Purification and Partial Characterization of a Cellular Carotenoid-binding Protein from Ferret Liver*

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Manjunath N. Rao, Pradeep Ghosh, and M. R. Lakshman‡

From the Department of Medicine, George Washington University, Washington, D. C. 20037 and the Lipid Research Laboratory, Department of Veterans Affairs Medical Center, Washington, D. C. 20422

A cellular carotenoid-binding protein was purified to homogeneity from β-carotene-fed ferret liver utilizing the following steps: ammonium sulfate precipitation, ion exchange, gel filtration, and affinity chromatography. The final purification was 607-fold. [14C]β-Carotene co-purified with the binding protein throughout the purification procedures. SDS-PAGE of the purified protein showed a single band with an apparent molecular mass of 67 kDa. Scatchard analysis of the specific binding of the purified protein to β-carotene showed two classes of binding sites, a high affinity site with an apparent KD of 56 × 10⁻⁹ M and a low affinity site with a KD of 32 × 10⁻⁶ M. The Bmax for β-carotene binding to the high affinity site was 1 mol/mol, while that for the low affinity site was 145 mol/mol. The absorption spectrum of the complex showed a 32-nm bathochromic shift in Amax with minor peaks at 460 and 516 nm. Except for α-carotene and cryptoxanthin, none of the model carotenoids or retinol competed with β-carotene binding to the protein. Thus, a specific carotenoid-binding protein of 67 kDa has been characterized in mammalian liver with a high degree of specificity for binding only carotenoids with at least one unsubstituted β-ionone ring.

The protective effects of β-carotene and, possibly, other carotenoids against certain chronic diseases such as cancer (1), coronary heart disease (2), and erythropoietic protoporphyria (3) are well documented, although a controversy has been raised recently (4). In addition, carotenoids serve as biological antioxidants that are known to absorb intact β-carotene by low density lipoproteins in the form of beadlets (Hoffman-La Roche, Nutley, NJ; β-carotene, 10% w/w, product code 65661) and 10 g of taurocholate/kg, and the animals were fed ad libitum for a period of 4 weeks. The animals were euthanized by nortic exsanguination under pentobarbital anesthesia (50 mg/kg, intraperitoneally), and the livers were saved for CCBP isolation.

EXPERIMENTAL PROCEDURES

Animals and Diet—Male ferrets (M. putoris furo) (body weight ~600 g) were procured from Marshall Farms, North Rose, NY. After 1 week of quarantine, they were maintained on a high protein ferret diet (Purina, St. Louis, MO) for 1 week. This diet had 40% protein, 13% fat, and 22% carbohydrate. This diet was fortified with 2 g of β-carotene in the form of beadlets (Hoffman-La Roche, Nutley, NJ; β-carotene, 10% w/w, product code 65661) and 10 g of taurocholate/kg, and the animals were fed ad libitum for a period of 4 weeks. The animals were euthanized by nortic exsanguination under pentobarbital anesthesia (50 mg/kg, intraperitoneally), and the livers were saved for CCBP isolation.

Chemicals—All chemicals, solvents, and reagents were of analytical or ultrapure grade.

Carotenoid Protein Complex Isolation—The procedure was performed essentially according to that described by us previously for rat liver (10). Briefly, each liver was homogenized using a Polytron homogenizer (Brinkman Instruments, Westbury, NY.) with 10 volumes of the homogenizing buffer (50 mM MES, 1 mM EDTA, 20% glycerol, 0.2% n-octyl-β-D-glucopyranoside, 5 mM CHAPS, 0.5% Triton X-100, 50 μg/ml butylated hydroxytoluene, and 1 μg/ml each of the following protease inhibitors: phenylmethylsulfonyl fluoride, aprotinin, and leupeptin). Unless otherwise specified, all procedures were carried out under F40 Gold fluorescent light at 4 °C. Following ultracentrifugation at 100,000 × g, the supernatant fraction was subjected to 0–50% ammonium sulfate (AS) fractionation. The AS fraction was redissolved in elution buffer (50 mM Tris-HCl, 50 mM ammonium bicarbonate buffer, pH 7.2, containing 0.5% Triton X-100 and 1 μg/ml each of the following: phenylmethylsulfonyl fluoride, aprotinin, and leupeptin). After dialysis against the elution buffer, this fraction was incubated with [14C]β-carotene (2 × 10⁶ dpm) for 1 h at 25 °C.

