Actin affinity chromatography in the purification of human, avian and other mammalian plasma proteins binding vitamin D and its metabolites (Gc globulins)

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The human plasma protein binding vitamin D and its metabolites (Gc globulin; group-specific component) has been isolated from human plasma by column affinity chromatography on gels to which monomeric actin was covalently attached. Rabbit skeletal-muscle G-actin was covalently coupled to amino-agarose gels before the application of human plasma. At actin/protein molar ratios of 4–8:1, excellent recovery (~58%) of purified binding protein was achieved. After 0.75 M-NaCl washes, the binding protein was eluted from the columns in 3 M-guanidinium chloride, dialysed and analysed. These eluates contained the binding protein as 34–100% of the total protein, reflecting a 130-fold average purification in this single step. In the presence of Ca^{2+} , gelsolin (another plasma protein that binds actin) was apparently retained by the affinity column, but this was prevented by chelation of plasma Ca^{2+} . The actin affinity step also was effective in the isolation of the binding protein from rat, rabbit and chicken plasma, as indicated by autoradiographs of purified fractions analysed by gel electrophoresis after incubation with 25-hydroxy[26,27- ^3H]cholecalciferol. Further isolation by hydroxyapatite chromatography yielded a purified binding protein which displayed characteristic binding activity toward vitamin D metabolites and G-actin, and retained its physicochemical features. This brief purification sequence is relatively simple and efficient, and should prove to be useful to investigators studying this interesting plasma protein.

The plasma protein binding vitamin D and its metabolites (DBP) has been isolated from the blood of man (Haddad & Walgate, 1976a; Bouillon *et al.*, 1976; Imawari *et al.*, 1976), rat (Botham *et al.*, 1976; Bouillon *et al.*, 1978), chicken (Bouillon *et al.*, 1980) and rabbit (Haddad *et al.*, 1981b). In general, the purification procedures have involved extensive preparative chromatographic steps (Haddad, 1980). With antiserum to human DBP available, one group has reported immunoaffinity chromatography of the protein (Bouillon & Van Baelen, 1982), and sterol–ligand affinity chromatography has also been described (Haddad *et al.*, 1981a).

Abbreviations used: DBP, plasma protein binding vitamin D and its metabolites; Hepes, 4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate; 25(OH)[^3H]D₃, 25-hydroxy[26,27- ^3H]cholecalciferol.

In recent years, another property of DBP has received attention. DBP binds to monomeric actin to produce a characteristic 5–6S protein–protein complex observed after sucrose-gradient ultracentrifugation (Haddad & Birge, 1975; Cooke *et al.*, 1979a,b; Van Baelen *et al.*, 1980; Haddad, 1982). The force of attraction between these proteins has been estimated to be quite strong (Cooke *et al.*, 1979b; Lees *et al.*, 1983), and could serve as the basis for a preparative actin-affinity-chromatography step in the isolation of DBP. We now report our experience with such a procedure. Our results indicate that this affinity-chromatography procedure is a superior method for the preparative isolation of this plasma binding protein. Since the DBP–actin association does not appear to be species-specific, this approach should be useful in the isolation and characterization of DBP from species in which it circulates in low titre.

Experimental procedures

Materials

All chemicals used were of reagent grade or the best grade available. High-resolution hydroxyapatite was purchased from Calbiochem and Coomassie Blue R250 was from Miles Laboratories. *N*-Hydroxysuccinimide-derivatized agarose (Affigel-15), Low-Molecular-Weight Kit, acrylamide, bisacrylamide and protein assay reagent (Bradford, 1976) were purchased from Bio-Rad. SDS, disodium EDTA, β -mercaptoethanol, grade I guanidinium chloride and low-electro-endosmosis agarose were obtained from Sigma. en^3Hance was purchased from New England Nuclear Corp. 25-Hydroxy[26,27- ^3H]cholecalciferol (sp. radioactivity 8.5 Ci/mmol) [$^{25}(\text{OH})[^3\text{H}]\text{D}_3$] was purchased from Amersham International.

