (7)

[a] An LKB HPLC system with an LKB-DEAE-5PW column was used for ion-exchange chromatography for the apo and holo forms of both CRBP and CRBP(II) as described under Experimental Procedures. Size-exclusion chromatography was carried out on a TosoHaas TSK-Gel G3000SW$_{XL}$ column as described under Experimental Procedures. [b] Ion-exchange elution time significantly different from holo-CRBP ($p < 0.001$) (Student's t-test). [c] Ion-exchange elution time significantly different from holo-CRBP(II) ($p < 0.001$) (Student's t-test). [d] Size-exclusion elution time significantly different from holo-CRBP ($p < 0.001$) and significantly different from apo- and holo-CRBP(II) ($p < 0.001$) (Statworks). [e] Size-exclusion elution time significantly different from holo-CRBP(II) ($p < 0.001$) and apo- and holo-CRBP ($p < 0.001$) (Statworks). [f] Each value is the average of the number of determinations (n) ± standard deviation. [g] Not determined.

Second, ligand binding altered the elution time for the two proteins when subjected to size-exclusion chromatography (Table II). The two holo binding proteins had identical retention times, as might be expected from their considerable similarities in molecular weight [15 430 for CRBP(II) versus 15 700 for CRBP] and expected similarities in overall conformation. However, compared to the corresponding apoproteins, the binding of retinol (formula weight 286.5) to the proteins had altered elution times and in an opposite manner. Apo-CRBP proved to have a longer retention time (i.e., appeared smaller) than the holoproteins, while apo-CRBP(II) had a shorter retention time (i.e., appeared larger) than the holoproteins. The retention times for a particular day were extremely reproducible, yet there was obvious variation between trials on separate days. As a result, two-way analysis of variance (ANOVA) using the Statworks statistical program was employed to demonstrate that the differences in elution times between apo- and holo-CRBP, between apo- and holo-CRBP(II), and between the two apoproteins were significant. Additional trials using other size-exclusion columns produced differences similar to those reported here (data not shown). The apparent differences in Stokes radii detected by size-exclusion chromatography observed here, as well as the charge differences detected by ion-exchange chromatography, indicated that binding of ligand had altered the physical properties of both proteins, possibly providing the means by which LRAT might discriminate between the apo and holo forms of the proteins without actually recognizing the bound retinol, per se.

DISCUSSION

Regulation of Vitamin A Storage. The liver plays a central role in maintaining vitamin A homeostasis. Newly ingested vitamin A is transported as retinyl esters to the liver via the chylomicrons. The esters are then hydrolyzed to retinol which is either stored, after esterification by LRAT, or secreted to the blood, bound to retinol binding protein (Blomhoff et al., 1991). Balancing the extent to which newly ingested vitamin A is either stored in the liver or secreted to the circulation would appear to be an important part of vitamin A homeostasis. The demonstration that apo-CRBP was a potent inhibitor of liver LRAT provides a mechanism by which production of retinyl esters for storage would decrease when the supply of retinol is low, insufficient to keep CRBP highly saturated. That apo-CRBP *inhibits* LRAT is in contrast to, but works in concert with, the recent observation that apo-CRBP *stimulates* the cholate-independent retinyl ester hydrolase present in liver microsomes (Boerman & Napoli, 1991). Thus, the synthesis and hydrolysis of retinyl esters in the liver, two activities intimately involved in maintaining retinol homeostasis, are both directly affected by levels of apo-CRBP, which in turn is sensitive to the vitamin A status of the animal. Interestingly, oxidation of retinol–CRBP to retinal, the first step in retinoic acid synthesis, is insensitive to apo-CRBP (Posch et al., 1991). Consequently, production of one of the hormonal forms of vitamin A would not be adversely affected by lack of saturation of CRBP.

To eliminate the possibility that hydrolysis of endogenous ester might have perturbed our measurement of LRAT activity here, we treated liver microsomes with diisopropyl fluorophosphate (DFP) (1 mM), a serine hydrolase inhibitor which has no effect on LRAT activity (Herr et al., 1991) but does inhibit the esterase (Boerman & Napoli, 1991). Results with the DFP-treated microsomes were identical to nontreated microsomes (data not shown), indicating that under the conditions employed here hydrolysis of any endogenous ester present had not perturbed our determination of LRAT activity.

Prolonged vitamin A deficiency has been shown to actually result in a decrease of liver microsomal LRAT levels which would inhibit storage in the chronically deficient state (Randolph & Ross, 1991). The attenuation of liver LRAT activity by apo-CRBP provides a mechanism to respond more rapidly to changes in intracellular retinol levels. As retinol levels decreased, the increase in apo-CRBP levels, relative to holo-CRBP levels, would inhibit the esterification and subseqeunt storage of retinol, but increase hydrolysis. Thus, there would be an immediate response sensitive to CRBP saturation levels, followed by an ensuing decrease in enzyme level with prolonged deficiency, allowing the animal to control esterification of retinol in times of vitamin A deficiency.