DEAE-Sepharose Column Chromatography—The dialyzed [14C]β-carotene-labeled AS fraction from the previous step was further fractionated on a DEAE-Sepharose column (1.5 cm × 25-cm bed, Sigma), equilibrated with elution buffer. The column was initially washed with two bed-volumes of the elution buffer containing 10 mM NaCl, and then with two bed-volumes of the same buffer containing 100 mM NaCl. Finally, the carotenoid-protein complex was eluted from the column as a yellow band with the elution buffer containing 350 mM NaCl at a flow rate of 0.4 ml/min. The eluted fractions were monitored for their absorption at 280 and 465 nm in a Shimadzu UV-visible spectrophotometer (Shimadzu UV-160). Fractions 9–19 corresponding to the peak of [14C]radiolabel were pooled, concentrated by the use of a Speed-Vac Concentrator (Forma Scientific, Inc., Marietta, OH), and stored at 4 °C for further studies.

Sephadex G-75 Column Chromatography—The concentrated fraction...
containing CCBP from the previous step was subjected to gel filtration chromatography on a Sephadex G-75 column (1 cm × 25 cm) equilibrated with the elution buffer. The eluted fractions were monitored for their absorption at 280 and 465 nm. Fractions 20–25 corresponding to the major peak of [14C] radioactivity were collected, concentrated by the use of a Speed-Vac concentrator, and stored at 4 °C for further studies.

Release of the Apoprotein Component from the Complex—The yellow concentrated CCBP fraction obtained from the Sephadex-G75 column chromatography step was treated with an equal volume of cold acetone (−20 °C), shaken gently, and left aside at −20 °C for 20–30 min, followed by centrifuging at 1300 × g for 20 min. The organic supernatant fraction was removed, while the apoprotein pellet was dissolved in the original volume of TAB buffer (50 mM Tris-HCl, 50 mM ammonium bicarbonate buffer, pH 7.2). The apoprotein was reprecipitated with an equal volume of cold acetone as before, and the pellet again was redissolved in the original volume of TAB buffer. Its protein content was estimated (17).

Affinity Chromatography—Since CCBP showed high affinity for β-carotene, we decided to explore whether it was possible to make an affinity column with immobilized β-carotene as the affinity ligand. However, β-carotene is a long chain hydrocarbon with no functional groups, which makes its immobilization difficult, if not impossible. Therefore, we took a novel approach of using the Pharmalink immobilization kit (Pierce), which is based on the principles of the Mannich reaction to immobilize the ligand. The Mannich reaction consists of the condensation of formaldehyde or any other aldehyde with ammonia, primary or secondary amines, and a ligand molecule having possibly an active hydrogen. The Pharmalink gel included in the kit is immobilized diaminodipropylamine, which can be used as a source of the primary amine. The 5,6-ethylenic bond on the diaminodipropylamine, which can be used as a source of the primary amine. The 5,6-ethylenic bond on the β-ionone ring, especially with the activator methyl group at C-5 position, can sufficiently activate the hydrogen atom at C-4 or C-4′ to participate in this reaction, as shown in Fig. 1. However, the ligand binding to the matrix can also occur at other positions since β-carotene has a number of conjugated double bonds with adjacent methyl groups to activate a hydrogen atom for participation in this reaction.