Human plasma was obtained from normal volunteers. Blood was collected into tubes containing 2 mM-EDTA on most occasions. Plasma was similarly prepared from blood collected from Holtzman rats and New Zealand White rabbits. Chicken serum (Gibco) was treated with 2 mM-EDTA.

Reference pure human DBP was prepared by physicochemical methods previously reported (Haddad & Walgate, 1976a). Rabbit antiserum to human DBP was prepared as previously reported (Haddad & Walgate, 1976b). Reference sterols were generously provided by Dr. John Babcock (Upjohn Co.) and Dr. Milan Uskokovic (Hoffman-La Roche).

Methods

G-Actin preparation. Back and leg skeletal muscles from New Zealand White rabbits were used for the preparation of the acetone-dried powder from which G-actin was purified by the method of Spudich & Watt (1971). The F-actin was depolymerized in a depolymerizing buffer consisting of 0.01 M-Tris, 0.2 mM- CaCl_2 , 0.5 mM-sodium ATP, 1 mM- NaN_3 and 0.2 mM-dithiothreitol, pH 8.0.

Actin affinity support. Affigel-15 gel was washed with cold deionized water (200 ml) on a glass filter (Ace Glass, Vineland, NJ, U.S.A.; pore size C). G-actin was dialysed against 0.01 M-Hepes, pH 7.5, at 4°C overnight, and the protein concentration was adjusted to 5 mg/ml by ultrafiltration on a PM-10 membrane (Amicon). The moist gel [1 ml of gel/ml of the G-actin (5 mg/ml) solution] was added and the entire mixture was gently rotated in a capped glass tube at 4°C overnight. The gel was then washed extensively in the depolymerizing buffer and packed into glass columns before chromatography. Gel washes always contained less than 10% of the protein exposed to the

gel. These gels were always used within 48 h of their preparation.

Actin affinity chromatography. Moist Affigel-15-G-actin support was rinsed into 5 or 10 ml glass pipette columns before sample application at room temperature. Plasma from blood collected in 10 ml Sarstedt Monovette syringes containing 15 mg of potassium EDTA was applied to the column, rinsed with depolymerizing buffer and then re-applied to the column. The second rinse was continued until the A_{280} of the eluant stream reached baseline levels. The third rinse was carried out with 0.75 M-NaCl in depolymerizing buffer. Similarly, rinsing was continued until eluate A_{280} declined. Finally, a fourth elution was carried out with 4 column volumes of 3 M-guanidinium chloride. The column eluates were collected directly into Spectropor dialysis bags and dialysed against 75 vol. of 0.01 M-Tris, pH 8.0, overnight at 4°C, with one change of diffusate. The dialysed eluates were saved for analysis of total protein, DBP content and other analytical procedures.

Hydroxyapatite column chromatography. Hydroxyapatite was swollen and degassed in 0.01 M-sodium phosphate, pH 7.0, at 4°C and packed into a 10 ml syringe barrel. The dialysed guanidinium chloride eluates from the actin-Affigel-15 columns were applied to the columns after dialysis in 0.01 M-sodium phosphate, pH 7.0 (<15 mg of protein/g of dry gel). The column was washed with 0.01 M-phosphate, pH 7.0, until the A_{280} returned to baseline before starting the salt gradient. A linear gradient was obtained by using a Pharmacia gradient mixer containing 150 ml of 0.01 M-sodium phosphate, pH 7.0, and 150 ml of 0.20 M-sodium phosphate, pH 7.0. The column flow rate was 22 ml/h at 4°C and 3.7 ml fractions were collected. All buffers and samples were degassed before use.

