Journal of Chromatography, 482 (1989) 107–115
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 837

CHROMATOGRAPHIC METHOD FOR THE PREPARATION OF APO-CELLULAR RETINOL-BINDING PROTEIN AND APO-CELLULAR RETINOIC ACID-BINDING PROTEIN FROM THEIR HOLO-TYPES

FUMIO FUKAI*, HIROYUKI SUZUKI and TAKASHI KATAYAMA
Department of Patho-Physiology, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Ichigaya Funagawara-Machi, Shinjuku-ku, Tokyo 162 (Japan)
(First received March 7th, 1989; revised manuscript received June 20th, 1989)

SUMMARY

A chromatographic method is described for the preparation of apo-cellular retinol-binding protein (CRBP) and apo-cellular retinoic acid-binding protein (CRABP) from their corresponding holoproteins. Elimination of retinoids from either purified CRBP or CRABP holoprotein complex could be performed quantitatively by DEAE-cellulose chromatography without any alteration in the inherent properties of the native proteins. In contrast, the usual methods, involving UV irradiation or acetone precipitation, resulted in some modification of these binding proteins. This chromatographic method was also applicable to the preparation of apo-fatty acid-binding protein (FABP) from FABP–palmitic acid holoprotein complex.

INTRODUCTION

In addition to circulating retinol-binding protein (so-called RBP)[1], some retinoid-binding proteins exist in tissues, including cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP)[2,3]. These binding proteins are distributed in various organs with relatively high concentrations and play a role in transporting their corresponding ligands to appropriate intracellular locations[3]. It has been presumed that interaction of retinoids with CRBP and CRABP would be a prerequisite for the storage or metabolism of retinoids and for the expression of retinoid action[2–4]. For the correct understanding of such interactions, it would be necessary first to obtain both apo- and holo-types of these retinoid-binding proteins having their inherent properties.

The purification of specific binding proteins, including CRBP and CRABP, is usually performed after occupying their binding sites with the corresponding ligands, as these proteins are generally unstable when free from ligands. Accordingly, in order to obtain their apoproteins, it is necessary to purify holoproteins and then to strip the ligands from them. CRBP and CRABP apoproteins have previously been prepared from their holo-types by acetone precipitation[5] and UV irradiation[5,6] methods, but there is little information about whether or not apoproteins prepared by these methods

0021-9673/89/$03.50

still maintain their own properties. In the acetone precipitation method, the proteins may be denatured in acetone solution. And also in the UV irradiation of holoproteins, some possible modifications of the proteins by decomposition products of retinoids are suspected. We describe here a method for the preparation of apo-CRBP and apo-CRABP from their corresponding holoproteins without their denaturation. A comparison of this method with the previous method is reported.

EXPERIMENTAL

Materials

[11,12(*n*)-^{3}H]Vitamin A, free alcohol (60 Ci/mmol), was purchased from Amersham International and [11,12-^{3}H(N)]retinoic acid (52.5 Ci/mmol) from New England Nuclear. All-*trans*-retinol and all-*trans*-retinoic acid were obtained from Sigma. DEAE-cellulose (DE 52) was purchased from Whatman.

Purification of CRBP, CRABP and FABP holoproteins

Purification of CRBP and CRABP holoproteins from rat testes cytosol was carried out according to the method of Ong and Chytil[7,8]. During purification, CRBP and CRABP were labelled with [^{3}H]retinol and [^{3}H]retinoic acid, respectively, instead of non-radioactive retinoids. Purification of the fatty acid-binding protein (FABP)–[^{3}H]palmitic acid holoprotein complex was performed as described previously[9]. Each purified holoprotein was shown to be apparently homogeneous as judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Preparation of CRBP and CRABP apoproteins

CRBP and CRABP free from retinoids were prepared using three different methods, as follows.