Preparation of Pharmalink-immobilized β-carotene—All operations were carried out in the dark or, where necessary, under F40 gold fluorescent light to minimize the oxidation of β-carotene. The procedure described below was essentially according to manufacturer’s specifications using their kit, which contained all the coupling reagents. Briefly, the storage solution in the Pharmalink column (2-ml prepacked column) was drained completely, and the gel was equilibrated with the coupling buffer (2 ml of buffer plus 2 ml of dimethyl sulfoxide (MeSO)). The coupling buffer was also drained as before. Purified β-carotene (10 mg) dissolved in 2 ml of MeSO containing butylated hydroxytoluene (50 μg/ml) was then mixed with the Pharmalink gel, followed by the addition of Pharmalink coupling reagent in the reaction bottle initially at 37 °C for 1 h, followed by incubation at 4 °C for 24 h with gentle mixing end over end. The column matrix was washed thoroughly with 30 ml of the TAB buffer containing 50% ethanol until no more unbound β-carotene was eluted from the column (evidenced by an absorption spectrum). It was found that 90% of the added β-carotene was bound to the matrix. The ligand binding to the affinity matrix increased to 100% when only 0.2 mg of β-carotene was used for binding. The washed column with immobilized β-carotene was ready for affinity chromatography after equilibration with five volumes of TAB buffer. Fig. 1 shows the proposed structure of immobilized β-carotene using the Pharmalink gel, although other sites of attachment of the chromophore may be possible as indicated previously.

Affinity Chromatographic Purification of CCBP—CCBP apoprotein fraction, isolated by cold acetone treatment of the complex fraction from Sephadex-G75 column chromatography step (~3 mg of protein), was subjected to affinity chromatography on the Pharmalink-immobilized β-carotene column prepared above. After applying the protein, the column was initially washed with 20 ml of the TAB buffer to remove the unbound protein (evidenced by the decrease in absorbance at 280 nm). The bound protein was then eluted from the affinity column with 20 ml of the TAB buffer containing 250 mM NaCl and collected as 1-ml fractions. Aliquots of fractions showing protein peaks were tested for their homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel (Bio-Rad). Fractions showing a single homogeneous band were pooled, concentrated in a Speed-Vac concentrator, dialyzed against TAB buffer, and stored at 4 °C. This purified protein was tested for its binding affinity to various carotenoid ligands as described below.

Binding Assay—The standard binding assay (unless specified otherwise) was as follows; 30 μg of purified CCBP or a nonspecific protein like bovine serum albumin (BSA) or the 43-kDa protein from ferret liver in 950 μl of TAB buffer was incubated with 8 nmol of β-carotene in 50 μl of acetone at 37 °C for 60 min. This was followed by thorough extraction of each reaction mixture with 8 ml of light petroleum five times to remove the unbound carotenoid. The absorption spectrum of each resulting aqueous reaction mixture was determined. In addition to β-carotene, the binding of the following ligands to CCBP was tested under standard binding conditions: α-carotene, cryptoxanthin, zeaxanthin, lycopene, astaxanthin, and retinol.

Competitive Binding Assay of CCBP with Alternate Ligands—The competitive binding assay was similar to the standard binding assay described above except for the following details. (i) [14C]β-Carotene (specific activity 168,000 dpm/nmol) was used instead of unlabeled β-carotene. (ii) The incubation with labeled β-carotene was carried out both in the absence and presence of 20-fold excess of the following unlabelled ligands: β-carotene, α-carotene, cryptoxanthin, astaxanthin, lycopene, and retinol. At the end of the incubation period, 100 μl of the reaction mixture was loaded on a Sephadex G-25 column matrix (Bio- Rad disposable column with an Empendorf collection tube; 0.85 × 3 cm, packed dimensions, Bio-Rad) pre-equilibrated with TAB buffer. The column was centrifuged at 1100 × g for 5 min, and the [14C] radioactivity in the eluted fraction was determined using a Beckman LS-6500 liquid scintillation spectrometer, which showed a 14C counting efficiency of 95%. This procedure resulted in the quantitative recovery of CCBP-bound [14C]β-carotene in the Empendorf collection tube, whereas the unbound [14C]β-carotene was completely trapped on the column. Control experiments with (i) labeled β-carotene only and (ii) labeled β-carotene incubated with a nonspecific protein, BSA, showed negligible recovery of the label in the eluate.

Binding Assay for Scatchard Analysis—The method was identical to the competitive binding assay described above, except that the CCBP (62 nm) was incubated with increasing concentrations of [14C]β-carotene (62.5–3200 nm) both in the presence and absence of 20-fold excess of unlabeled β-carotene and the amount of [14C]β-carotene bound was determined from the spin column procedure. The specific binding (total minus nonspecific) was subjected to Scatchard analysis using the LIGAND computer program.