Polyacrylamide-gel electrophoresis. This was carried out in the absence or presence of SDS to characterize the eluates from the affinity and hydroxyapatite columns. SDS/polyacrylamide-gel electrophoresis was performed in 0.75 mm-thick slab gels with 8 cm 7% (w/v)-polyacrylamide separating gels and a 1.5 cm 4.5%-polyacrylamide stacking gels. Sample proteins were denatured at 100°C for 5 min in 2% (w/v) SDS/2.5% β -mercaptoethanol/5% (v/v) glycerol/0.2 M-Tris, pH 6.8. Protein, 10 μg in 30–50 μl , was applied to the gels and run at 20 mV constant current. The gels were stained in acetic acid/methanol/water (2:3:15, by vol.) containing 1.2 g of Coomassie Blue/litre overnight, and destained in the same solution without the stain. Destained gels were dried on Whatman 3 MM chromatography paper with a Hoeffer slab gel dryer.

Polyacrylamide gel electrophoresis in the ab-

sence of SDS was performed in 1.5 mm slab gels with 9.5 cm 10% separating gels and 1 cm 4.5% stacking gels. Protein (10 µg in 0.07 ml of 0.01 M-Tris, pH 8.0) was incubated at 4°C overnight with 5.3 pmol of N₂-dried 25(OH)[³H]D₃ without or with 10 nmol of non-radioactive 25(OH)D₃. Sterol not bound to protein was removed before sample application by adsorption to 0.5% charcoal/0.05% dextran in sodium phosphate buffer (0.025 ml) at 4°C. After centrifugation, the volume applied to the gel was 80 µl. Reference proteins were run under the same conditions minus sterol and charcoal additions. The gels were run overnight at 4°C at 5 mV constant voltage. Half of the gels were stained as outlined above. The gels containing the 25(OH)[³H]D₃ samples were immediately immersed in en³Hance autoradiography enhancer for 2 h at 4°C with gentle agitation. The gel was then washed in deionized water at room temperature with gentle agitation for 50 min to precipitate the fluorescent material inside the gel. The gel was then dried on Whatman 3 MM filter paper on the slab-gel dryer. Autoradiography was carried out at -70°C for 5 days with Kodak XAR-5 x-ray film.

Other methods. Total protein was determined by the method of Bradford (1976). Human DBP was quantified by rocket gel immunoelectrophoresis (Walsh & Haddad, 1982). Saturation analyses of sterol-binding capacity and affinity were carried out with charcoal-adsorbent techniques as previously described (Haddad *et al.*, 1976), and graphic analyses of binding were performed by the method of Rosenthal (1976). Liquid-scintillation counting was performed in Budget-Solve (Research Products International Corp.) fluor in a Packard Tri-Carb 460 CD spectrometer.

Results

In 11 preparations of covalently-bonded G-actin to Affigel-15 agarose, 90% efficiency was observed for all experiments employing the hypo-osmotic Hepes buffer system. Initial efforts revealed an

optimal ratio of covalently linked G-actin to DBP in raw human plasma for efficient recovery of DBP. At DBP/actin molar ratios of 1:3 or less, 22% recovery of DBP was observed. At ratios of 1:4–1:15, however, approx. 60% of the DBP in plasma was retained by the columns until eluted by 3 M-guanidinium chloride.

In the absence of EDTA, an additional 90 kDa plasma protein was retained by the G-actin affinity support. Retention of this protein, presumably gelsolin (Norberg *et al.*, 1979; Yin & Stossel, 1979; Harris & Gooch, 1981), was completely obviated by treating the plasma with EDTA before application on the column.