Chromatographic method using a DEAE-cellulose column. A 1-ml volume of the purified holoprotein solution at a concentration of 6 μM in 20 mM Tris–HCl buffer (pH 7.4) containing 10% glycerol (TG buffer) was applied to a DEAE-cellulose column (2 × 0.5 cm I.D.) equilibrated with TG buffer. The column was washed successively with 4 ml each of 10%, 20% and 50% ethanol in TG buffer, and then washed with the opposite series of ethanol concentrations in TG buffer. After washing further with 5 ml of TG buffer, the protein was eluted with 0.2 M sodium chloride in TG buffer and then dialysed with 20 mM potassium phosphate buffer (pH 7.4) containing 0.15 M sodium chloride (phosphate-buffered saline; PBS).

UV-irradiation method. UV irradiation was carried out as reported previously[5,6]. Each purified preparation of the holoproteins at a concentration of 6 μM in PBS was exposed to glass-filtered UV light (365 nm; intensity 0.5 $\mu W/cm^2$; UV lamp, Tokyo Kohgaku) below 4°C for over 12 h. Destruction of retinoids was assessed by monitoring the disappearance of the intrinsic absorption at 350 nm and fluorescence (excitation 350 nm, emission 480 nm) of these holoproteins[6–9]. The irradiated sample was subjected to gel filtration on a Sephadex G-25 column (9.5 × 1.5 cm I.D.) in PBS and the protein fractions were pooled.

Acetone precipitation method. This method was performed as reported by MacDonald and Ong[5]. A 1-ml volume of the purified holoprotein solution at a concentration of 6 μM in PBS was added dropwise to 20 ml of acetone at −20°C in an

acetone–dry-ice bath. After stirring for 10 min, the precipitated protein was recovered by centrifuging at 5000 rpm (3000 *g*) for 5 min. The supernatant was discarded and the pellet was resuspended in 8 ml of acetone at −20°C. Following centrifugation, the precipitate was carefully dried under a gentle stream of argon. The pellet was dissolved in 1 ml of PBS and subjected to gel filtration on a Sephadex G-25 column as described above.

The protein concentration in each preparation was determined by the method of Lowry *et al.*[10].

Apparent binding activity of CRBP and CRABP

Each sample containing 100–150 n*M* protein in PBS (1-ml volume) was incubated with 300 n*M* of tritium-labelled retinoid ([^{3}H]retinol or [^{3}H]retinoic acid) at 4°C for 3 h in the dark. The sample mixture was then subjected to gel filtration as described above. Radioactivity in the protein fraction was measured as the total binding activity. To measure the non-specific binding activity, parallel incubations were carried out in the presence of a 100-fold molar excess of the corresponding non-radioactive retinoids. The specific binding activity was calculated by subtracting the non-specific binding activity from the total binding activity.

Dissociation constant

Apoprotein solution was incubated with the corresponding tritium-labelled retinoids at concentrations from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-6}$ *M* in the presence or absence of a 100-fold molar excess of non-radioactive retinoids. After the incubation, each sample was subjected to gel filtration (Sephadex G-25 column, 9.5 × 1.5 cm I.D.) as described above. Binding data were plotted according to Scatchard[11].

RESULTS

Purified CRBP–[^{3}H]retinol holoprotein complex adsorbed quantitatively on the DEAE-cellulose column at pH 7.4. When the column was washed with increasing concentrations of ethanol (10–50% in TG buffer), most of the applied radioactivity was eluted (Fig. 1). As protein was not detected in this eluate, it was considered that only retinol was released from the column, leaving the protein on the gel. After washing off the ethanol with TG buffer, about 80% of the applied CRBP, which was nearly free from retinol, could be recovered by eluting with 0.2 *M* sodium chloride in TG buffer (Fig. 1 and Table I). Similarly, the preparation of apo-CRABP from the CRABP–[^{3}H]retinoic acid holoprotein complex was achieved in the same way with over 80% yield as protein. Whether or not these apoproteins maintain their inherent properties was analysed as described below.

