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^{19}F-NMR studies of retinol transfer between cellular retinol binding proteins and phospholipid vesicles

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Abstract The cellular retinol binding proteins, CRBP and CRBP II, are implicated in the cellular uptake of retinol and intracellular trafficking of retinol between sites of metabolic processing. ^{19}F-NMR studies of retinol transfer between CRBP and CRBP II and phospholipid vesicles, using either fluorine-labeled ligand or protein, demonstrated that there was significantly more transfer of retinol from CRBP II to lipid vesicles than from CRBP. Differences in how readily protein-bound retinol is released to lipid bilayers may lead to differences in how these two proteins modulate intracellular retinol metabolism.

Key words: Cellular retinol binding protein; Cellular retinol binding protein II; Retinol; Liposome; ^{19}F-NMR

1. Introduction

Vitamin A is an essential nutrient required for growth, reproduction, differentiation of epithelial tissues and vision [1]. The small intestinal epithelium, esterifies ~90% of dietary retinol (the alcohol form of vitamin A), and packages the retinyl esters into chylomicrons prior to delivery to the liver. Most of the retinyl acetate metabolites recovered from the intestinal mucosa are in the form of retinyl esters and retinol, however, a small fraction is found in the form of retinoic acids, retinoyl glucuronides and polar metabolites [2]. Following hepatic uptake of chylomicron remnants, the retinol esters are hydrolyzed and either complexed with serum retinol binding protein (RBP) and released to the circulation, or reesterified with long-chain fatty acids and stored within the liver.

It has been proposed that specific intracellular carrier proteins participate in the cytoplasmic trafficking of these hydrophobic compounds between sites of metabolic processing [3]. Cellular retinol binding protein II (CRBP II) is a 15.5 kDa intestinal protein that binds all-*trans*-retinol and all-*trans*-retinal but not all-*trans*-retinoic acid [4]. It is closely homologous (sharing 56% amino acid identity) to cellular retinol binding protein (CRBP), which is present in many tissues but is particularly abundant in liver, kidney and testes [5,6]. The intestinal localization of CRBP II suggests that it is uniquely adapted for the intestinal absorption and metabolism of retinol. Both CRBP and CRBP II have been implicated in delivering retinol to lecithin:retinol acyltransferase based on in vitro studies of retinol esterification [7–10]. Overexpression of either rat CRBP or rat CRBP II in stably transfected Caco-2 cells resulted in increased uptake of retinol and increased esterification of retinol in vivo [11]. The addition of apoCRBP has been observed to stimulate hydrolysis of retinyl esters in vitro [12–14]. Holo-CRBP complexed with retinol serves as the substrate for a microsomal NADP-dependent dehydrogenase which converts retinol to retinal [13,15]. Differential recognition of the apo- and holo-CRBPs by enzymes involved in the metabolism of vitamin A has been proposed as a mechanism by which these retinol binding proteins restrict the metabolism of the bound retinol to certain metabolic pathways, based on analyses of the kinetic parameters obtained when unbound retinol, CRBP-retinol or CRBP II-retinol are used as substrates [7,10,13]. These analyses were made with the assumption that both holo-CRBP and holo-CRBP II were stable when incubated in the presence of membranes. Previous studies measuring transfer of radiolabeled retinol or decreases in protein bound retinol fluorescence, indicate that little dissociation of the CRBP-retinol complex takes place when coincubated with membranes [10,16]. The stability of the CRBP II-retinol complex in the presence of membranes has not been described thus far.

We have previously shown that the distribution of retinol between CRBP and CRBP II is difficult to predict based on a comparison of previously determined dissociation constants for these two proteins [17]. This may be due in part to the difficulty of accurately measuring dissociation of retinol from CRBP and CRBP II in water, given the extremely hydrophobic properties of retinol. By fluorometric titration, the dissociation constants for CRBP-retinol and CRBP II-retinol in an aqueous buffer have been previously determined to be roughly equivalent at 10^{-8} M [18,19]. Subsequent ^{19}F-NMR studies of CRBP and CRBP II isotopically labeled with 6-fluorotryptophan, however, demonstrated that retinol complexed to CRBP II is readily transferred to CRBP, whereas retinol complexed to CRBP is not transferred to CRBP II [17]. In contrast, retinal is transferred readily in both directions between CRBP and CRBP II [17]. These studies suggest that under conditions of limiting retinol concentrations CRBP ligand-binding sites will be preferentially populated over those of CRBP II.

