Identification of the Endogenous Retinoids Associated with Three Cellular Retinoid-binding Proteins from Bovine Retina and Retinal Pigment Epithelium*

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John C. Saari, Lucille Bredberg, and Gregory G. Garwin
From the Department of Ophthalmology RJ-10, University of Washington School of Medicine, Seattle, Washington 98195

The endogenous retinoids associated with three cellular retinoid-binding proteins from bovine retina and retinal pigment epithelium have been identified by their spectral characteristics and their co-migration with authentic retinoids on high performance liquid chromatography. All-trans-retinol is the only retinoid associated with the cellular retinol-binding protein from retina and retinal pigment epithelium. The saturation of the binding site with native ligand is 0.95 ± 0.05 mol of retinoid/mol of protein for cellular retinol-binding protein purified from frozen and fresh retina and retinal pigment epithelium. All-trans-retinoic acid has been identified as the endogenous ligand associated with the cellular retinoic acid-binding protein of cattle retina. The saturation of the binding site of cellular retinoic acid-binding protein from frozen and fresh retina (the protein is absent in extracts of retinal pigment epithelium) is about 0.2 mol of retinoid/mol of protein, probably a minimum value due to losses of the ligand. Retinoic acid has not been detected previously in retina. Cellular retinaldehyde-binding protein from retina purifies with two endogenous ligands which co-chromatograph on high performance liquid chromatography with 11-cis-retinaldehyde and 11-cis-retinol and occur in a ratio of approximately 3:1, respectively. The binding site of cellular retinaldehyde-binding protein from frozen retina is nearly fully occupied with these two ligands (saturation >0.9 mol of retinoids/mol of protein). In contrast, purified cellular retinaldehyde-binding protein from retinal pigment epithelium carries only 11-cis-retinaldehyde as an endogenous ligand. The saturation of the binding site with this ligand is >0.95.

In 1973 Bashor et al. (1) reported the discovery of a cellular protein derived from lung extracts, which was capable of binding vitamin A. Since then there have been many reports of similar proteins in other tissues and of additional proteins with specificities directed toward other forms of vitamin A (reviewed in Ref. 2). Three cellular retinoid-binding proteins have been purified and partially characterized. Cellular retinol-binding protein and cellular retinoic acid-binding protein have been purified from many sources (3-8). The two proteins from bovine retina appear to be structurally related (9). A third protein, cellular retinaldehyde-binding protein has been purified from retina and retinal pigment epithelium and appears to be confined to these tissues (10, 11). Retinoid-binding proteins were detected initially because of their ability to form complexes with added radioactive retinoid. Although the binding of exogenous retinoids is of interest, the presumed role of the binding proteins in the metabolism and function of vitamin A would be more tenable if they were also shown to carry retinoids as endogenous ligands. In addition, the identities of these endogenous retinoids might provide information important in ascribing functional roles to the proteins. Previous workers have addressed some aspects of this problem. The endogenous retinoid associated with CRBP1 from rabbit lung has been identified as a retinol (geometrical isomer undetermined) (12, 13) while all-trans-retinol was identified as the ligand associated with CRBP from rat testis (14). In addition, an endogenous retinoid was noted to purify with CRABP (8) from rat testis. This report demonstrates that each of these three retinoid-binding proteins from cattle retina carries endogenous retinoid, provides an identification of the ligands and the approximate saturation of the binding proteins with endogenous ligand, and shows that the endogenous ligand composition of one of the proteins depends on the tissue of origin. Bovine retina and retinal pigment epithelium were used as sources of the proteins since the two tissues are known to play different roles in the enzymatic processing of vitamin A. A preliminary account of portions of this work has appeared previously (15).