The spectral properties were examined (Fig. 2). The original holo-CRBP revealed a dominant absorption peak at 350 nm due to bound retinol. After the DEAE-cellulose chromatography, the peak at 350 nm almost disappeared. This absorption peak reappeared in holo-CRBP reconstituted by incubating the apoprotein with retinol. The ratio of the absorbance at 350 nm to that at 280 nm (A_{350}/A_{280}) in the reconstituted holo-CRBP (1.68) was higher than that in the original holo-CRBP (1.62). The absorption spectrum of the reconstituted holo-CRABP was similar to that of the original holoprotein, and the A_{350}/A_{280} values for the original and reconstituted

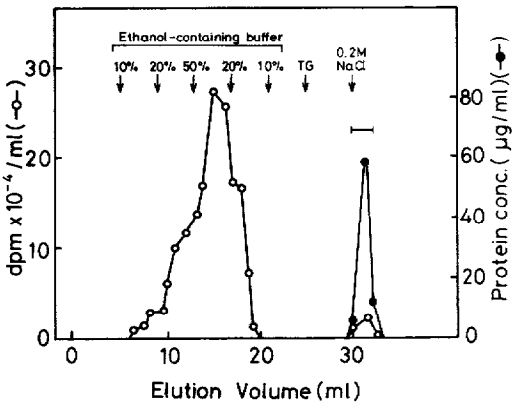

Fig. 1. Preparation of apo-CRBP from CRBP-[^{3}H]retinol holoprotein complex by DEAE-cellulose chromatography. Purified preparation (1 ml) of the holoprotein (6 μM) was applied to a DEAE-cellulose column (2 × 0.5 cm I.D.) equilibrated with TG buffer, and the column was washed with 4 ml each of the indicated concentrations of ethanol solution in TG buffer. After further washing with 5 ml of TG buffer, elution was carried out with TG buffer containing 0.2 M sodium chloride. Eluates were examined for (○) radioactivity and (●) protein concentration. Fractions eluted with 0.2 M sodium chloride were pooled (solid bar) as the preparation of apo-CRBP.

holo-CRABP were 1.72 and 1.76, respectively. These results indicate that the conformational changes of these binding proteins during the chromatography may be negligible.

The binding characteristics of these apoproteins were next examined. The binding activity of the apo-CRBP with retinol increased apparently up to 1.5 times that of the original holo-CRBP (Table I). Such an increase in apparent binding activity was also observed with the apo-CRABP obtained by the chromatographic method, and its

TABLE I

COMPARISON OF METHODS FOR THE PREPARATION OF CRBP AND CRABP APOPROTEINS

Method	*Apoprotein*	*Radioactivity (dpm · 10^{-4})*		
		Original holoprotein	*After treatment*[a]	*Reconstituted holoprotein*[b]
DEAE-cellulose chromatography	CRBP	133.8	3.8	202.0 (151%)[c]
	CRABP	68.5	2.1	90.4 (132%)
UV irradiation	CRBP	133.8	18.7	61.4 (46%)
	CRABP	68.5	10.3	28.0 (41%)
Acetone precipitation	CRBP	133.8	1.8	22.7 (17%)
	CRABP	68.5	1.2	17.1 (25%)

[a] Radioactivity remaining after the treatment.

[b] Apparent binding activity as determined by incubation of each apoprotein with 300 nM of the corresponding tritium-labelled retinoids as described under Experimental.

[c] Recovery of binding activity through the treatment expressed as a percentage of that of the original holoprotein is given in parentheses.

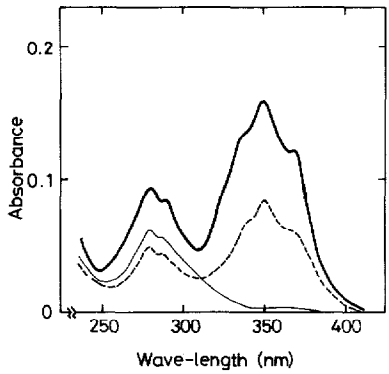

Fig. 2. Absorption spectra of CRBP. Bold line, original holo-CRBP; thin line, CRBP after the chromatography; dotted line, reconstituted holo-CRBP. Each sample was subjected to gel filtration on a Sephadex G-25 column and absorption spectra of the protein fractions were measured.