Intracellular retinoid flux also involves movement of reti-

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Abbreviations: CRBP, cellular retinol binding protein; CRBP II, cellular retinol binding protein II; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; D_2O, deuterium oxide; $CDCl_3$, chloroform-d; 6-FTrp, 6-fluorotryptophan; TMS, trimethylsilane; TFA, trifluoroacetic acid; FABP, fatty acid binding protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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noids between membranes and the CRBPs. To model the distribution of retinol between CRBP and CRBP II and membranes, a fluororetinol analog which exhibits distinct resonances when complexed to CRBP, CRBP II and when associated with lipid vesicles, was used as an ^{19}F-NMR probe to measure partitioning of retinol between CRBP, CRBP II and lipid vesicles. Transfer of all-*trans*-retinol between 6-FTrp-CRBP, 6-FTrp-CRBPII and lipid vesicles was also measured. We report here the results of these studies.

2. Materials and methods

2.1. Materials

6-Fluorotryptophan, deuterium oxide (D_2O), all-*trans*-retinol and cholesterol were purchased from Sigma (St. Louis, MO). Plasmids and bacterial strains used for expression of rat CRBP II and rat CRBP in *E. coli* have been described in earlier publications [19–21]. Egg lecithin phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) were purchased from Lipid Products Co., UK.

2.2. Preparation of (all-E)-9-(2-methoxy-6-trifluoromethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (1, Fig. 1)

The acid derivative of **1** (from Hoffmann-La Roche) was esterified with diazomethane and then reduced with diisobutylaluminum hydride to **1** using the procedure reported previously [22]. Ligand **1** was characterized by NMR spectroscopy. ^{1}H-NMR ($CDCl_3$) 7.27 (2H,m), 7.08 (1H, d, J=16 Hz), 7.07 (1H, d, J=10 Hz), 7.01 (1H, dd, J=15, 11 Hz), 6.78 (1H, dd, J=16, 1.5 Hz), 6.33 (1H, d, J=15 Hz), 6.29 (1H, d, J=11 Hz), 5.79 (1H, s), 3.88 (3H, s), 3.70 (1H, s), 2.35 (3H, d, J=1.5 Hz), 2.06 (3H, s) ppm from trimethylsilane (TMS). ^{19}F-NMR ($CDCl_3$), 14.38 (s) ppm relative to trifluoroacetic acid (TFA).

2.3. Phospholipid vesicle preparation

Small sonicated PC vesicles were prepared from co-dried lipids as previously described [23], except a D_2O buffer containing 10 mM potassium phosphate pH 7.4, 5 mM 2-mercaptoethanol and 0.05% sodium azide was employed. The concentration of phospholipid in the stock vesicle preparation was measured by measuring total phosphate, and correcting for inorganic phosphate, as described by Ames [24].

2.4. Production of rat CRBP II and CRBP in E. coli

Unlabeled rat apoCRBP and apoCRBP II were expressed in *E. coli* strain JM101 harboring the appropriate recombinant expression vectors as described previously [19,20]. 6-Fluorotryptophan was incorporated into the *E. coli* tryptophan auxotroph, W3110 trpA33 [25] by inducing expression of recombinant protein in media containing the analog and purified as described previously [17,21].

