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Binding affinities of CRBPI and CRBPII for 9-*cis*-retinoids

Maureen A. Kane [a,1], Frank V. Bright [b], Joseph L. Napoli [a,*]

[a] Department of Nutritional Science and Toxicology, University of California, Berkeley; Berkeley, CA 94720-3104, USA
[b] Department of Chemistry, University at Buffalo, The State University of New York; Buffalo, NY 14260-3000, USA

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ABSTRACT

Background: Cellular retinol binding-protein I (CRBPI) and cellular retinol binding-protein II (CRBPII) serve as intracellular retinoid chaperones that bind retinol and retinal with high affinity and facilitate substrate delivery to select enzymes that catalyze retinoic acid (RA) and retinyl ester biosynthesis. Recently, 9-*cis*-RA has been identified *in vivo* in the pancreas, where it contributes to regulating glucose-stimulated insulin secretion. *In vitro*, 9-*cis*-RA activates RXR (retinoid × receptors), which serve as therapeutic targets for treating cancer and metabolic diseases. Binding affinities and structure–function relationships have been well characterized for CRBPI and CRBPII with all-*trans*-retinoids, but not for 9-*cis*-retinoids. This study extended current knowledge by establishing binding affinities for CRBPI and CRBPII with 9-*cis*-retinoids.
Methods: We have determined apparent dissociation constants, K'_d, through monitoring binding of 9-*cis*-retinol, 9-*cis*-retinal, and 9-*cis*-RA with CRBPI and CRBPII by fluorescence spectroscopy, and analyzing the data with non-linear regression. We compared these data to the data we obtained for all-*trans*- and 13-*cis*-retinoids under identical conditions.
Results: CRBPI and CRBPII, respectively, bind 9-*cis*-retinol (K'_d, 11 nM and 68 nM) and 9-*cis*-retinal (K'_d, 8 nM and 5 nM) with high affinity. No significant 9-*cis*-RA binding was observed with CRBPI or CRBPII.
Conclusions: CRBPI and CRBPII bind 9-*cis*-retinol and 9-*cis*-retinal with high affinities, albeit with affinities somewhat lower than for all-*trans*-retinol and all-*trans*-retinal.
General significance: These data provide further insight into structure–binding relationships of cellular retinol binding-proteins and are consistent with a model of 9-*cis*-RA biosynthesis that involves chaperoned delivery of 9-*cis*-retinoids to enzymes that recognize retinoid binding-proteins.

1. Introduction

Specific binding-proteins, enzymes, and receptors control flux through retinoid pathways, and ultimately produce retinoic acids (RA), active forms of vitamin A that control numerous physiological processes [1–4]. RA occurs in several isomeric forms *in vivo* with different biological actions [5]. Two isomers activate type II nuclear receptors: all-*trans*-RA activates RA receptors and peroxisome proliferators-activated receptors δ/β, whereas 9-*cis*-RA activates RA receptors and RXR (retinoid × receptors) [5]. 9-*cis*-RA activation of RXR may exert additional biological effects through dimerization of RXR with an array of other type II nuclear receptors [6]. A body of literature describes all-*trans*-retinoid metabolism *in vitro* and *in vivo*, but gaps exist in understanding 9-*cis*-retinoid metabolism [7]. Interest in 9-*cis*-RA synthesis and function *in vivo* has been motivated by its pleiotropic effects on numerous biological pathways activated by RXR, and the potential therapeutic uses of RXR ligands, including for treating cancer and metabolic diseases [8,9]. Renewed interest in 9-*cis*-RA biosynthesis has been prompted by a recent report that 9-*cis*-RA occurs *in vivo* in the pancreas, where it contributes to regulating glucose-stimulated insulin secretion [10].

