Chromatofocusing in the purification and separation of apo- and holo-(vitamin D-binding protein)

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Chromatofocusing was used to purify the vitamin D-binding protein (DBP) from pig plasma in a procedure that consisted of an initial DEAE-cellulose chromatography followed by DEAE-Sephadex chromatography, with final purification by chromatofocusing. The protein was purified 184-fold over its concentration in plasma. When the plasma was labelled with a tracer concentration of [³H]caldiol, it was apparent that holo- and apo-DBP did not co-chromatograph on chromatofocusing. The separation of these two forms of DBP on chromatofocusing was verified by using purified apo-DBP mixed with either a tracer or a saturating concentration of caldiol. This separation was consistent with differences observed in their isoelectric points. The ability to separate apo and holo forms of DBP should permit the study of their specific interactions with other binding proteins and help determine the physiological relevance of these interactions.

Vitamin D-binding protein (DBP) transports vitamin D and its hydroxylated metabolites in serum. It is a glycoprotein with an M_r of 52000, with one high-affinity binding site for the ligand. This protein has also been referred to as group-specific component, G_c , a frequently used genetic marker (Daiger *et al.*, 1975; Bouillon *et al.*, 1976). The DBP concentration in plasma is normally relatively invariant between 5 and 10 μ M, whereas the concentration of the main circulating form of vitamin D, caldiol (25-hydroxyvitamin D₃), is in the range 10–100 nM. The reason for the large molar excess of apo-DBP is not known. It has variously been proposed to protect against vitamin D toxicity (Bouillon & Van Baelen, 1979), to prevent oxidation of the bound ligands (Surarit & Svasti, 1980), to promote cholecalciferol (vitamin D₃) conversion from (6Z)-tacalcinol (pre-vitamin D₃) in skin (Holick *et al.*, 1980), and to depolymerize actin released into plasma (Van Baelen *et al.*, 1980).

An important distinction between apo- and holo-DBP may be their different conformations. Svasti & Bowman (1978) observed that the binding of cholecalciferol by apo-DBP resulted in the lowering of the isoelectric point of DBP, and they

proposed that it resulted from a conformational change in the protein. Surarit & Svasti (1980) confirmed that such changes occurred by the use of c.d. and fluorescence spectroscopy. Because of these conformational differences apo- and holo-DBP may interact differently with specific binding proteins. The separation of these two forms should facilitate the study of these interactions. Chromatofocusing separates proteins with different isoelectric points (Sluyterman & Elgersina, 1978). In the present paper we describe its successful utilization to separate apo- and holo-DBP. Furthermore, we report that this technique is a useful adjunct in the purification of DBP, decreasing the number of chromatographic steps required.

Materials and methods

Materials

DEAE-cellulose (DE-52) was obtained from Whatman, Clifton, NJ, U.S.A. DEAE-Sephadex, Polybuffer Exchanger, low- M_r protein standards and Polybuffer, pH 4.0, were from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. [26,27-³H]Caldiol (22 Ci/mmol) was from Amersham Corp., Arlington Heights, IL, U.S.A. All chemicals, of reagent grade, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Abbreviation used: DBP, vitamin D-binding protein.

Purification of DBP

Pig plasma (600 ml) was labelled with $1.7 \mu\text{Ci}$ of [^3H]caldiol. After dialysis against 10 mM-sodium phosphate buffer, pH 5.9, the plasma was centrifuged at $15000g_{\text{max}}$ for 20 min and the precipitate was discarded. The supernatant was applied to a DEAE-cellulose column ($3.8 \text{ cm} \times 33 \text{ cm}$), and the radioactivity was eluted with 4 litres of a linear gradient of 0.01–0.1 M-sodium phosphate buffer, pH 5.9, at a flow rate of 40–60 ml/h. The fractions containing the peak of radioactivity were combined and adjusted to a $(\text{NH}_4)_2\text{SO}_4$ concentration of 55% with the addition of solid $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation at $15000g_{\text{max}}$ for 20 min and was dialysed against 0.1 M-NaCl/0.05 M-Tris/HCl buffer, pH 9. The non-diffusible material was then applied to a DEAE-Sephadex column ($3.8 \text{ cm} \times 14.5 \text{ cm}$), and the DBP was eluted with 600 ml of a linear salt gradient of 0.1–0.2 M-NaCl in 0.05 M-Tris/HCl buffer pH 9.0, at a flow rate of 50–70 ml/h. The fractions containing radioactivity were pooled, dialysed against 0.1 M- NH_4HCO_3 and then freeze-dried. The freeze-dried powder was dissolved in 25 mM-piperazine, pH 5.5, and applied to a chromatofocusing column ($1.6 \text{ cm} \times 50 \text{ cm}$). The column was eluted with Polybuffer 74 that had been diluted 1:10 and adjusted to pH 4 with 0.5 M-HCl, at a flow rate of 30–40 ml/h, thereby generating a pH 5–4 gradient. The desired protein fractions were pooled and dialysed against 0.1 M- NH_4HCO_3 and then freeze-dried.