2.5. NMR measurements

0.5 ml samples containing holo-CRBP I or holo CRBP II (final concentration of 0.2 mM) in 10 mM potassium phosphate, pH 7.4, 5 mM 2-mercaptoethanol, and 0.05% sodium azide were mixed with varying amounts of vesicles. ^{19}F-NMR spectra were recorded at 470.3 MHz without proton decoupling on a Varian VXR-500 (Varian Associates, Palo Alto, CA) spectrometer after mixing (~5–10 min) and 24–48 h after mixing. The chemical shift was referenced to an internal standard (potassium salt of trifluoroacetic acid in solution). All studies were conducted at 37°C. The T_1 values for **1** complexed with CRBP II, CRBP and PC liposome were 0.31, 0.29 and 0.36 s, respectively, as measured by inversion-recovery experiments. A delay of 1.5 s between successive transients was employed to ensure complete relaxation and thus quantitation of ^{19}F signals. The integrated intensities of the F resonances were used to measure the relative mole quantities of vesicle-bound ligand **1** and of protein-bound ligand **1**. Measurement of the net transfer of ligand **1** from the CRBPs to the PC vesicles represent the average of two separate experiments, using two preparations of phospholipid vesicles and of protein. The measurements of the net transfer of retinol from 6-FTrp-labeled CRBPs each represent a single NMR experiment.

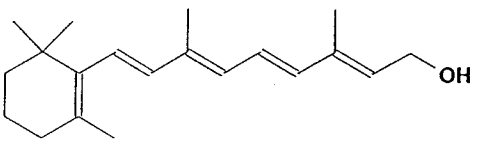
3. Results

3.1. ^{19}F-NMR spectra of ligand 1 complexed with CRBP II, CRBP and PC vesicles (see Fig. 2)

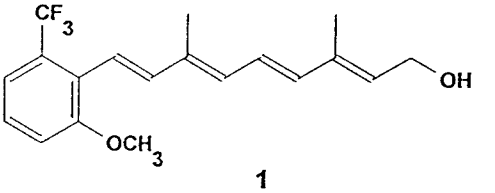
Ligand **1** is a fluororetinol analog (see Fig. 1) that binds CRBP and CRBP II with a stoichiometry of 1:1 with apparent dissociation constants that are $< 10^{-8}$M as determined by fluorometric titration. The F resonance corresponding to ligand **1** exhibited distinct chemical shifts when associated with PC vesicles (16.6 ppm from TFA, see Fig. 2A,B), complexed with CRBP II (17.1 ppm from TFA, see Fig. 2C) and complexed with CRBP (19.6 ppm from TFA, see Fig. 2D). Advantages of using a fluorine probe include both increased sensitivity of this nucleus and absence of background signal due to natural abundance of the isotope. The ^{19}F-NMR spectra of CRBP II and CRBP complexed with one equivalent of ligand **1**, and then mixed with increasing amounts of PC phospholipid vesicles are shown in Fig. 2E–H. The relative distribution of **1** between the proteins and vesicles was calculated from the integrated areas under the resonances at 19.6 ppm (corresponding to **1** complexed with CRBP), 17.1 ppm (corresponding to **1** complexed with CRBP II), and 16.6 ppm (corresponding to **1** associated with phospholipid vesicles). At a protein to phospholipid molar ratio of 1:5, 22±4% (mean±S.D.) of ligand **1** originally bound to CRBPII partitioned to phospholipid vesicles (see Fig. 2E), but there was no detectable transfer of **1** from CRBP to phospholipid vesicles (see Fig. 2F). At a protein to phospholipid molar ratio of 1:50, 62±3% of ligand **1** originally bound to CRBP II partitioned into phospholipid vesicles (see Fig. 2G), and only 6±6% of ligand **1** originally bound to CRBP partitioned into phospholipid vesicles (see Fig. 2H). The relative distribution of **1** between protein and vesicles in spectra (data not shown) representing the initial 16 transients accumulated, obtained approx. 5 min after mixing, is the same as the distribution observed in spectra shown in Fig. 2 (~1–4 h after mixing) as well as that observed in spectra obtained 24–48 h after mixing (data not shown).