RA biosynthesis involves reversible and rate-limiting dehydrogenation of retinol into retinal, catalyzed by membrane-bound, short-chain retinol dehydrogenases/reductases, followed by irreversible dehydrogenation of retinal by soluble retinal dehydrogenases [1,7]. A number of these enzymes are active with 9-*cis*-retinoids [11,12]. Dietary 9-*cis*-β-carotene generates 9-*cis*-retinoids *via* cleavage into 9-*cis*-retinal and all-*trans*-retinal [13]. Liver of chow-fed wild-type mice contain 9-*cis*-retinol [14]. This indicates that the intestine, a major site of β-carotene cleavage, reduces 9-*cis*-retinal into 9-*cis*-retinol, similar to its reduction of all-*trans*-retinal into all-*trans*-retinol, and esterifies 9-*cis*-retinol for transport to liver, i.e. 9-cis-retinol is a naturally occurring retinoid in vertebrates. Physiologically, intracellular retinol occurs bound to CRBP (cellular retinol binding-protein) [15]. Thus, the 15 kDa cytosolic CRBP function as intracellular retinoid chaperones by binding retinol and retinal, but not RA, with high affinity [16]. CRBP assists in regulating retinoid homeostasis by protecting retinoids from non-specific oxidation, facilitating delivery of retinoids to specific enzymes, and regulating retinyl ester synthesis and hydrolysis [17].

* Corresponding author. Tel.: +1 510 642 5202; fax: +1 510 642 0535.
E-mail address: jna@berkeley.edu (J.L. Napoli).
[1] Current address: Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland; Baltimore, MD 21201, USA.

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CRBPI is widely expressed in the adult and is essential for retinol storage in the liver [18,19]. CRBPII is expressed mainly in the adult small intestine, where it functions in vitamin A absorption/uptake [20,21]. Expression patterns, conformational changes upon binding, affinities for retinoids, and interactions with enzymes and membranes unique to each binding-protein suggest distinct functions for CRBPI and CRBPII [22,23].

Affinity and structure–function relationships have been well characterized for CRBPI and CRBPII binding with all-*trans*-retinol and all-*trans*-retinal, but not for 9-*cis*-retinol or 9-*cis*-retinal [16,24–27]. Here we report affinities of CRBPI and CRBP II for 9-*cis*-retinoids, and compare the data to results with all-*trans*- and 13-*cis*-retinoids.

2. Materials and methods

2.1. Materials

Retinoids were purchased from Sigma-Aldrich (St. Louis, MO), except 9-*cis*-retinol, which was prepared as described by reduction of 9-*cis*-retinal with $NaBH_4$, followed by identity verification using spectrophotometry and chromatography [13]. Retinoid solutions were prepared in ethanol on the day of use. Concentrations were verified spectrophotometrically.

2.2. CRBP preparation

CRBPI and CRBPII were expressed in *E. Coli* with the vectors pMONCRBP and pMONCRBPII, respectively, and were isolated and quantified as described [24,26]. CRBPI and CRBPII were purified further by fast protein liquid chromatography as described [23,28]. CRBPI/II concentrations were determined from absorbance at 280 nm using published ε values [23,28]. The A340/A280 ratios, used to assess purity, were at least 1.6 for FPLC-purified preparations. Stock and binding assay solutions of CRBPI and CRBPII were prepared in 20 mM KH_2PO_4, 100 mM KCl, pH = 7.4 (buffer A). K_d value determinations were performed with 150 nM CRBPI or CRBPII, except 13-*cis*-retinol, where 300 nM CRBPI and CRBPII were used. CRBPI and CRBPII have amino acid sequences that are highly conserved across species (>95% for CRBPI and ~91% for CRBPII), and share 56% amino acid sequence homology [15]. CRBPI/CRBPII tryptophan residues (responsible for the intrinsic protein fluorescence) are located at positions 9, 89, 107, and 110.

2.3. Fluorescence measurements

Data were generated with a SLM-8100 fluorometer with a 450-watt Xe lamp. Measurements were made at 25 °C. CRBPI and CRBPII were excited at 280 nm. Emission was monitored at 340 nm with spectral band passes of 4 nm. Retinoid solutions were added with a glass Hamilton syringe, gently mixed, and equilibrated for 5 min, before measuring the solution fluorescence. For titrations with retinol, increasing retinol fluorescence was monitored at 490 nm with excitation at 350 nm, as a function of added retinoid. Retinal and RA do not fluoresce; therefore, only CRBP fluorescence was monitored when evaluating these retinoids. The excitation shutter was closed between measurements and retinoid solutions (kept shielded from light) were added in the dark. Inner filter effects were negligible at the protein and retinoid concentrations used and were not corrected. Excitation and emission spectra were corrected using the appropriate blank. Blank contributions were <2% of total intensity. Data were analyzed by non-linear least square fit according to Eq. (1), where F is the observed fluorescence, F_f is the fluorescence of free protein, F_b is the fluorescence of bound protein, P_t is the total protein concentration, R_t is the total retinoid concentration, and K'_d is the apparent dissociation constant [27]. Data were fit to Eq. (1) using GraphPad Prism (version 5.0). Before adding retinoids, apo-CRBPI and apo-CRBPII exhibited no detectable emission at wavelengths >400 nm, indicating absence of retinol. Binding experiments were done at least three times.