Table 1 lists the steps used in the affinity-chromatography procedure. After application of plasma, the column was washed with phosphate-buffered 0.15 M-NaCl until eluates exhibited no absorbance at 280 nm. The second rinse, utilizing 0.75 M-NaCl, eluted a small amount of additional proteins. The final elution, with 3 M-guanidinium chloride, gave an eluate considerably enriched with DBP. These latter eluates were shown to contain DBP/total protein ratios which were 68–200-fold higher than those in raw plasma. In addition to immunoassay of column eluates, the efficiency of purification was also estimated by SDS/polyacrylamide-gel electrophoresis (Fig. 1). In comparison with the starting plasma, the 3 M-guanidinium chloride-eluted fractions were clearly enriched with protein of 58 kDa. Variable amounts of a 43 kDa protein were also observed in the denaturing salt fractions. Since this material was eluted from affinity supports not exposed to plasma, we concluded that small amounts of G-actin, not washed away in the depolymerizing buffer, were removed from the support by the 3 M-guanidinium chloride. It, and other contaminating proteins, were removed from the DBP by gradient elution from hydroxyapatite columns (Fig. 2). The early eluting protein from such columns proved to be further-enriched DBP (Fig. 2).

The affinity-purified proteins from human,

Table 1. *Efficiency of G-actin-agarose affinity chromatography in the isolation of DBP from human plasma*

The results shown are from those columns employed at an actin/DBP ratio of 4–8:1. Preparation of support, plasma applications and elutions are described under 'Methods' in the Experimental procedures section. Column eluates were analysed for total protein and human DBP content. The results are expressed as means ± S.E.M. for five separate experiments. The plasma volumes applied ranged from 5 to 20 ml.

Step	Yield (% of total plasma protein applied)	DBP/total protein (%)
Human plasma	(100)	0.5 ± 0.1
NaCl rinses		
0.15 M	96 ± 4	0.2 ± 0.1
0.75 M	3 ± 1	< 0.01
Final elution with 3 M-guanidinium chloride	0.46 ± 0.1	65 ± 7

Fig. 1. SDS/polyacrylamide-slab-gel electrophoresis of human plasma and eluates from the agarose-G-actin affinity chromatography and hydroxyapatite chromatography columns

Polyacrylamide-gel (7%, w/v) electrophoresis and staining were carried out as described under 'Methods'. Purified proteins, plasma or molecular-mass markers (10 μ g) were analysed in each gel. 1, Molecular-mass markers [phosphorylase *b* (93 kDa), bovine serum albumin (67 kDa) and egg albumin (45 kDa); 2, human plasma (0.15 μ l or 10 μ g of protein); 3, 0.75 M-NaCl rinse; 4, elution with 3 M-guanidinium chloride. In a separate analysis: 5, markers as described for 1; 6, hydroxyapatite column protein eluted after salt gradient (10 μ g). The anodal front (bottom) was tracked with Bromphenol Blue (BPB).

Fig. 2. Hydroxyapatite chromatography of dialysed eluates of the guanidinium chloride-eluted fractions from the agarose-G-actin affinity chromatography procedure. After dialysis in 0.01 M-sodium phosphate, pH 7.0, 15 mg of protein was applied to the column and gradient elution was performed as described under 'Methods'. The effluent stream was monitored for A_{280} (—) and fractions were assessed for their conductivity (----). The protein eluting first after initiation of the salt gradient (arrow) was human DBP, as assessed by sterol binding (Haddad *et al.*, 1976) and by immunoelectrophoresis (Walsh & Haddad, 1982).

avian, rat and rabbit plasma were examined for their sterol-binding properties by competitive binding assay and autoradiography of proteins pre-incubated with 25(OH)[3 H]D $_3$ and separated by 10%-polyacrylamide-gel electrophoresis (Figs. 3 and 4). As shown, the characteristic post-albumin proteins were capable of specifically binding the [3 H]sterol-ligand in all instances. Saturation analyses revealed affinities ($K_d \sim 5 \times 10^{-8}$ M) for this sterol that were similar to those observed with dilutions of the raw plasmas, and binding capacities consistent with more-than-100-fold purification as suggested by the stains of the native gels (Figs. 3 and 4). The slower migration of the avian protein was expected, since its pI has been reported to be higher than for mammalian DBP species (Bouillon *et al.*, 1980).