Polyacrylamide-gel electrophoresis

Proteins were separated by electrophoresis on sodium dodecyl sulphate/8%-(w/v)-polyacrylamide slab gels as described by Laemmli (1970). Proteins were stained with Coomassie Blue in accordance with the procedure of Fairbanks *et al.* (1971).

Isoelectric focusing

To determine pI, isoelectric focusing was conducted in polyacrylamide gels in a FBE 3000 flat-bed apparatus (Pharmacia). The gel was prepared from a solution consisting of 15 ml of a stock acrylamide solution (24.25 g of acrylamide, 0.75 g of bisacrylamide, 2.5 g of Amberlite MB-1 and 250 ml of distilled water), 1.9 ml of Pharmalytes (Pharmacia), pH 4.0–6.5, 4 ml of glycerol and 9.1 ml of distilled water. The anode solution consisted of 40 mM-DL-glutamic acid, and the cathode solution was 0.2 M-L-histidine. Samples (1 mg/ml) were run at 2000 V for 15 h. The gel was fixed with a solution containing 5% (w/v) sulphosalicylic acid and 10% (w/v) trichloroacetic acid for 60 min. The gel was placed in a destaining solution of methanol/acetic acid/water (3:1:6, by vol.) for 30 min, and then in a 0.2% Coomassie Brilliant

Blue stain for 3–6 h. After the staining, the gel was returned to the destaining solution until the background was clear. Isoelectric points of samples were assessed by comparison with a low-pI calibration kit (pH 2.5–6.5) purchased from Pharmacia.

Results

Purification of DBP from pig plasma in a procedure involving the use of chromatofocusing

A tracer amount of [^3H]caldiol was added to pig plasma to enable the fractionation of DBP to be monitored during its isolation. Plasma was not equilibrated with non-radioactive caldiol sufficient to saturate DBP because this resulted in significant amounts of non-specific binding, presumably to albumin and lipoproteins. The determination of specific radioactivities after the saturation of plasma with caldiol was thus an inaccurate indicator during DBP isolation and the pattern of elution was complex.

Three columns were used: DEAE-cellulose, DEAE-Sephadex and chromatofocusing. The elution profiles of protein and radioactivity for DEAE-cellulose are illustrated in Fig. 1(a). This column removed the bulk of the total plasma protein, resulting in a 22-fold purification (Table 1). DBP was eluted between 0.07 M- and 0.09 M-phosphate in a 0.01–0.1 M-phosphate gradient.

After concentration of the proteins in the fraction by $(\text{NH}_4)_2\text{SO}_4$ fractionation and subsequent dialysis, the sample was loaded on a DEAE-Sephadex column. DBP was eluted in 200 ml between 0.14 M- and 0.16 M-NaCl in a 0.1–0.2 M-NaCl gradient (Fig. 1b). The cumulative purification after this step was 87.5-fold (Table 1).

After freeze-drying and dialysis the fraction was applied to a chromatofocusing column. With a pH 5–4 gradient, major and minor protein peaks, fractions 83–104 and 130–160, and one major radioactive peak, fractions 110–125, were obtained (Fig. 1c).

