Expression of Cellular Retinoic Acid Binding Protein (CRABP) in *Escherichia coli*

**CHARACTERIZATION AND EVIDENCE THAT HOLO-CRABP IS A SUBSTRATE IN RETINOIC ACID METABOLISM**

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Cellular retinoic acid binding protein (CRABP) has been expressed efficiently in *Escherichia coli* from the cDNA of bovine adrenal CRABP and characterized, especially with respect to affinity for endogenous retinoids and a role for it in retinoic acid metabolism. The purified *E. coli*-expressed CRABP was similar to authentic mammalian CRABP in molecular weight (~14,700), isoelectric point (4.76), absorbance maxima (apo-CRABP, 280 nm; holo-CRABP, 350 and 280 nm with the ratio A350/A280 = 1.8), and in fluorescence excitation (350 nm) and emission spectra (475 nm). The equilibrium dissociation constant, Kd, of *E. coli*-derived CRABP and all-trans-retinoic acid was 10 ± 1 nM (mean ± S.D., n = 4) by retinoid fluorescence and 7 ± 1 nM (mean ± S.D., n = 3) by quenching of protein fluorescence, but neither retinol nor retinal bound in concentrations as high as 7 μM. All-trans,cyclohexyl ring derivates of retinoic acid (3,4-didehydro-, 4-hydroxy-4-oxo-, 16-hydroxy-4-oxo-, 18-hydroxy-) had affinities similar to that of all-trans-retinoic acid, whereas 13-cis-retinoic acid and 4-oxo-13-cis-retinoic acid had ~25-fold lower affinity. Holo-CRABP was a substrate for retinoic acid catabolism in rat testes microsomes by three criteria: 1) the rate of retinoic acid metabolism with CRABP in excess of retinoic acid exceeded the rate supported by the free retinoic acid; 2) increasing the apo-CRABP did not decrease the rate as predicted if free retinoic acid were the only substrate; and 3) holo-CRABP had a lower Michaelis constant (1.8 nM) for retinoic acid elimination than did free retinoic acid (49 nM). These data indicate a direct role for CRABP in retinoic acid metabolism and suggest a mechanism for discriminating metabolically between all-trans- and 13-cis-retinoids.

Retinoic acid, a physiologically occurring metabolite of retinol, is the paradigm for retinoids, a class of naturally occurring and synthetic compounds that are potent modulators of transcription (for reviews see Refs. 1–3). Retinoic acid is the most active naturally occurring retinoid and most likely is responsible for mediating directly many vitamin A-dependent processes (4). Retinoic acid is the major endogenous ligand recovered from CRABP5 (5, 6), a specific retinoic acid binding protein that is distributed widely throughout retinoid target tissues (7, 8).

The function of CRABP is not well defined, and CRABP does not seem to be obligatory for gross retinoid responsiveness. CRABP does not appear to be a retinoic acid receptor: it lacks the DNA binding zinc finger common to many DNA binding proteins (9); it does not bind chromatin (10); and it is 3-fold smaller than and fundamentally different in amino acid sequence from the members of two distinct families of retinoic acid receptors (11–16). Nor is there a simple relationship between the affinity of retinoids for CRABP and their biological activities in diverse assays (17–22). At least one retinoid-responsive cell line, HL-60, expresses no CRABP yet differentiates when treated with low concentrations of retinoic acid (23). Reducing the steady-state concentration of CRABP in F9 embryonal carcinoma cells with antisense RNA does not prevent the nuclear localization of retinoic acid and subsequent F9 cell differentiation (24). A role for CRABP has been suggested in the transfer of retinoic acid from the cytosol to nuclear retinoic acid receptors, but this is contraindicated by the differential distribution of CRABP mRNA and the mRNA of at least some of the retinoic acid receptors in developing mouse limbs (25).

The function of CRABP may be simply to modulate the steady-state concentrations of retinoic acid and thereby alter cellular responses to retinoic acid (26, 27). If so, then involvement of CRABP directly in retinoic acid metabolism, as a substrate and/or a regulatory factor, may be an aspect of this function. To provide readily accessible apo- and holo-CRABP for structural, analog, and metabolic studies, we constructed an *Escherichia coli* CRABP expression vector. This paper reports the expression, purification, and characterization of *E. coli*-derived CRABP and presents evidence that the binding protein-ligand complex, holo-CRABP, is a substrate in retinoic acid metabolism.