binding activity with retinoic acid was about 130% of the original value for holo-CRABP (Table I). As mentioned above, with both CRBP and CRABP the A_{350}/A_{280} value of the original holoprotein was smaller than that of the reconstituted holoprotein. Therefore, the increase in the apparent binding activity might be attributed to both original holoproteins not being fully saturated by their corresponding ligands. To assess the recovery of binding activity in the chromatographic method more quantitatively, Scatchard analysis was then performed (Fig. 3). Apo-CRBP obtained with our method showed a dissociation constant ($K_d = 1.6 \cdot 10^{-9}$ M) of the same order as that of apo-CRBP in rat testes cytosol ($2.5 \cdot 10^{-9}$ M). The total binding sites in the preparation after the chromatography were calculated by extrapolation of the linear plot to be about 5 μM. As the protein concentration of this preparation was about 5 μM as CRBP, the results indicate that most of the protein was

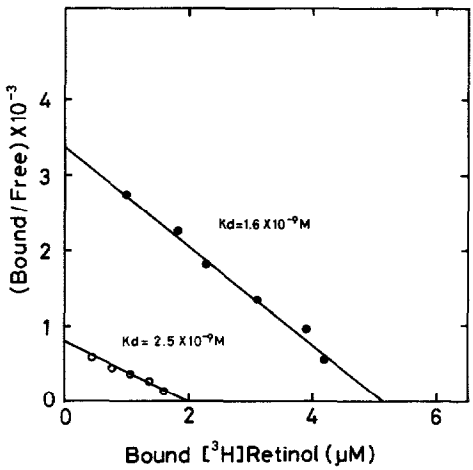

Fig. 3. Scatchard analysis of binding of retinol with CRBP. (○) Rat testes cytosol; (●) apo-CRBP obtained by the chromatographic method.

recovered as an active protein that displays a high affinity toward retinol. On the other hand, binding of the apo-CRBP with [^{3}H]retinol was interrupted by a 100-fold molar excess of non-radioactive retinol, but not by retinoic acid (Fig. 4). Apo-CRABP obtained by our method also retained its own binding affinity and specificity (data not shown). In addition, the molecular parameters of both CRBP and CRABP (Stokes radius 17 Å, sedimentation coefficient 2.3S) were not changed after the chromatography. It was therefore concluded that either CRBP or CRABP apoprotein could be prepared by the chromatographic method without the loss of their own characteristics.

In this chromatographic method, an increase in the ethanol concentration of the washing buffer caused a decrease in binding activity. For example, the binding activity of apo-CRBP prepared using 80% ethanol solution was 65% of that in the standard method. Utilization of another organic solvent having a lower polarity, such as a 50% solution of isopropanol or acetone, also resulted in some decrease in their binding activities (data not shown). In contrast, ethylene glycol was shown to have the ability to dissociate retinol from holo-CRBP. Apo-CRBP could be obtained quantitatively with the chromatographic method by utilizing 50% ethylene glycol solution instead of ethanol. However, elimination of retinoic acid from holo-CRABP by this solution was hardly detectable. On the other hand, DEAE-cellulose could be used as an adsorbent in this method instead of a hydroxyapatite gel. Both CRBP and CRABP adsorbed on the gel, and retinoids were removed effectively with the ethanol-containing buffer in the same manner. However, the recoveries of both proteins were very low (20–40% of the applied protein).

Our chromatographic method was also applicable to the preparation of apo-FABP from the FABP–[^{3}H]palmitic acid holoprotein complex. As FABP has a basic p*I* (8.5), as reported previously[12], it was necessary to carry out the DEAE-cellulose chromatography at pH 9.5. Elimination of the ligand and elution of the resulting apo-FABP could be performed as described above. The binding activity of apo-FABP thus obtained was about 90% of that of the original holoprotein. The Stokes radius (16 Å) and sedimentation coefficient (2.3S) did not change after the chromatography.