Gel Electrophoresis—All proteins were tested by 10% SDS-PAGE or 6% native PAGE essentially as described by Laemmli and Favre (18), and stained with Silver Stain Plus (Bio-Rad) or Coomassie Blue stain.

Labeling of Apo-CCBP with 125I—Purified apo-CCBP (50 μg) was labeled with 125I using Bolton and Hunter’s reagent essentially as described (19). After extensive dialysis the specific activity of the 125I-apo-CCBP was 2 × 106 cpm/μg, of which 95% was trichloroacetic acid-precipitable (15% w/w).
Results and Discussion

A 67-kDa protein has been purified to homogeneity from ferret liver, which showed a high degree of specificity to β-carotene. The purification steps involved ion exchange, gel filtration, and affinity chromatography, which are described below.

**DEAE-Sephacel Chromatography**—The elution profile of labeled β-carotene complex during ion exchange chromatography of the crude AS fraction of the 100,000×g fraction of ferret liver homogenate in a detergent-containing buffer is shown in Fig. 2. The yellow fraction that was eluted with the elution buffer containing 0.35 M NaCl showed the characteristic β-carotene absorption spectrum (data not shown). It was also found that 78% of the 14C radioactivity applied to the column was associated with this protein complex. SDS-PAGE on 10% gel of this fraction is shown in Fig. 3. It revealed the existence of four major bands and several minor bands when stained with Coomassie Blue. The peak fraction from DEAE-Sephacel column had an absorbance of 0.156 at 280 nm and 0.284 at 465 nm (A280/A465 ratio 1.92).

**Gel Filtration on Sephadex G-75 Column**—The peak labeled β-carotene complex fraction isolated from the DEAE-Sephacel chromatography step was subjected to Sephadex-G75 gel filtration chromatography. Several early fractions were eluted exhibiting minor radioactive peaks, but none of them had β-carotene spectrum (data not shown). However, fractions 20–25 showed a major radioactive peak along with a characteristic β-carotene spectrum. Fractions 20–25 accounted for 74% of the 14C radioactivity applied to the column. The peak fractions 21 and 22 had 14C/protein ratios of 6×105 and 6.1×105 dpm/280 nm absorbance unit, respectively. The peak fraction had an absorbance of 0.24 at 280 nm and 0.46 at 465 nm (A280/A465 ratio 1.92). SDS-PAGE of this fraction showed a major band of 67 kDa and several minor bands of ~50 kDa (data not shown). It was clear that the β-carotene-binding protein was still not homogeneous. A 43-kDa protein band was extracted with TAB buffer and saved for binding assay as a nonspecific ferret liver protein. To purify further, the apoprotein fraction was isolated from the pooled fractions 20–25 by removing the chromophore with cold acetone as described under “Experimental Procedures.”

**Affinity Chromatography**—The apoprotein fraction, isolated from the complex after Sephadex-G75 chromatography, when subjected to affinity chromatography on the Pharmalink-immobilized β-carotene column, yielded a fraction that was eluted with TAB buffer (no detergent) containing 250 mM NaCl. SDS-PAGE of an aliquot of this fraction on 10% gel showed a single homogeneous band with a molecular mass of 67 kDa (Fig. 4). Significantly, no detergent-containing buffer was used to elute the apoprotein from the affinity column. Thus the apoprotein is totally water-soluble. This affinity-purified protein fraction was used for all subsequent analyses. The elution profile of BSA, a nonspecific protein, was tested to demonstrate the specificity of this affinity column. It was found that BSA was completely eluted in the void volume when chromatographed on the affinity column under identical conditions. This experiment proves beyond doubt the specificity of the affinity column to a specific carotenoid-binding protein.