3.2. Net transfer of all-trans-retinol between 6-FTrp-CRBP II and 6-FTrp-CRBP and phospholipid vesicles

Isotopic labeling of CRBP and CRBP II with 6-fluorotryp-



all-trans-retinol



1

Fig. 1. Structures of all-*trans-retinol and its analog.*

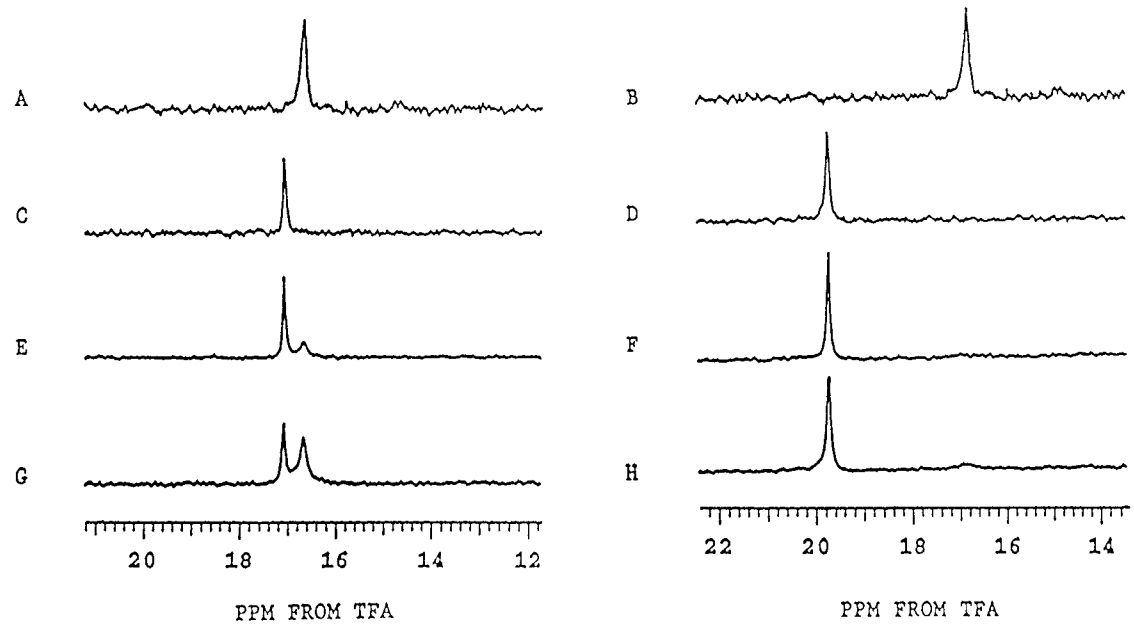


Fig. 2. ^{19}F-NMR spectra of ligand **1** and CRBP, CRBP II and PC vesicles. The un-decoupled 470.3-MHz ^{19}F-NMR spectra were collected as described under Section 2. Chemical shifts are referenced to the ^{19}F signal for TFA. 20 Hz line broadening was applied for all spectra, 400–6000 transients were accumulated for all spectra. (A,B) 0.2 mM **1**+50 mM PC; (C) 0.2 mM holo-CRBPII-**1**; (D) 0.2 mM holo CRBP-**1**; (E) 0.2 mM holo CRBP II-**1**+1 mM PC; (F) 0.2 mM holo CRBP-**1**+1 mM PC; (G) 0.2 mM holo-CRBP II-**1**+10 mM PC; (H) 0.2 mM holo CRBP-**1**+10 mM PC.