**MATERIALS AND METHODS**

*General*—[11,12-*H*]Retinoic acid (23.3 Ci/mmol) was purchased from Amer sham Corp. and was purified by reverse-phase HPLC. Retinoid standards were a gift from Dr. Peter Sorter, Hoffmann- LaRoche, Nutley, NJ. Reagents used for oligonucleotide synthesis were purchased from American Bionetics Inc. Restriction enzymes and DNA ligase were purchased from New England BioLabs Inc.

1 The abbreviations used are: CRABP, cellular retinoic acid binding protein; HPLC, high performance liquid chromatography; FPLC, fast-protein liquid chromatography; IPTG, isopropyl-β-D-thiogalactoside; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)- propane-1,3-diol; SDS, sodium dodecyl sulfate; FAGE, polyacrylamide gel electrophoresis.
Beverly, MA. Components of the bacterial medium were purchased from the microbiological division of Becton Dickinson, Cockeysville, MD. Other chemicals were purchased from Sigma. Retinoic acid metabolism experiments and HPLC analyses were done under yellow fluorescent lights.  

Construction of a CRABP Expression Vector—Two single-stranded complementary oligonucleotides homologous to the first 15 amino acids of CRABP with an NdeI site at the start codon were synthesized with a Biosearch DNA Synthesizer (Millipore Corp.): 5'-TATGCCCCAACTTGCGTGACACGAGGAAAGCGCAGCGAAATTTT-3', and 3'-ACGGTTGGAGCCGCTTGAATCTTACGCGTCGCTGCTTAAAGC-5'. The single-stranded oligonucleotides were purified by 15% PAGE and the two strands were hybridized. This oligonucleotide was used to construct an NdeI site at the 5' terminus of CRABP cDNA. The cDNA of bovine adrenal CRABP was obtained from Dr. Anne M. McNamara (National Institute on Aging) as a PstI/BamHI restriction fragment in pUC18 (28). The PstI/BamHI cDNA fragment was isolated and then was digested with TaqI to provide a 633-base pair fragment that encoded encoded amino acids 16 through the carboxyl terminus of CRABP. The 633-base pair TaqI fragment (100 ng) was ligated with a 3-fold molar excess of the synthetic oligonucleotide to recreate the complete coding sequence of CRABP cDNA. This NdeI/BamHI CRABP cDNA was ligated into the unique NdeI and BamHI sites in the vector PET-3a. The ligation mixture was used to transform E. coli strain TB-1 by the CaCl2 precipitation method. Ten randomly chosen colonies were analyzed by restriction endonuclease analysis and all contained plasmid pET-3a/CRABP cDNA. One of these was designated pET-3a/CRABP and was used in subsequent studies.  

Expression of CRABP in E. coli—The plasmid pET-3a/CRABP was used to transform E. coli K12 strain BL21(DE3)/pLyssX (F ompT rfa1). The overnight culture (1 ml) was inoculated with 300-μl aliquots of the concentrate to an A595 of 0.6 at 595 nm. At this time, to induce the synthesis of T7 RNA polymerase, IPTG was added at a concentration of 0.4 mM. The mixture was centrifuged at 10,780 × g for 10 min. The pellet was discarded, and the supernatant was centrifuged at 110,000 × g for 1 h. The microsomal pellet was rehomogenized on 10 ml of buffer C with a PE type tissue grinder. The microsomal protein (25 mg/ml) was stored at −70 °C. Protein was determined by the dye-bonding method with bovine serum albumin as standard (32).  