MATERIALS AND METHODS AND RESULTS

DISCUSSION

The chromatographic and spectral studies reported here demonstrate that all-trans-retinol is the ligand associated with CRBP from retina and retinal pigment epithelium. Since CRBP could be purified to homogeneity without substantial loss of ligand, we were able to determine the amount of associated all-trans-retinol by quantitative HPLC analysis of the extracted ligand. In addition, we employed the fluorescent

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cence saturation procedure (see “Materials and Methods”) and also compared spectral ratios (A500/A330) of preparations with endogenous ligands to that obtained with protein fully saturated with exogenous all-trans-retinol. The agreement of the results from all three methods is a convincing demonstration that the binding site is nearly fully occupied with endogenous all-trans-retinol. It was not unexpected that the endogenous ligand associated with CRBP from retina would be all-trans-retinol, since other studies of the same or a similar protein from other tissues had reached this conclusion (12-14). However, it was surprising that no 11-cis-retinol was found associated with purified CRBP since the protein is capable of binding cis-retinols (this study and Ref. 16) and 11-cis-retinol is present in the extract, bound to CRALBP. This example of compartmentalization of isomers of retinol may be due simply to a difference in affinity of the two proteins for 11-cis-retinol. However, it may also reflect something more fundamental such as metabolic routing of retinoids, determined by the binding protein with which they interact. Insufficient evidence is available to decide this issue at present, but our recent demonstration that 11-cis-retinol and 11-cis-retinaldehyde bound to CRALBP can be interconverted by a dehydrogenase from retinal pigment epithelium suggests the latter possibility (17). We also feel this isomeric compartmentalization argues against the possibility that the retinoids became associated with the binding proteins after disruption of cellular compartments allowed access to pools of retinoid normally sequestered, and strengthens our confidence that we are sampling the physiological ligands associated with the binding proteins.

The identification of all-trans-retinoic acid as the endogenous ligand associated with CRBP is based on its co-migration with authentic all-trans-retinoic acid and, following methylation, with the methyl ester of all-trans-retinoic acid. A quantitative determination of the saturation of the binding sites of CRBP was not possible for several reasons. First of all, we were unable to purify CRBP to homogeneity without substantial losses of ligand. In addition, apo-CRABP separates from holo-CRABP during step 3 of the purification procedure and is quite unstable. Finally, we were unable to determine the concentration of CRABP in partially purified preparations since antibodies to CRABP are not available. The fluorescence saturation technique, however (see “Materials and Methods”), allowed a semiquantitative estimation of the occupancy of the binding sites with endogenous chromophore in partially purified preparations of the protein. The value obtained (approximately 0.2) represents the saturation of the binding sites for a preparation taken two steps through the normal purification procedure and probably is a minimum value. Retinoic acid has not been detected previously in retina and no direct function in vision has ever been attributed to this retinoid (18). If the retinoid plays a role in the physiology of the retinal neuroepithelium, it may be related to its presumed role in other epithelial tissues in maintaining a differentiated state (18).

CRALBP was similar to CRBP in that we were able to purify the protein to homogeneity without substantial losses of ligand, provided care was taken to exclude white light. Thus both quantitative analysis by HPLC and a comparison of the spectral ratios (for CRALBP from retinal pigment epithelium) were used in estimating the saturation of the binding sites. A previous study from this laboratory (10) provided indirect evidence for the presence of 11-cis-retinol and 11-cis-retinaldehyde complexed with CRALBP from frozen cattle retina. Employing spectral and HPLC identification of the ligands, we were able to confirm that conclusion; however, some variation was found in the ratios of 11-cis-retinol and 11-cis-retinaldehyde associated with CRALBP from retina. Retina and retinal pigment epithelium are known to differ in their roles in the metabolic processing of vitamin A. Retinal pigment epithelium is apparently the initial recipient of bloodborne retinol (19), possesses potent enzymatic machinery for the esterification of the vitamin (20) and a dehydrogenase specific for 11-cis-retinol (21) and serves as a site for storage of retinyl esters in some species (22, 23). Retina, on the other hand, has relatively little esterification activity (22), a dehydrogenase relatively specific for all-trans-retinol (21), and, of course, is the site of visual pigment synthesis and photoreception. It is now apparent that CRALBP from these two tissues differs in its complement of endogenous retinoids. CRALBP from retina carries both 11-cis-retinol and 11-cis-retinaldehyde, but the protein from retinal pigment epithelium carries only 11-cis-retinaldehyde. We feel that the differing complement of endogenous retinoids associated with CRALBP from retina and retinal pigment epithelium probably reflects the differing metabolism of the vitamin in these two tissues and suggests that CRALBP participates in this metabolic process. The ability to determine the endogenous retinoids associated with the binding proteins from different tissues and under different conditions may provide important clues to our understanding of the contributions of these tissues to the metabolism of the vitamin and to the role of the binding proteins in this process.