**Summary of Purification**—Table I shows the summary of purification of CCBP. It must be pointed out that because the crude liver homogenate failed to show any high affinity binding...
Table I

Summary of purification of CCBP

| Fraction     | Protein | β-Carotene bound | Specific binding | Purification |
|--------------|---------|------------------|-----------------|-------------|
| AS-Fraction  | 3500    | 77.8             | 0.022           | 1           |
| DEAE-Sephacel| 500     | 166.8            | 0.33            | 15          |
| Sephadex-G75 | 245     | 162.3            | 0.66            | 30          |
| Affinity     | 50      | 667              | 13.35           | 607         |

with labeled β-carotene the true -fold purification of the homogeneous CCBP should be much higher than that reported. Thus, taking the crude AS fraction to have a relative binding of 1 arbitrary unit, CCBP was purified 15-fold at DEAE-Sephacel chromatography step, 30-fold at Sephadex G75 chromatography step, and, finally, 607-fold after the affinity chromatography step. The final yield of the purified protein was approximately 500 μg from 5 g of liver.

Ligand Binding—The purified protein showed a high affinity binding with β-carotene with its characteristic absorption spectrum (Fig. 5) consisting of a shoulder peak at 460 nm and two prominent peaks at 482 and 516 nm, apart from a protein peak at 280 nm. There was a 32-nm bathochromic shift of its λmax compared with that of β-carotene in light petroleum. The bound chromophore could be extracted from the CCBP complex with light petroleum only after it was treated with an equal volume of acetone. In contrast, the nonspecific proteins BSA and ferret liver 43-kDa protein showed low affinity binding to β-carotene as evidenced by the lack of the characteristic β-carotene absorption spectrum in the standard binding assay. This was because the weakly bound β-carotene was completely extracted with light petroleum even without denaturation with acetone.

Scatchard Analysis of Specific Binding Data—Fig. 6 shows the Scatchard plot of the specific binding of β-carotene to CCBP as a function of increasing concentration of β-carotene. The nonspecific binding ranged from 7 to 13% of the total binding at the ligand concentrations tested. The analysis of the specific binding data by LIGAND program showed two classes of binding sites, with an apparent Kd of 56 ± 10⁻⁶ M for the high affinity site and an apparent Kd of 32 ± 10⁻⁶ M for the low affinity site. The nonspecific binding ranged from 7 to 13% of the total binding at the ligand concentrations tested.
Purified 125I-apo-CCBP (10

KDa

-217

-130

-72

1 2 3

Fig. 7. Autoradiogram of native PAGE profile of purified apo-CCBP and holo-CCBP. Purified 125I-apo-CCBP (10 μg; specific activity 2 × 106 cpm/μg) in 100 μl of TAB buffer was incubated with 2.7 nmol of β-carotene for 1 h at 37 °C and subjected to gel filtration on Sephadex G-25 spin column. 125I-Holo-CCBP was isolated in the eluate. Both holo- and apo-CCBP were mixed with glyceral/bromocresol purple loading dye and electrophoresed on a 6% native polyacrylamide gel as described under “Experimental Procedures.” Later, the gel was dried and exposed to autoradiographic film for 3 h. A single slow moving radioactive band can be clearly seen in lane 1 for holo-CCBP, while a faster moving band can be seen in lane 2 for apo-CCBP. However, no molecular size can be assigned to these bands based on their mobility on native PAGE. Bio-Rad Kaleidoscope prestained molecular size markers, consisting of myosin (217 kDa), β-galactosidase (130 kDa), BSA (72 kDa), carbonic anhydrase (42 kDa), soybean trypsin inhibitor (31 kDa), and lysozyme (18 kDa), were run on lane 3. The 217-, 130-, and 72-kDa markers can be seen as diffused bands.

Comparable to that obtained from the Scatchard analysis. Thus, it is reasonable to conclude that CCBP binds β-carotene mole per mole at the high affinity site. In contrast, the calculated Bmax of 145 mol/mol by LIGAND program for the low affinity site may not have physiological relevance because of its very high Ks of 32 μM.