Retinoic Acid Metabolism—Incubations were done in duplicate at 37 °C in a shaking water bath. The 0.5-ml incubation mixture contained 150 mM KCl, 5 mM MgCl2, 50 mM Tris-HCl (pH 7.4), and an NADPH regenerating system (2.5 units of glucose-6-phosphate dehydrogenase, 500 nmol of NADP, and 0.5 μmol of glucose-6-phosphate) and microsomal protein as noted in the figure legends. The reaction was initiated by adding [3H]retinoic acid or a preferred apo-CRABP/iso-CRABP mixture containing [3H]retinoic acid. Immediately before quenching the reactions retinoid standards were added in 20 μl of methanol: 4-oxo-retinoic acid, 18-hydroxy retinoic acid, 4-hydroxyretinoic acid, all-trans-retinoic acid. The reactions were quenched by placing the tubes in Dry Ice/2-propanol. The samples were lyophilized and each residue was extracted with 2 ml of 2 N methanolic HCl. The methanolic extract was treated with 2 ml of 50 mM sodium acetate buffer. The mixture was centrifuged at 10,780 × g for 30 min. The supernatant was applied to a Sephadex G-50 column (5 × 50 mm) equilibrated with 200 mM NaCl, 50 mM Bis-Tris-HCl (pH 7.0), 2 mM β-mercaptoethanol, and 0.05% sodium azide. The first 15 ml of effluent was collected and analyzed for retinoid content. The second 15 ml of effluent was collected and analyzed for retinoid content. This fraction was used for the ligand binding assays as a PstI/BamHI restriction fragment in pUC18 (28). The transformed cells were suspended in 1 ml of buffer A containing 125 mM NaCl (buffer B). CRABP was eluted with 125 mM NaCl, 50 mM bis-Tris-HCl (pH 7.0), 2 mM β-mercaptoethanol, and 0.05% sodium azide. A plot of Po versus Ro (a/l) where Po = initial fluorescence intensity, yields a straight line with a slope of 1/n and a y intercept of Kd/n.  

Preparation and Quantification of Holo-CRABP—Purified CRABP (150 μg in 0.5 ml of buffer B) was incubated with a 1.5 molar excess [3H]retinoic acid (1.4 μCi/ml) at room temperature for 1 h. To remove excess unbound [3H]retinoic acid, the mixture was eluted from a Superose-12 column with buffer B at a flow rate of 0.5 ml/min. The fractions (1 ml each) containing CRABP were identified by liquid scintillation counting of aliquots for tritium. Generally, four fractions were pooled and concentrated in a Centricon 10 filter at 5000 × g for 30–60 min. The amount of holo-CRABP in the concentrated fractions was determined by obtaining the absorbancespectra between 500 and 220 nm. A molar absorbity of 50,000 at A595 was used to determine the amount of holo-CRABP.  

Preparation of Testes Microsomes—Male Sprague Dawley rats (3-5 per group) were killed by decapitation. The testes were removed, and homogenized (3 ml of tissue) in 10 mM Tris-HCl, 250 mM sucrose (pH 7.4) (buffer C) in a Potter-Ehlejhem type tissue grinder with a Teflon pestle. The homogenate was centrifuged at 10,000 × g for 10 min. The pellet was discarded, and the supernatant was centrifuged at 110,000 × g for 1 h. The microsomal pellet was rehomogenized on 10 ml of buffer C with a PE type tissue grinder. The microsomal protein (25 mg/ml) was stored at −70 °C. Protein was determined by the dye-bonding method with bovine serum albumin as standard (32).
produces a modest amount of T7 lysozyme, a T7 polymerase inhibitor which prevents constitutive expression of T7 promoter-dependent genes. This inhibition is overcome by IPTG, which induces synthesis of relatively large amounts of T7 polymerase (29, 30). E. coli transformed with the vector pET-3a/CRABP produced a species with the molecular mass of CRABP (~14.7 kDa) upon induction with IPTG; the protein was not detected in uninduced cells (Fig. 2), nor was it detected in induced cells that had been transformed with pET-3a (not shown). About 25% of the CRABP was soluble. The remainder precipitated in the pellet formed by low-speed centrifugation.

Purification of E. coli-synthesized CRABP—The CRABP in the supernatant was purified by chromatography through a Sephadex G-50 column (Fig. 2). The yield was 50–100 mg of CRABP/500 mg of supernatant protein, depending upon the specific batch. Pretreatment of the whole cell lysate with DNase before preparation of the supernatant was crucial to recovery. Attempts to purify CRABP in the absence of DNase treatment were unsuccessful. The Sephadex G-50-purified CRABP was analyzed by anion-exchange chromatography as described in detail under “Materials and Methods.” The new sequence vector pET-3a/CRABP.