When these peaks were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the major 280 nm-absorbing and radioactive peaks contained a single polypeptide migrating with an apparent M_r of 52000 (Fig. 2), suggesting these were apo and holo forms of the protein. The high degree of purity of the DBP was demonstrated by overloading the gel (lane 3 in Fig. 2) with DBP.

Combining the main protein and radioactivity fractions indicated that 184-fold purification was achieved (Table 1). This is consistent with the maximum degree of purification possible, as the concentration of DBP in humans, rats and pigs ranges from 1/150th to 1/250th of the total plasma protein. This degree of purification corresponds to

Fig. 1. Elution profiles during the purification of DBP.

(a) A 35.3g portion of dialysed pig plasma was loaded on a 3.8cm $\times$ 33cm DEAE-cellulose column. A linear phosphate gradient (----) was used for elution, generated with 2 litres of 0.01 M-sodium phosphate buffer, pH 5.9, and 2 litres of 0.1 M-sodium phosphate buffer, pH 5.9, and 20 ml fractions were collected. (b) The radioactive peak in (a) was loaded on a 3.8cm $\times$ 14.5cm DEAE-Sephadex column, and it was eluted with a linear gradient (----) generated with 300 ml of 0.1 M-NaCl/0.05 M-Tris/HCl, pH 9.0, and 300 ml of 0.2 M-NaCl/0.05 M-Tris/HCl, pH 9.0, and 3 ml fractions were collected. (c) The radioactive peak from (b) was fractionated on a 1.6cm $\times$ 50cm column containing Polybuffer Exchanger 94, equilibrated with 25 mM-piperazine, pH 5.5. Fractions of volume 4 ml were eluted with Polybuffer 74 that had been diluted 1:10 and adjusted to pH 4.0 with 0.5 M-HCl, and a pH gradient (----) was used for elution. Protein (○) and radioactivity (●) were monitored, in each case.

a 7 μ M concentration of DBP in the initial pig plasma.

Verification of the separation of apo- and holo-DBP

To demonstrate the separation of holo- and apo-DBP, 15 mg of presumptive apo-DBP was incubated with [3 H]calcidiol in a 1:1 molar ratio. After incubation for 24 h to allow complete binding, the DBP was loaded on a chromatofocusing column. The elution profile is shown in Fig. 3(a). The protein peak and the radioactive peak were eluted together, consistent with the formation of holo-DBP. The elution profile of apo-DBP loaded on the chromatofocusing column after incubation with a tracer amount of [3 H]calcidiol is shown in Fig. 3(b). Two separate peaks were present: a protein peak, which was eluted first, followed by a radioactive peak. The radioactive peak was eluted in the same fractions as the apparent holo-DBP shown in the lower profile. This confirms that the protein peak in this profile represents apo-DBP. Final verification that these two forms separate on a chromatofocusing column was obtained by combining the

presumptive apo and holo forms (Fig. 3c). Two protein peaks were obtained, with radioactivity being co-eluted with one of the protein peaks, clearly indicating that chromatofocusing separates the apo and holo forms (Fig. 3c).

Isoelectric points of apo- and holo-DBP

The separation of apo- and holo-DBP on chromatofocusing suggests that a change in pI occurs after apo-DBP binds ligand. Isoelectric focusing on polyacrylamide gels indicated that apo-DBP had pI 4.93 and holo-DBP pI 4.80. This is consistent with the results reported by Svasti & Bowman (1978).

Binding of calcidiol to isolated DBP

The chromatofocusing technique exposes DBP to a low pH for a short period of time. To determine whether this altered the interaction of calcidiol with DBP, we conducted a Scatchard analysis of binding. A single class of high-affinity binding sites was observed, with a calculated dissociation constant of 1.53×10^{-7} M and a bind-

Table 1. *Purification of DBP*
For full experimental details see the text.