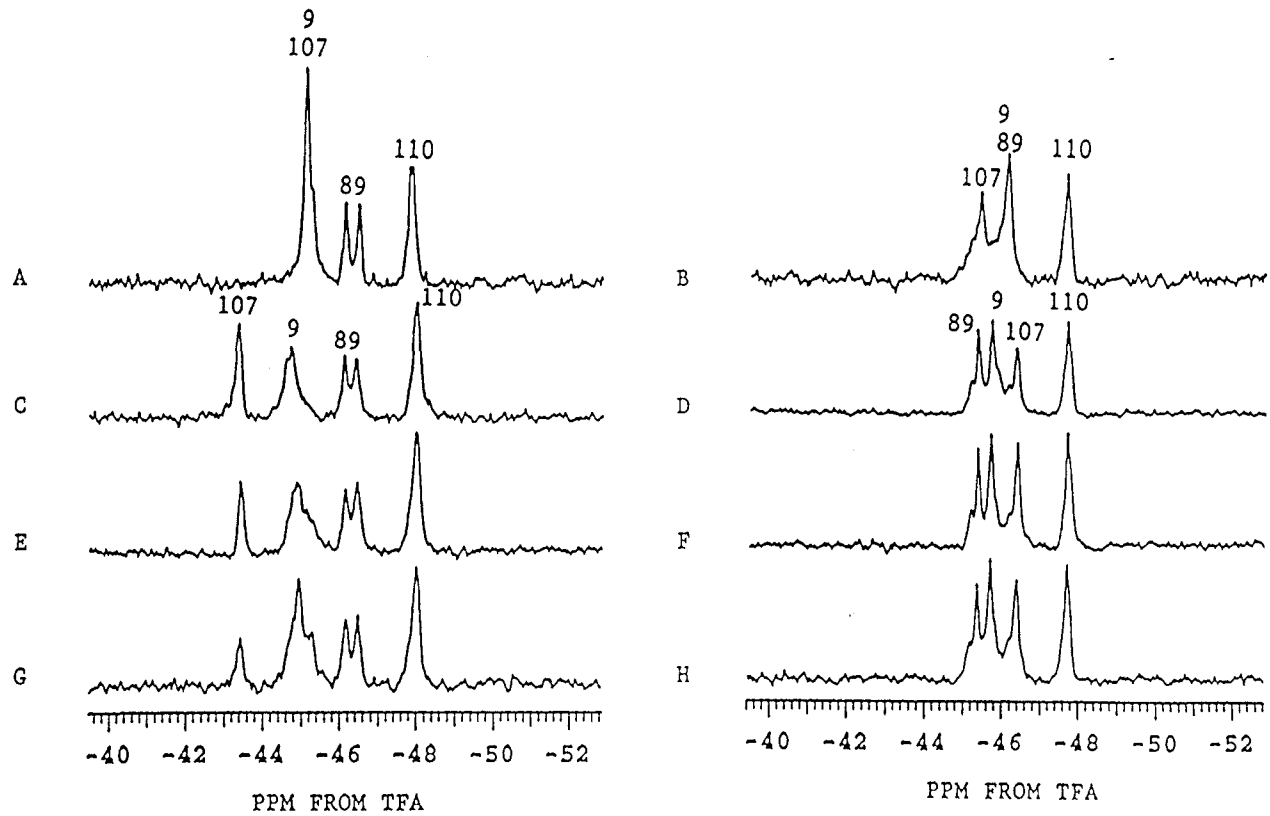


Fig. 3. ^{19}F-NMR spectra of 6-FTrp-labeled holo-CRBP II and 6-FTrp-labeled holo-CRBP complexed with all-*trans*-retinol after the addition of PC liposomes. Varying amounts of PC vesicles were added to 6-FTrp-holo-CRBP and 6-FTrp-holo-CRBP II complexed with all-*trans*-retinol. 470.3 MHz ^{19}F-NMR spectra of (A) 0.2 mM apo-6-FTrp-CRBP II+1 mM PC; (B) 0.2 mM apo 6-FTrp-CRBP+1 mM PC; (C) 0.2 mM holo 6-FTrp-CRBPII; (D) 0.2 mM holo 6-FTrp-CRBP; (E) 0.2 mM holo 6-FTrp-CRBPII+1.0 mM PC; (F) 0.2 mM holo 6-FTrp-CRBP+1 mM PC; (G) 0.2 mM holo-6-FTrp-CRBP II+10 mM PC. (H) 0.2 mM holo 6-FTrp-CRBP+10 mM PC. Chemical shifts are referenced to the ^{19}F signal for TFA. 20 Hz line broadening was applied for all spectra. 400–6000 transients were accumulated for all spectra. The numbers indicate the resonance assignments for the four tryptophan residues.

tophan, provides residue specific NMR probes for the four tryptophan residues at positions 9, 89, 107 and 110 [17]. The addition of phospholipid vesicles to 6-FTrp-apoCRBP II and 6-FTrp-apoCRBP at a protein to phospholipid molar ratio of 1:5 resulted in a slight line broadening (6 Hz) of the F resonances but did not significantly perturb the chemical shifts. Further addition of vesicles resulted in further broadening (10–20 Hz) (data not shown).