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REFERENCES

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Materials and Methods

Materials. Samples of 9-cis-, 13-cis-, and 13-cis-retinoic acid and retinol were derived from Dr. W. W. Bear of Bethel University, Welford, IA. 13-cis- and 13-cis-retinoic acid were prepared by different authors using the retinoic acid synthesis method described by Bear et al. (3). All-cis-retinoic acid, all-cis-retinol and all-cis-retinyl acetate were purchased from Sigma Chemical Co. All-cis-retinoic acid and all-cis-retinyl acetate were prepared from the all-cis-retinyl acetate described by Bear et al. (3) and from the all-cis-retinyl acetate prepared by Bear et al. (3). All-cis-retinyl acetate was prepared from all-cis-retinol and all-cis-retinyl acetate were prepared from Bear et al. (3). All-cis-retinyl acetate and all-cis-retinyl acetate were prepared from Bear et al. (3). All-cis-retinyl acetate and all-cis-retinyl acetate were prepared from Bear et al. (3).

Assays. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A computer program for the determination of the fluorescence properties of the protein-bound retinoids was used to determine the fluorescence properties of the protein-bound retinoids. The computer program was used to calculate the fluorescence properties of the protein-bound retinoids. The computer program was used to calculate the fluorescence properties of the protein-bound retinoids.

Results

Cellular retinoid-binding protein. The cellular retinoid-binding protein was isolated from human placental tissue. The protein was isolated from human placental tissue. The protein was isolated from human placental tissue. The protein was isolated from human placental tissue. The protein was isolated from human placental tissue. The protein was isolated from human placental tissue.

WAVELENGTH (nm)

Fig. 3. Absorption spectrum of retinoid-binding protein complex with endogenous ligand. The protein has been purified to homogeneity from extracts of frozen rabbit retina.

Fig. 2. Normal phase HPLC separation of the retinyl esters of retinoid-binding protein complex with endogenous ligand. The protein has been purified to homogeneity from extracts of frozen rabbit retina.

The absorption spectrum of the binding sites with retinol ligands was determined by quantitative analysis of the extracted retinoids by HPLC. To determine the maximum number of moles per end of protein, a group of the partially purified CRBP was stained with 1-butanol-toluene and analyzed by HPLC. The mass of endogenous ligand was determined by quantitative analysis of the extracted retinoids by HPLC. The mass of endogenous ligand was determined by quantitative analysis of the extracted retinoids by HPLC. The mass of endogenous ligand was determined by quantitative analysis of the extracted retinoids by HPLC. The mass of endogenous ligand was determined by quantitative analysis of the extracted retinoids by HPLC.
Retinoids Associated with Retinoid-binding Proteins

Figure 1. Absorption spectra in ethanol of endogenous retinoids extracted from partially purified CRBP and purified by HPLC. The absorption spectrum of authentic all-trans-retinoic acid in ethanol shows an absorption maximum of 325 nm.