Characterization of E. coli-generated CRABP—Proteins I and II were subjected to isoelectric focusing on an agarose gel (Fig. 4). Protein I was homogeneous and had an isoelectric point of 4.76. Protein II contained a major band with an isoelectric point of 4.79 and a barely detectable band with an isoelectric point of 4.83. The isoelectric point of bovine CRABP has not been published. The value for protein I, determined by Edman degradation, was 16574 residues because it contained a blocked amino-terminal methionine residue. Because protein I was the major product and

FIG. 1. Strategy for the construction of the CRABP expression vector pET-3a/CRABP. The gray box in the CRABP cDNA represents the complete coding region of CRABP spanning amino acids 1–136. The sequence CCATGCCC of the native cDNA was changed by digestion at the Taq I site and ligation of a 48-base pair synthetic nucleotide containing the sequence TATGCC, as described in detail under “Materials and Methods.” The new sequence was ligated into pET-3a to create the pET-3a/CRABP vector. This vector has the translation initiation signal (the Shine Delgarno box) s10 between the unique Nde I site and the 410 T7 promoter, a φT transcription terminator downstream of a BamH cloning site, and confers resistance to ampicillin. The stippled box in the pET-3a/CRABP vector represents the complete coding region and the 3′-flanking sequence of the cDNA of CRABP.

FIG. 2. Analysis of the expression and purification of E. coli CRABP. Samples were fractionated by electrophoresis on a 15% SDS-polyacrylamide gel. Lane 1, molecular mass markers; lane 2, whole cell lysate of transformed E. coli not treated with IPTG (100 μg); lane 3, whole cell lysate of transformed E. coli induced with IPTG (100 μg); lane 4, 10,780 × g supernatant of the induced cell lysate (120 μg); lane 5, 10,780 × g pellet of the induced cell lysate (125 μg); lane 6, Sephadex G-50 of the supernatant analyzed in lane 4 (2 μg); lane 7, protein I from anion-exchange chromatography of the proteins recovered from the Sephadex G-50 analyzed in lane 6 (2.8 μg); lane 8, Superose-12 chromatography of protein I recovered from anion-exchange chromatography analyzed in lane 7 (1.5 μg). The numbers on the ordinate represent the apparent molecular mass of the protein standards (kilodaltons). The bands were visualized by Coomassie Blue staining.

FIG. 3. FPLC analysis of E. coli-generated CRABP. An aliquot of the pool from Sephadex G-50 chromatography was analyzed by anion-exchange chromatography as described in detail under “Materials and Methods” (left panel). Two peaks eluted: protein I at 21 min and 100 mM NaCl and protein II at 23 min and 140 mM NaCl. Integration of the area under the peaks indicated that protein I contained 60% of the total of proteins I and II. The dashed line indicates the NaCl gradient. Protein I was re-analyzed by size-exclusion chromatography (right panel).
homogeneous and had the characteristics of authentic mammalian CRABP, all subsequent work was done with CRABP from protein I, unless noted otherwise.

The UV/vis spectra of *E. coli*-derived apo-CRABP had an absorbance maximum at 280 nm and no absorbance beyond 320 nm (Fig. 5). Saturation of apo-CRABP with all-trans-retinoic acid and separation of the excess retinoic acid by size-exclusion FPLC-produced holo-CRABP that had a spectrum with maxima at 350 and 280 nm. The ratio \( A_{350}/A_{280} \) was 1.8, indicating pure CRABP (39-42). The fluorescence excitation spectra of *E. coli*-holo-CRABP had a maximum at 350 nm with a shoulder at 355 nm and a smaller peak at 290 nm. The excitation spectrum of holo-CRABP was less complex with a single peak at 475 nm. Both the UV/vis spectra and the fluorescence spectra of *E. coli*-derived CRABP are practically identical to the spectra of CRABP purified from rat testes (39, 40), bovine retina (41, 42), and human placenta (35).