The F resonances corresponding to Trp^{9} and Trp^{107} undergo ligand-dependent perturbations in chemical shifts when 6-FTrp-CRBP and 6-FTrp-CRBP II bind all-*trans*-retinol [17]. The F resonances corresponding to the Trp^{89} and Trp^{110} are not perturbed by the addition of all-*trans*-retinol. We have previously shown that the 'doublet' appearance of some of the tryptophan resonances is due to the presence of protein retaining the initiator methionine and protein in which this residue has been removed [21]. The transfer of all-*trans*-retinol from the CRBPs to phospholipid vesicles was monitored by measuring the conversion of 6-FTrp-holoCRBPs to 6-FTrp-apoCRBPs after the addition of phospholipid vesicles. The fraction of 6-FTrp-CRBPII which was occupied by ligand was calculated from the integrated area under the Trp^{107} F resonance at −43.3 ppm, which corresponds to only the holo-form of 6-FTrp-CRBP II, relative to the integrated area under the Trp^{110} F resonance at −47.8 ppm, which corresponds to both the apo and holo form of 6-FTrp-CRBP II (see Fig. 3A,C,E,G,I). At a protein to phospholipid molar ratio of 1:5, 40% of the all-*trans*-retinol partitioned away from 6-FTrp-CRBP II (see Fig. 3E). At a protein to phospholipid molar ratio of 1:50, 65% of the all-*trans*-retinol partitioned away from 6-FTrp-CRBP II (see Fig. 3G). At a 6-FTrp-CRBP to phospholipid molar ratio of 1:50, ^{19}F-NMR spectra (see Fig. 3H) remained virtually identical to that of the holoprotein, indicating that there was minimal transfer of all-*trans*-retinol from CRBP to the lipid vesicles. The studies of all-*trans*-retinol partitioning between 6-FTrp-CRBP II and 6-FTrp-CRBP and phospholipid vesicles, thus yielded similar results to the studies on the partitioning of the fluororetinol analogs between the unlabeled proteins and PC vesicles.

4. Discussion

In these ^{19}F-NMR studies the net transfer of protein bound retinol to model membranes can be directly observed without separation of donor and acceptor complexes. This approach is similar to previously described experiments using ^{13}C-NMR to measure partitioning of ^{13}C-enriched oleic acid between liver fatty acid binding protein (FABP) and unilamellar PC vesicles [26] and between albumin and unilamellar PC vesicles [27], but has the advantage of utilizing the fluorine nucleus which can be detected with higher sensitivity. These ^{19}F-NMR studies demonstrate a marked difference in the net transfer of protein-bound retinol when complexed with CRBP as opposed to CRBP II from phospholipid vesicles. The observation that holo-CRBP was stable when incubated in the presence of phospholipid vesicles is consistent with previous studies where virtually no decrease in retinol fluorescence upon excitation of CRBP was observed when holo-CRBP was incubated with membranes [10], and transfer of only 10% of radiolabeled retinol from holo-CRBP to membranes was measured [16]. In contrast there was significantly more dissociation of holo-CRBP II when incubated in the presence of phospholipid vesicles.

Because of the relatively long time (5 min to collect the initial 6–16 transients) required to obtain NMR spectra with good signal to noise, the kinetics of transfer could not be analyzed. The observation that the resonances for ligand **1** exhibit the same chemical shift in mixtures of protein with vesicles as in the isolated systems indicate that exchange is slow on the NMR time scale ($< 500\ s^{-1}$ for exchange of ligand **1** between CRBP II and PC vesicles and $< 3000\ s^{-1}$ for exchange between CRBP and PC vesicles).

The precise functional roles for CRBP and CRBP II remain to be defined. It has been proposed that these proteins play a role in cellular uptake of retinol, cytoprotection, and modulation of intracellular retinol metabolism [5,11]. These NMR studies suggest that CRBP will be more effective than CRBP II in serving a cytoprotective function. Since bound retinol is oriented within the binding pockets of both CRBP and CRBP II with the alcohol moiety situated innermost, this alcohol moiety of bound retinol is not immediately accessible for modification [5]. Differences in how readily retinol is released from the binding pocket to lipid bilayers may lead to differences in how CRBP-retinol and CRBP-II retinol interact with various sites of metabolic processing within the cell.

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