Native PAGE of the Purified CCBP Complex—The purified 125I-apo-CCBP was complexed with β-carotene under standard conditions, and both the holo- and apo-CCBP were subjected to native PAGE on a 6% polyacrylamide gel followed by autoradiography. Fig. 7 shows that both holo-CCBP (lane 1) and apo-CCBP (lane 2) moved as homogeneous bands, although apo-CCBP moved faster than the holo-CCBP. Since the mobility of proteins in native PAGE is by their net charge, native PAGE is not a reliable method to assess the molecular size of any protein (21). Significantly, 125I-apo-CCBP moved as a single sharp band with a molecular size of 67 kDa on a 10% SDS-PAGE gel (data not shown) and thus confirmed our finding of the mobility of unlabeled apo-CCBP (Fig. 4).

Competition by Alternate Ligands—Among the alternate ligands tested, only α-carotene and cryptoxanthin showed any binding as evidenced by their corresponding absorption spectra (data not shown). In contrast, β-ionone ring substituted carotenoids such as zeaxanthin or astaxanthin, exhibit an absorption spectrum without an intact β-ionone ring like lycopene, and a shortened molecule with one intact β-ionone ring like retinol showed no binding, as evidenced by the lack of their characteristic absorption spectra.

Competitive binding of [14C]β-carotene by 20-fold excess of each alternative ligand was determined, and the results are shown in Table II. Each value is the average of triplicate experiments. It is obvious that, while α-carotene and cryptoxanthin inhibited labeled β-carotene binding by 94% and 84%, respectively, none of the other ligands tested showed any competition. Thus, CCBP showed a high degree of specificity toward carotenoids with at least one unsubstituted β-ionone ring but not toward other carotenoids, or retinol.

It is significant to point out that in contrast to a molecular size of 67 kDa for the mammalian CCBP found in this study, carotenoproteins from various lower organisms vary widely between 18 and 350 kDa in their sizes. Thus, α-crustacyanin, an astaxanthin-binding protein from carapace of the lobster, is a 350-kDa protein (22), whereas a lutein-binding protein isolated from the mid-gut of silkworm Bombyx mori is a 36-kDa protein (23). The plant and the bacterial carotenoproteins are 18- and 35-kDa proteins, respectively (24, 25). On the other hand, mammalian retinoid-binding proteins are in the 15-kDa range (11, 12).

CCBP from the mammalian source in the present study exhibits three peaks at 460, 482, and 516 nm (Fig. 5) with a 32-nm bathochromic shift in its λmax compared with the absorption spectrum of β-carotene in light petroleum. Interestingly, carotenoid-binding proteins from Mangifera indica (26) and from cyanobacterium (25) also had a similar absorption spectra with λmax at 498 and 476 nm, respectively. However, crustacyanin, the carotenoprotein from lobster carapace, showed a 160-nm bathochromic shift in its λmax compared with the absorption spectrum of the parent carotenoid, astaxanthin (27).

Possible Roles of CCBP in Biological Systems—The physiological role(s) of CCBP remains to be defined. In view of the possible protective roles of carotenoids against cancer, heart disease, and erythropoietic porphyria, a potential role for a specific binding protein may become central in the mechanism of action of carotenoids. Thus, CCBP may play a major role in the storage, transport, and targeting of β-carotene in mammalian systems. It may also act as the natural substrate for many of the metabolic reactions of β-carotene. By virtue of forming a stable high affinity complex, it may protect the carotenoid from degradation. As a result, carotenoids bound to CCBP may be better antioxidants compared with free carotenoids and thus protect the system from oxidative damage.

In conclusion, a specific CCBP has been purified to homogeneity with a molecular mass of 67 kDa from a mammalian liver. It exhibits high affinity binding to β-carotene in the molar ratio of 1:1, with an apparent dissociation constant of 56 × 10⁻⁹ M. The complex exhibits the characteristic carotenoid absorption with the λmax at 482 nm. Alternate ligands, with one intact β-ionone ring like α-carotene and cryptoxanthin, compete with β-carotene binding by 94% and 84%, respectively. However, CCBP does not bind astaxanthin, zeaxanthin, lycopene, or retinol.

### Table II

| Ligand                  | Inhibition % |
|------------------------|-------------|
| β-Carotene             | 100         |
| α-Carotene             | 94          |
| Cryptoxanthin          | 84          |
| Astaxanthin            | 5           |
| Lycopene               | +13         |
| Retinol                | 0           |
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