Step	Total protein (mg)	$10^{-3} \times$ Total radioactivity (d.p.m.)	Cumulative recovery		Specific radioactivity (d.p.m./mg)	Cumulative purification (-fold)
			Protein (%)	Radioactivity (%)		
Starting material: plasma	40670	3806	100	100	94	
1. After DEAE-cellulose chromatography	1071	2241	2.6	59	2902	22
2. After DEAE-Sephadex chromatography	239	1956	0.59	51.4	8184	87.5
3. After chromatofocusing	67	1154	0.16	30	17225	184

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of DBP purified by chromatofocusing* Electrophoresis was carried out at 20°C with the discontinuous buffer system described by Laemmli (1970), with a 5%-acrylamide stacking gel and an 8%-acrylamide separating gel. Standard and sample proteins were prepared under reducing conditions in a buffer containing 2% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol. Protein bands were detected by Coomassie Blue staining. The lanes contain the following: lane 1, M_r standards, which include, from top to bottom, phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000) and carbonic anhydrase (M_r 30000); lane 2, pig plasma; lane 3, DBP.

ing capacity of 6.2×10^{-7} M. This represents 0.86 mol of calcidiol bound/mol of DBP. This is within the range reported by other investigators (Nilsson *et al.*, 1972; Bouillon *et al.*, 1976; Haddad & Walgate, 1976; Imawari *et al.*, 1976), indicating that binding properties were preserved in the isolated DBP.

Discussion

The established purification procedures for DBP are lengthy, involving up to six chromatographic steps (e.g. Bouillon *et al.*, 1978). Two innovations that have been reported to decrease the number of steps required are Cibacron Blue binding (Chapius-Cellier *et al.*, 1982) and actin affinity chromatography (Haddad *et al.*, 1984). We

Fig. 3. Chromatofocusing of apo- and holo-DBP

(a) Chromatofocusing of presumptive apo-DBP on a 1 cm $\times$ 30 cm column containing Polybuffer Exchanger 94, equilibrated with 25 mM-piperazine, pH 5.5. Fractions of 1 ml volume were eluted with Polybuffer 74 that had been diluted 1:10 and adjusted to pH 4.0 with 0.5 M-HCl, and a pH gradient (----) was used for elution. The apo-DBP had been incubated for 24 h with [^3H]calcidiol in a 1:1 molar ratio. (b) Chromatofocusing of presumptive apo-DBP that had been incubated with only a tracer amount of [^3H]calcidiol. (c) Chromatofocusing of the combined holo and apo forms of DBP from (a) and (b). Protein (○) and radioactivity (●) were monitored in each case.

have shown in the present paper that chromatofocusing is also a valuable technique for the isolation of DBP, permitting its purification in three chromatographic steps.

Several chromatographic procedures appear to separate apo- and holo-DBP, including the use of Cibacron Blue binding (Chapius-Cellier *et al.*, 1982), isoelectric focusing (Svasti & Bowman, 1978) and sulphopropyl-Sephadex cation-exchange chromatography (Imawari & Goodman, 1977). This separation offers an explanation for the low yield in some early isolation procedures when radioactive calcidiol was used to monitor the purification of the protein. None of these chromatographic procedures have been exploited to isolate the two forms successfully, however. We have shown in the present paper that chromatofocusing is a useful technique for separating the apo and holo forms of DBP. With the development of h.p.l.c. columns to exploit chromatofocusing principles, this technique should permit an extremely rapid separation (Wagner & Regnier, 1982).

Chromatofocusing separates proteins primarily on the basis of their isoelectric points (Sluyterman & Elgersina, 1978), and an analysis of the isoelectric points of apo- and holo-DBP by isoelectric focusing confirmed that they differed by

0.13 pH unit. This difference is in agreement with the results reported by Svasti & Bowman (1978), who observed a 0.15 pH unit shift in the pI of DBP with ligand binding, and by Van Baelen *et al.* (1978) who observed a 0.07 pH change. This difference in pI of apo- and holo-DBP accounts for their separation on chromatofocusing. This shift in pI with ligand binding indicates that the conformational changes associated with binding create physical changes in the properties of the protein. Surarit & Svasti (1980) have demonstrated, by using c.d. and by examining tryptophan fluorescence, that binding of cholecalciferol to DBP results in a change in the 'backbone' conformation of the protein.

In experiments where holo-DBP is prepared from apo-DBP by equilibration with an ethanolic solution of ligand, it is possible that interpretation of results is complicated by the presence of ethanol, free ligand and apo-DBP. The successful separation of apo- and holo-DBP should limit such complications, and may facilitate the study of their interactions with specific binding proteins.

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