**Affinities of Retinoids for *E. coli*-generated CRABP** —The affinities of retinoids for the *E. coli*-derived CRABP were measured by two methods: increased retinoid fluorescence upon binding, for those retinoids that did increase fluorescence upon binding, and quenching of CRABP fluorescence upon retinoid binding, because not all retinoids increase their fluorescence upon binding to retinoid binding proteins. Representative data for both methods are shown for all-trans-retinoic acid, 13-cis-retinoic acid, retinal, and retinol (Fig. 6). The equilibrium dissociation constants, \( K_d \) values, and number of binding sites, \( n \), were calculated as described under "Material and Methods" by linearizing the fluorescence data and determining the slopes and intercepts by least squares regression analyses. Each method yielded similar results for those retinoids that could be evaluated by both approaches. By either method the number of binding sites for retinoids on CRABP (protein I) was 0.9. The \( K_d \) values determined by retinoid fluorescence were not different markedly from those determined by quenching of CRABP fluorescence (Table I). The \( K_d \) of 7-10 nM for protein I and all-trans-retinoic acid was in good agreement with the value of 4.2 nM determined by retinoic acid fluorescence for CRABP isolated from rat testes (39, 40). The \( K_d \) of protein II was also determined to be (average of duplicate determinations) 10 nM by retinoic acid fluorescence and 19 nM by quenching of protein fluorescence. Neither retinol nor retinal bound to *E. coli*-produced CRABP (protein I). These data confirm that *E. coli*-derived CRABP is similar to mammalian CRABP in its ability to bind retinoic acid and discriminate against retinol and retinal. It is important to note that retinal was used soon after purification by HPLC. Retinal stored longer than 1 week had binding affinity midway between retinol and 13-cis-retinoic acid, presumably resulting from accumulation of retinoic acid through artificial oxidation, even though it had been stored at ~20 °C.

Although IC\(_{50}\) values have been obtained for partially purified CRABP and various retinoids, \( K_d \) values have not been determined for pure CRABP and retinoids other than all-trans-retinoic acid. The \( K_d \) values obtained here for 4-hydroxyretinoic acid and 4-oxo-retinoic acid indicated about 3-fold less affinity than all-trans-retinoic acid. The reported IC\(_{50}\) values of the C(4)-oxidized retinoids range from 2-fold more potent to 2-fold less potent than radioinert all-trans-retinoic
The results of the limitations of the experimental methodology, whether acid in competition with \(^{3}H\)retinoic acid for rat testes or work) or competition with all-trans-retinoic acid for binding to semi-purified mixtures of holo-CRABP and apo-CRABP were summarized in Table I.

CRABP with various retinoids by measuring quenching acid by each method are similar to those obtained with the C(4)-derivatized retinoids and indicate that catabolic oxidation of the cyclohexyl ring results with the C(18) and C(16)-derivatized retinoids are little effect on binding and that vitamin A derivatives (i.e., Didehydro-all-trans-retinoic acid, not reported previously, reduces, but does not eliminate, affinity for CRABP. 3,4-16-hydroxy-4-oxo-retinoic acid have not been reported previously and were assayed here because they are possible endogenous retinoids. The metabolism in the presence of free retinoic acid in equilibrium with that of all-trans-retinoic acid, 4-oxo-13-cis-retinoic acid was tested to corroborate the central role of the double bond nearest the carboxyl terminus in lowering the affinity for CRABP. 4-Oxo-13-cis-retinoic acid had an affinity 26-fold lower than all-trans-retinoic acid and 9-fold lower than 4-oxo-all-trans-retinoic acid, reinforcing the conclusion that changes in the carboxyl terminus, i.e. either oxidation state or geometry, affect affinity more than changes in the cyclohexyl ring. The \( K_d \) of 13-cis-retinoic acid has not been reported previously using pure CRABP or binding between ligand and apo-CRABP, and IC\(_{50}\) data are in substantial disagreement.