Retinol Esterification in the Intestine. The lack of substantial inhibition of intestinal LRAT activity by apo-CRBP(II) also meets the physiological needs of the animal. Efficient intestinal processing of vitamin A requires that intestinal LRAT be able to esterify ingested vitamin A especially during periods of low intake. The level of CRBP(II) in the intestine is quite high compared to the level of CRBP in the liver (1% vs 0.02% of soluble protein) (Ong, 1987). Consequently, significant inhibition of LRAT by apo-CRBP(II) would impede vitamin A processing dramatically during low intake. The insensitivity of LRAT activity to the apo-CRBP(II) level works in concert with the report that the intestinal LRAT level, in contrast to the liver LRAT level, remains unchanged during prolonged periods of vitamin A deficiency (Randolph & Ross, 1991). Therefore, the effects of apo binding proteins on LRAT activity observed here complement the previous study on regulation of the overall LRAT levels in both liver and intestine.

CRBP(II) Expression in Liver during the Perinatal Period. Our previous work has shown that CRBP(II), normally

restricted to the absorptive cell of the small intestine, is transiently expressed in rat liver during the perinatal period (Levin et al., 1987; Ong et al., 1988). CRBP(II) appears at about day 19 of gestation, and levels peak several days after birth, being higher than CRBP levels during this period. The amount of CRBP(II) then gradually declines until undetectable around day 14. This transient expression of CRBP(II) parallels an increase in the retinyl ester level in the liver, which is apparently then mobilized in the midsuckling period (Ismadi et al., 1982; Zachman et al., 1984; Levin et al., 1987). CRBP, in contrast, is present in the liver as early as the 10th day of gestation (Kato et al., 1985) and remains throughout life. Interestingly, immunohistochemical studies on the perinatal liver have revealed that the two proteins have different patterns of expression. Staining for CRBP(II) is primarily in hepatocytes near the central vein, at the center of the liver lobule (Ong et al., 1988), while, during this time period only, staining for CRBP in hepatocytes is higher for the hepatocytes at the periphery of the lobule (Kato et al., 1985; Ong et al., 1988). The reason for the expression of CRBP(II) during the perinatal period and the differential lobular distribution of the two retinol binding proteins may now be clear. Circulating vitamin A, upon entering the hepatic tissue, would first encounter and be taken up by hepatocytes at the periphery of the lobule, cells containing CRBP. Unless cellular retinol levels achieved were sufficient to saturate CRBP, esterification by LRAT in these cells would be inefficient due to the presence of apo-CRBP. As the remaining circulating vitamin A (presumably reduced in level) approached the center of the lobule, it would now be taken up by cells containing CRBP(II). Because apo-CRBP(II) does not inhibit esterification of retinol–CRBP(II) by LRAT, the internalized retinol would be available for esterification even if levels achieved were low. In this manner, the perinatal liver, due to the transient expression of CRBP(II), would be able to increase retinyl ester stores even when the supply of retinol might be insufficient to saturate all binding protein. Such stores could then be utilized during the transition from placental delivery of nutrients to mammary gland delivery during suckling.

Other Intracellular Lipid Binding Proteins. Studies on the fatty acid binding proteins [within the same superfamily as CRBP and CRBP(II)] have revealed properties similar to those observed here. Ligand binding has been shown to alter the conformation of liver fatty acid binding protein although intestinal fatty acid binding protein remained unchanged (Nemecz et al., 1991). In addition, ligand binding altered the ability of an adipocyte fatty acid binding protein, 422-(aP2), to interact with the insulin receptor. There, fatty acid binding allowed certain residues to become available for phosphorylation by the insulin receptor tyrosine kinase and the K_m for phosphorylation to decrease 60-fold (Hresko et al., 1990). This phosphorylation is thought to be important for insulin action on the adipocyte (Yang et al., 1989). Thus, members of the FABP family can exhibit conformational change upon ligand binding, and in one case, such change alters the interaction with another cellular component, similar to what was observed here for the cellular retinol binding proteins.

Roles of CRBP and CRBP(II). CRBP and CRBP(II) are products of separate, closely-linked genes on the same chromosome (Demmer et al., 1987). Undoubtedly derived from a common ancestor, they have retained only 56% sequence identity (Li et al., 1986) yet do display remarkable similarities in retinoid binding specificities (MacDonald & Ong, 1987; Levin et al., 1991). We report here that the two apoproteins interact quite differently with LRAT. The physiological need for two separate cellular retinol binding proteins may lie, therefore, not in differences in their interactions with bound ligand but instead in differences in recognition and interaction of the proteins with the enzymes directly involved in the metabolism of the bound ligand. The high degree of conservation of sequence between species for each protein would argue such interactions are very important in the overall roles of these two proteins.

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