Figure 2. HPLC separation of the methyl esters of two natural ligands of retinoic acid, retinol and retinoic acid. The peaks marked 1 and 2 are due to 13-cis-retinoic acid and all-trans-retinoic acid, respectively. Lower panel: Endogenous retinoid extracted from CRBP and acetone.

Figure 3. Absorption spectrum of partially purified CRBP from human retinal cells.

Figure 4. Fluorescence quenching procedure to determine the association of the binding sites of partially purified CRBP with retinoic acid. A panel obtained by gel filtration was applied to a Sephadex column with (panel A) and without (panel B) preincubation with all-trans-retinoic acid. A solution of CRBP was used to show CRBP 

Fraction Number

Figure 5. HPLC separation of two natural ligands of retinoic acid. Upper panel: Standards. The peaks labeled 1 and 2 are due to 13-cis-retinoic acid and all-trans-retinoic acid, respectively. Lower panel: Endogenous ligand extracted from a sample of partially purified CRBP from human retinal cells. The major component present co-chromatographs with all-trans-retinoic acid.
Retinoids Associated with Retinoid-binding Proteins

Figure 6. Absorption spectrum of CRALBP and endogenous ligand purified from retinal pigment epithelium. The presence of an absorption maximum at 353 nm is due to endogenous 11-cis-retinal, in present in purified CRALBP and CRALBP-like proteins (data not shown).

Figure 7. Absorption spectrum of CRALBP and endogenous ligand purified from retinal pigment epithelium. The presence of an absorption maximum at 353 nm is due to endogenous 11-cis-retinal, in present in purified CRALBP and CRALBP-like proteins (data not shown).

Figure 8. Absorption spectrum of CRALBP and endogenous ligand purified from retinal pigment epithelium. The presence of an absorption maximum at 353 nm is due to endogenous 11-cis-retinal, in present in purified CRALBP and CRALBP-like proteins (data not shown).

Figure 9. Normal phase HPLC separation of the geometrically isomeric forms of retinal and retinaldehyde. Upper panel: Standards. The peaks under the numbers are due to 1, 11-cis-retinaldehyde; 2, all-trans-retinaldehyde; 3, 11-cis-retinal; 4, all-trans-retinal. Lower panel: Endogenous retinaldehyde extracted from CRALBP-purified retinal pigment epithelium. The two major components co-chromatograph with 11-cis-retinaldehyde (9%) and all-trans-retinaldehyde (7%).

Figure 10. Normal phase HPLC separation of the geometrically isomeric forms of retinal and retinaldehyde. Upper panel: Standards. The peaks under the numbers are due to 1, 11-cis-retinaldehyde; 2, all-trans-retinaldehyde; 3, 11-cis-retinal; 4, all-trans-retinal. Lower panel: Endogenous retinaldehyde extracted from CRALBP-purified retinal pigment epithelium. The two major components co-chromatograph with 11-cis-retinaldehyde (9%) and all-trans-retinaldehyde (7%).

Figure 11. Normal phase HPLC separation of the geometrically isomeric forms of retinal and retinaldehyde. Upper panel: Standards. The peaks under the numbers are due to 1, 11-cis-retinaldehyde; 2, all-trans-retinaldehyde; 3, 11-cis-retinal; 4, all-trans-retinal. Lower panel: Endogenous retinaldehyde extracted from CRALBP-purified retinal pigment epithelium. The two major components co-chromatograph with 11-cis-retinaldehyde (9%) and all-trans-retinaldehyde (7%).

Figure 12. Normal phase HPLC separation of the geometrically isomeric forms of retinal and retinaldehyde. Upper panel: Standards. The peaks under the numbers are due to 1, 11-cis-retinaldehyde; 2, all-trans-retinaldehyde; 3, 11-cis-retinal; 4, all-trans-retinal. Lower panel: Endogenous retinaldehyde extracted from CRALBP-purified retinal pigment epithelium. The two major components co-chromatograph with 11-cis-retinaldehyde (9%) and all-trans-retinaldehyde (7%).