The usual methods for the preparation of apo-CRBP, with either UV irradiation

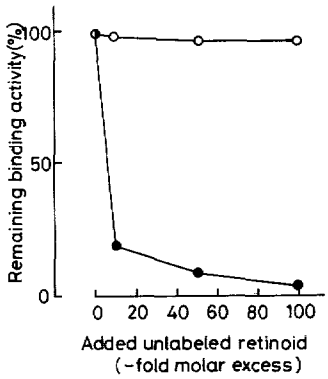

Fig. 4. Binding specificity of apo-CRBP prepared by the chromatographic method. Aliquots of the apo-CRBP preparation were incubated with 300 n*M* of [^{3}H]retinol with the addition of the indicated molar excess of (●) retinol or (○) retinoic acid.

or acetone precipitation, were compared with our chromatographic method. When purified CRBP–[^{3}H]retinol holoprotein complex was exposed to long-wavelength UV light, the characteristic absorption peak at 350 nm decreased in size and had almost completely disappeared after irradiation for over 12 h. However, a considerable amount of radioactivity still remained in the protein fraction (12% of the original) (Table I). The apparent binding activity of the protein with retinol was less than 50% of that of the original holo-CRBP (Table I). Neither the radioactivity remaining in the protein nor the binding activity was altered even when the irradiation was prolonged. The remaining radioactivity could not be released by extraction with *n*-hexane. Some decomposition products of retinol might bind covalently to the protein. Similarly, the absorption at 350 nm of holo-CRABP disappeared on irradiation, and the treatment was accompanied by loss of its binding activity with retinoic acid (Table I). Some radioactivity (about 15% of the original) also remained in the preparation (Table I).

In contrast to the UV-irradiation method, the radioactivity of retinoids was effectively removed from the holoproteins by acetone precipitation (Table I). However, treatment with acetone caused a considerable decrease in their binding activities (Table I). Apo-CRBP prepared by this method retained only 17% of the binding activity of the original holo-CRBP, although nearly 80% of the protein was recovered. This means that about 80% of the recovered protein was inactivated during the treatment. When holo-CRBP reconstituted by relabelling the protein with [^{3}H]retinol was subjected to sucrose density gradient centrifugation analysis, most of the radioactivity migrated to the bottom of the centrifuge tube (over 10S). The results show clearly that CRBP was aggregated by treatment with acetone. Acetone precipitation of CRABP also resulted in a marked decrease in its binding activity (Table I) and in aggregation of the protein.

DISCUSSION

A chromatographic method utilizing a DEAE-cellulose column for the preparation of CRBP and CRABP apoproteins was established. This method enabled both apoproteins to be obtained quantitatively without any loss of their inherent properties. The principle of this method is substantially similar to that of the acetone precipitation method, *i.e.*, both methods are based on the fact that hydrophobic ligands are easily released from binding with protein in an organic solvent. In the utilization of these methods, protein denaturation should therefore be taken into account. Indeed, acetone precipitation resulted in denaturation of CRBP and CRABP. As in our method the proteins are bound to DEAE-cellulose and retinoids can be eliminated by relatively low concentrations of ethanol (less than 50%), denaturation of the proteins may be avoided. The similarity of the spectral properties of the holoproteins prior to and after passage through a DEAE-cellulose column supports the assumption that the conformational changes of the proteins may be kept to a minimum in our method.

Retinoid-binding proteins free from retinoids have been prepared exclusively by the UV-irradiation method, taking advantage of the photo-lability of retinoids[6,13]. We tried to examine this method. The characteristic absorption peak at 350 nm of holo-CRBP or holo-CRABP disappeared on UV irradiation, indicating degradation of the retinoids. However, the binding activity had also decreased by about half after

the irradiation. Ong and Chytil[6] reported that retinol bound to CRBP could be destroyed by long-wavelength UV light (intensity 0.5 $\mu W/cm^2$) for 10–15 h without detectable damage to the protein. Their irradiation conditions were almost the same as those in this work. In contrast, MacDonald and Ong[5] treated CRBP and CRBP II holoproteins with UV light for only 25 min to obtain the corresponding apo-proteins[5]. The reduction in binding activity in our experiment may be related to the prolonged time of UV irradiation. However, the fact that considerable amounts of radioactivity were bound covalently to the proteins after the irradiation would be meaningful for assessing this method. In any case, the UV-irradiation method is suitable only for removing photo-labile ligands.