Using competition with all-trans-retinoic acid for semi-pure mixtures of apo-CRABP and holo-CRABP as a measure, 13-cis-retinoic acid has been reported to be as potent or slightly more potent that all-trans-retinoic acid with CRABP from rat testes and skin (18, 43), 7-fold less potent with CRABP from human skin (45), and 30- to 50-fold less potent with CRABP from rat skin (44) or embryonic hamsters (22). Inherent differences in the CRABP are not likely to be determining factors, because CRABP in highly conserved throughout species. The quality of the CRABP, the methods themselves, or the purity of the samples may have affected the results. For example, because there is chemical equilibrium between all-trans-retinoic acid and 13-cis-retinoic acid which favors the all-trans-isomer, samples of 13-cis-retinoic acid used without purification or stored for long periods could contain substantial amounts of all-trans-retinoic acid. 13-cis-Retinoic acid used in the present study was pure by HPLC.

Holo-CRABP as Substrate in Retinoid Acid Metabolism—Rat testes have one of the highest concentrations of CRABP, ~1.2 nmol/g tissue (40). Therefore testes microsomes were used to test whether CRABP is a substrate for retinoic acid catabolism. The metabolism of unbound \(^{3}H\)retinoic acid was compared with that of \(^{3}H\)retinoic acid bound to an excess of CRABP (Fig. 7). In both cases, HPLC analysis showed production of very polar metabolites that eluted in fractions 1–5 (peak I). A second peak was observed in fractions 7 and 8, with a polarity intermediate between the polar metabolites and retinoic acid that migrated with the 4-hydroxyretinoic acid standard (peak II).

\begin{table}
\caption{Retinoid binding properties of \( E.\) coli-derived CRABP}
\begin{tabular}{ll}
\hline
Retinoid & \( K_d' \) & \( F_{\text{retinoid}} \) & \( F_{\text{CRABP}} \) \\
\hline
All-trans-RA & 10 \pm 1\(^b\) & 7 \pm 1\(^b\) & \\
3,4-Didehydro-RA & 7 & 6 & \\
4-Hydroxy-RA & 9 & 17 & \\
4-Oxo-RA & - & 20 & \\
16-Hydroxy-4-oxo-RA & - & 20 & \\
18-Hydroxy-RA & 15 & 22 & \\
13-cis-RA & - & 156 \pm 8\(^c\) & \\
4-Oxo-13-cis-RA & - & 183 & \\
Retinal & - & NB\(^d\) & \\
Retinol & - & NB & \\
\hline
\end{tabular}
\end{table}

\(^a\) \( F_{\text{retinoid}} \) determined by fluorescence of the retinoid upon binding to CRABP. \( F_{\text{CRABP}} \) determined by quenching of CRABP fluorescence upon binding of the retinoid.

\(^b\) Means \( \pm \) S.D. \( n=4 \) (all-trans-RA) and (13-cis-RA)). All other values are averages of two independent determinations.

\(^c\) Retinoid did not fluoresce upon binding to CRABP.

\(^d\) NB, no binding with concentrations to 7 \( \mu M \).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Representative data for determining the ligand binding properties of \( E.\) coli-derived CRABP. Top panel, titration of 1 \( \mu M \) CRABP with all-trans-retinoic acid as detected by enhancement of retinoic acid fluorescence (filled circles) or quenching of CRABP fluorescence (open circles). Middle panel, titration of CRABP with various retinoids by measuring quenching of protein fluorescence: all-trans-retinoic acid (open circles), 13-cis-retinoic acid (open squares), retinol (filled squares), retinol (open triangles). Bottom panel, linearization of the data in the top panel by the method of Cogan et al. (31). The symbols represent the same data shown in the top panel. The \( K_d \) values were calculated from \( K_d = (y \text{ intercept}) \times \frac{1}{\text{slope}}. \) The slopes and \( y \text{ intercepts} \) were calculated by linear regression analysis of each set of data. The \( y \text{ intercept} \) for the retinoid fluorescence data was 0.015, and the slope was 1.11. The \( y \text{ intercept} \) for the CRABP quenching data was 0.112, and the slope was 1.25. The results of four independent determinations for all-trans-retinoic acid by each method are summarized in Table I.