$$\frac{F}{F_f} = 1 + \left(\frac{F_b}{F_f} - 1\right) \times \left(\frac{P_t + R_t + K'_d - \sqrt{(P_t + R_t + K'_d)^2 - 4P_tR_t}}{2P_t}\right) \quad (1)$$

2.4. Sephadex G-50 column

Sephadex G-50 (fine) was equilibrated in buffer A for several days and used to construct individual columns (~2–3″ long × ½″ diameter) in disposable 10 cm^3 syringes. Separate columns were used for each protein-substrate combination. Excitation spectra were collected for CRBP bound to retinol before and after passing through the Sephadex G-50 columns. Excitation spectra also were collected for an approximately equivalent amount of free retinol in buffer A.

3. Results and discussion

To determine if 9-*cis*-retinoids bind to CRBPI and/or CRBPII, we used a fluorescence method modified from Cogan et al. and Levin et al. to measure binding affinities [24,29]. Briefly, ligand binding quenches the intrinsic fluorescence of the apo-proteins by energy transfer from tryptophan residues. This causes a complimentary increase in retinol fluorescence that is red-shifted in comparison to free retinol. Data for binding of 9-*cis*-retinol, 9-*cis*-retinal, and 9-*cis*-RA, and for direct comparison, all-*trans*- and 13-*cis*-isomers to CRBPI and CRBPII were determined by calculating the apparent dissociation constant (K'_d) using non-linear least squares fit to Eq. (1) (Figs. 1 and 2) (Table 1). Recovered K'_d values were statistically equivalent, whether determined by quenching of protein fluorescence, or by increases in retinoid fluorescence.

Emission maxima for CRBPI and CRBPII are 340 nm, when excited at 280 nm (Fig. 1A, shown for 9-*cis*-retinol binding to CRBPI). Emission maxima for retinol bound to CRBPI or CRBPII are 490 nm when excited at 350 nm (Fig. 1B, shown for 9-*cis*-retinol binding to CRBPI). Free retinal and RA do not fluoresce under these experimental conditions, and therefore only protein quenching was monitored for these retinoids.

Protein quenching (Fig. 1C) and retinol fluorescence (Fig. 1D) are shown for 9-*cis*-retinol binding to CRBPI and CRBPII. The K'_d for 9-*cis*-retinol was 11 nM for CRBPI binding and 68 nM for CRBPII binding, indicating that CRBPI has ~6-fold stronger affinity for 9-*cis*-retinol compared to CRBPII. Both K'_d values are significantly below 9-*cis*-retinol concentrations *in vivo*, indicating that 9-*cis*-retinol would exist physiologically as does all-*trans*-retinol: bound to either CRBPI or CRBPII [13]. CRBPI exhibits ~3-fold lower affinity for 9-*cis*-retinol relative to all-*trans*-and 13-*cis*-retinol. CRBPII exhibits decreasing affinity for retinol ligands in the order: all-*trans*-retinol > 13-*cis*-retinol > 9-*cis*-retinol (Fig. 2A and D). Thus, CRBPI and CRBPII might foster somewhat different outcomes for 9-*cis*-retinol metabolism, based on their different affinities for this retinoid.

Fig. 1E shows binding of CRBPI or CRBPII to 9-*cis*-retinal by monitoring quenching of intrinsic tryptophan fluorescence. K'_d values are similar for CRBPI and CRBPII binding to 9-*cis*-retinal: 8 nM and 5 nM, respectively. Binding of 9-*cis*-retinal also is similar compared to all-*trans*-retinal for either CRBPI (9 nM) or CRBPII (11 nM) (Fig. 2E). 13-*cis*-retinal was not commercially available and its binding was not measured.

Neither 9-*cis*-RA (Fig. 1F) nor other RA isomers (all-*trans*- or 13-*cis*-) (Fig. 2F and G) exhibited significant binding to CRBPI or CRBPII, as expected from previous binding and structural analyses [15,17,24–27]. Instead, RA isomers are chaperoned by cellular retinoic acid binding-proteins, which do not bind retinal or retinol [1,5,16].