On examination of the washing buffer conditions in our chromatographic method, a unique phenomenon was observed. Elimination of retinol from holo-CRBP could also be achieved with 50% ethylene glycol solution, extended to retinoic acid was not dissociated from holo-CRABP under these conditions. Although the mechanism of this specific effect of ethylene glycol remains unclear, it may be possible that ethylene glycol specifically affects the interaction between CRBP and retinol. On the other hand, we adopted DEAE-cellulose as the adsorbent of the retinoid-binding proteins, but another adsorbent such as CM-cellulose or hydroxyapatite could be employed, depending on the characteristics of the desired protein. For instance, both CRBP and CRABP were adsorbed quantitatively on a hydroxyapatite gel and removal of the retinoids could be carried out effectively by washing the gel with an ethanol-containing buffer, although the proteins could not be eluted in good yields.

Recently, Gordon and co-workers[14–16] reported a comparison of the tertiary structures of FABP and CRBP II by X-ray diffraction. To obtain FABP and CRBP II apoproteins without denaturation, they expressed these apoproteins genetically in *Escherichia coli* and purified them from cell lysate. Our chromatographic method was shown to be applicable to the preparation of apo-FABP from the FABP–palmitic acid holoprotein complex by DEAE-cellulose chromatography at pH 9.5[9,12]. Similarly, apo-CRBP II may be obtained by the chromatographic method. Hence it can be expected that our chromatographic method will be widely applicable to the preparation of various apoproteins including other retinoid-binding proteins and also steroid hormone receptors if appropriate chromatographic conditions are chosen.

ACKNOWLEDGEMENT

The authors thank Dr. Sukeo Onodera, Science University of Tokyo, for his valuable suggestions.

REFERENCES

1 D. S. Goodman and W. S. Blander, in M. B. Sporn, A. B. Roberts and D. S. Goodman (Editors), *Biosynthesis, Absorption, and Hepatic Metabolism of Retinol, Vol. 2, The Retinoids*, Academic Press, New York, 1984, pp. 1–39.
2 J. C. Saari and A. H. Bunt-Milam, in M. I. Sherman (Editor), *Retinoids and Cell Differentiation*, CRC Press, Boca Raton, FL, 1986, pp. 1–15.
3 F. Chytil and D. E. Ong, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 38 (1979) 2510.
4 A. M. Jetten and M. E. Jetten, *Nature (London)*, 278 (1979) 180.
5 P. N. MacDonald and D. E. Ong, *J. Biol. Chem.*, 262 (1987) 10550.

6 D. E. Ong and F. Chytil, *Methods Enzymol.*, 67 (1980) 288.
7 D. E. Ong and F. Chytil, *J. Biol. Chem.*, 253 (1978) 828.
8 D. E. Ong and F. Chytil, *J. Biol. Chem.*, 253 (1978) 4551.
9 F. Fukai, T. Kase, T. Shidotani, T. Nagai and T. Katayama, *Biochem. Biophys. Res. Commun.*, 147 (1987) 899.
10 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
11 G. Scatchard, *Ann. N. Y. Acad. Sci.*, 51 (1949) 660.
12 F. Fukai, T. Kase, T. Shidotani, T. Nagai and T. Katayama, *Biochem. Int.*, 18 (1989) 1101.
13 Y. Takahashi, T. Nakajima, M. Washitake, T. Anmo, M. Sugiura and H. Matsumaru, *Chem. Pharm. Bull.*, 27 (1979) 12.
14 E. Li, B. Locke, N. C. Yang, D. E. Ong and J. I. Gordon, *J. Biol. Chem.*, 262 (1987) 13773.
15 J. C. Sacchettini, D. S. Khausen, E. Li, L. J. Banazak and J. I. Gordon, *J. Biol. Chem.*, 262 (1987) 15756.
16 E. Li, C. A. Demmer, D. A. Sweetser, D. E. Ong and J. I. Gordon, *Proc. Natl. Acad. Sci. U.S.A.*, 83 (1986) 5779.