Because the \( K_d \) of 13-cis-retinoic acid was 22-fold lower than that of all-trans-retinoic acid, 4-oxo-13-cis-retinoic acid was tested to corroborate the central role of the double bond nearest the carboxyl terminus in lowering the affinity for CRABP.

\begin{align*}
K_d &= (y \text{ intercept}) \times \frac{1}{\text{slope}} \quad \text{(1)} \\
F_{\text{retinoid}} &= \text{fluorescence data was 0.125, and the slope was 1.11. They intercept for the retinoid fluorescence data was 0.112, and the slope was 1.25. The results of four independent determinations for all-trans-retinoic acid by each method are summarized in Table I.}
\end{align*}
CRABP serving as substrate (8 nM in this experiment as calculated from the \( K_d \) of 10 nM). To distinguish between these two possibilities, the kinetic constants were determined for retinoic acid metabolism in the presence and absence of CRABP.

With either free retinoic acid as substrate or in the presence of CRABP, the rate of retinoic acid catabolism was substrate-dependent (Fig. 8). The kinetic constants (averages from two experiments) were 49, 53, and 44 nM for the \( K_a \), of total metabolites, peak I and peak II, respectively, from \([^{3}H]\)retinoic acid, with \( V_{max} \) values of 9.9, 6.7, and 3.3 pmol/min/mg of microsomal protein, respectively. With holo-CRABP as substrate, the \( K_a \) values (averages of two experiments) were 1.8, 0.8, and 2.4 nM for total metabolites, peak I and peak II, respectively, with \( V_{max} \) values of 2.6, 1.1, and 1.5 pmol/min/mg of protein, respectively. In the experiments that generated the latter data, a constant molar ratio of total CRABP to \([^{3}H]\) retinoic acid of 3/1 was used. Under these conditions, the concentration of unbound retinoic acid present would vary from 2.2 nM with a total retinoic acid concentration of 5 nM to 4.6 nM with a total retinoic acid concentration of 75 nM, calculated with a \( K_d \) of 10 nM. From these data and the kinetic constants of free retinoic acid, the rate of formation of total metabolites can be calculated as 0.4 and 0.8 pmol/min/mg protein, respectively. The rates actually observed were 3- to 4-fold higher: 1.2 and 3.3 pmol/min/mg protein, respectively. Similar results were obtained with the remaining concentrations of CRABP and total metabolites and with the peak I and peak II metabolites. Thus the rate of retinoic acid metabolism in the presence of an excess of CRABP is higher than can be accounted for by metabolism of only the free retinoic acid, i.e. holo-CRABP is substrate for retinoic acid catabolism and its affinity for catabolizing enzyme(s) is 27-fold higher than that of free retinoic acid (Michaelis constants of 1.8 versus 49 nM, respectively, for total metabolite production).

An independent indication that holo-CRABP is substrate was provided by determining the effect on retinoic acid metabolism of titrating increasing concentrations of apo-CRABP into a fixed concentration of \([^{3}H]\) retinoic acid. The effect of increasing the binding protein would be to markedly decrease the concentration of free ligand, with a negligible increase in the concentration of bound ligand. This would have a small effect on the rate of metabolism if holo-binding protein were substrate but a large effect if only free ligand were substrate. The rates observed for the production of total metabolites, or peaks I and II individually, relative to the rates at a 1/1 ratio of total CRABP/\([^{3}H]\) retinoic acid decreased by 35% with an increase in total CRABP to 600 nM, i.e. with 4-fold more apo-CRABP than holo-CRABP (Fig. 9). In contrast, if only free retinoic acid were substrate the rate should have decreased 8-fold over this range of apo-CRABP concentrations. The small decrease with the higher concentrations of apo-CRABP might reflect competition between apo-CRABP and holo-CRABP.

The time course of metabolism of 80 nM retinoic acid in the presence of 2 molar equivalents of total CRABP was determined, because this concentration of ligand is in the physiological range (3), and total CRABP is in excess of retinoic acid in vivo (40). For comparison, the time course was determined of the metabolism of a similar concentration of free \([^{3}H]\) retinoic acid (Fig. 10). The latter data were used to calculate the rate of metabolism from the amount of free retinoic acid in equilibrium with CRABP under these experimental conditions. The formation of total metabolites and peaks I and II individually were 2- to 5-fold greater in the presence of CRABP than can be accounted for by metabolism of only the free retinoic acid. These results strengthen the evidence that holo-CRABP itself is substrate for retinoic acid metabolism and suggest that in the presence of excess CRABP, as occurs physiologically in many retinoid target tissues, the quantitatively major route of retinoic acid catabolism proceeds through CRABP-bound retinoic acid, not free retinoic acid.

Concluding Summary—This work describes the efficient
production and purification of mammalian CRABP from E. coli. The CRABP purified from E. coli was identical, within experimental limits, to authentic mammalian CRABP in $M_r$, isoelectric point, sequence of the first 10 amino acids, UV/vis and fluorescence spectra, affinity for all-trans-retinoid acid, and its ability to discriminate against retinol and retinal. CRABP has been purified from several mammalian sources, but it is a low abundance protein typically requiring kilograms of tissue to provide several milligrams of pure protein, and its purification is tedious and costly, limiting the available quantities of pure protein (40). Moreover, CRABP recovered from mammalian sources is largely in the holo form, which limits the experiments that can be done with it. The procedures described here allow rapid and inexpensive production of this protein in the pure state in either the holo or apo forms.

Three criteria indicate that holo-CRABP is a substrate for retinoic acid metabolism: the rate of retinoic acid metabolism in the presence of CRABP was faster than the rate supported by the free retinoic acid in equilibrium with holo-CRABP; increasing the concentration of apo-CRABP did not result in the decrease in the rate of metabolism predicted if only free retinoic acid were substrate; and CRABP had higher affinity (lower $K_a$) for metabolizing enzymes than did free retinoic acid. These results show that there is no requirement for spontaneous retinoic acid release from holo-CRABP and diffusion through the aqueous medium to undergo catabolism and are consistent with direct transfer of retinoic acid from CRABP to the catabolizing enzyme(s).

CRABP has no established direct function in the mechanism of transcription modulation by retinoids, but it may buffer the concentration of free retinoic acid. Gradients of CRABP have been postulated to accentuate retinoic acid gradients in developing limbs and thereby affect the nature of retinoid-dependent responses, i.e. to allow retinoic acid to function as a morphogen (26). Alternatively, recent reports (46, 47) have indicated that retinoic acid is not a morphogen, because gradients of retinoic acid are not generated and are not integral to determining the nature of retinoic acid-induced responses during limb development. In this case the function of CRABP may be to protect sensitive cells from relatively high concentrations of retinoic acid (27). Our results show that CRABP modulates the concentrations of free retinoic acid not only by sequestering retinoic acid but also by serving as a conduit for its efficient catabolism.

The lower affinity of 13-cis-retinoids compared with all-trans-retinoids for CRABP is in agreement with the results of multiple binding studies, which have shown that changes in the C(15) terminus have large effects on ligand affinity whereas changes in the cyclohexyl ring have more modest effects (18, 22, 43–45). It is also consistent with what is known about the structure of CRABP. CRABP belongs to a family of lipid binding proteins which includes other retinoid binding proteins, such as cellular retinol binding protein (types I and II) and fatty acid binding protein (48). Each family member binds specific ligands and is characterized by a “β-clamp” pocket formed by orthogonal β-sheets. The binding pockets are in the interiors of these proteins, not on their surfaces, and ligands occupy the binding pockets with their functional groups, e.g. the carboxyl group of retinoic acid, facing the interior (49). It is reasonable to postulate that the differences in affinity between 13-cis-retinoic acid and all-trans-retinoic acid for CRABP contribute to the differences in their pharmacological effects. The major route of metabolism of pharmacological doses of 13-cis-retinoic acid in the rat begins with its conversion into all-trans-retinoic acid (50). This observation, the lesser biological activity of 13-cis-retinoic acid (51), its lower affinity for CRABP, and the relatively rapid rate of retinoid clearance from CRABP, considered together, suggest that 13-cis-retinoic acid provides a reservoir which relatively slowly releases the more active and toxic all-trans-retinoic acid for mediation of retinoid action and relatively rapid catabolism.

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REFERENCES