Actin-affinity-purified human DBP was analysed for its behaviour toward actin *in vitro*, and a preliminary account of this work has been pre-

Fig. 3. Polyacrylamide (10%) slab-gel electrophoresis of human plasma and dialysed guanidinium chloride eluates from agarose-G-actin columns charged with human plasma

The guanidinium chloride-eluted fractions were dialysed and incubated with 25(OH)[3 H]D $_3$ without or with unlabelled 25(OH)D $_3$ before analyses, as described under 'Methods'. After electrophoresis, gels were stained with Coomassie Blue (a) and analysed by autoradiography (b), also as described under 'Methods'. Purified proteins (10 μ g) were analysed in each gel. (a) Coomassie Blue staining: 1, human plasma proteins (1 μ l or 75 μ g of protein applied); 2, and 3, human plasma protein eluted from actin affinity support with guanidinium chloride. (b) Autoradiography: 1, human plasma incubated with 25(OH)[3 H]D $_3$ alone; 2, purified human DBP incubated with 25(OH)[3 H]D $_3$ alone; 3, purified human DBP incubated with 25(OH)[3 H]D $_3$ and a 200-fold excess of non-radioactive 25(OH)D $_3$. The anodal front (bottom) was tracked with Bromphenol Blue.

Fig. 4. Polyacrylamide (10% w/v) slab-gel electrophoresis of actin-affinity-column-purified fractions from rat, chicken and rabbit plasmas

Guanidinium chloride-eluted fractions were dialysed and incubated with $25(\text{OH})[^3\text{H}]\text{D}_3$ without or with a 200-fold excess of unlabelled $25(\text{OH})\text{D}_3$ as described under 'Methods'. After electrophoresis, the gels were stained (lanes 1–4) and analysed by autoradiography (lanes 5–10). Protein ($8\text{ }\mu\text{g}$) was applied to each gel. Staining: 1, purified human DBP was stained only; 2, rat plasma fraction; 3, chicken plasma fraction; 4, rabbit plasma fraction. Autoradiography: 5 and 6, rat plasma fractions incubated without and with an excess of unlabelled $25(\text{OH})\text{D}_3$ respectively; 7 and 8, chicken plasma fractions incubated without and with an excess of unlabelled $25(\text{OH})\text{D}_3$ respectively; 9 and 10, rabbit plasma fractions incubated without and with an excess of unlabelled $25(\text{OH})\text{D}_3$ respectively. Anodal fronts (bottom, not shown) were tracked with Bromophenol Blue.

sented (Lees *et al.*, 1983). In brief, these studies appeared to indicate that DBP sequestered actin monomers.

Discussion

The present method provides a rapid and relatively simple way to isolate the vitamin D-binding protein from avian and mammalian plasmas. The affinity step alone provides a more-than-100-fold purification of a protein that normally constitutes 0.5% of the plasma proteins in man. Since the reagents are relatively inexpensive and easily available, the procedure should prove useful to those investigators interested in the protein. Before settling on the use of a G-actin support, we tried coupling paraformaldehyde-treated F-actin to agarose. This approach was

suggested by previous studies that had revealed an association between fluorescent human DBP and paraformaldehyde-fixed microfilamentous actin (Haddad & Sanger, 1982). However, we did not obtain consistent results with the cross-linked material. Such an approach, if feasible, might provide a re-usable support which would likely have less tendency to shed actin moieties during elution under denaturing salt conditions. The recovery of human DBP from the guanidinium chloride eluates was associated with sterol-binding function, as reported by Cooke *et al.* (1979a,b) and Kawakami & Goodman (1981), as well as with actin-binding properties (Lees *et al.*, 1983).

In previous studies (Haddad & Birge, 1975; Cooke *et al.*, 1979a,b), an association between DBP and a cellular constituent, later shown to be actin (Van Baelen *et al.*, 1980; Haddad, 1982), was

suggested. The stability of the protein-protein complex in 0.5M-KCl and the requirement of denaturing salt or ionic detergent (Haddad & Birge, 1975; Cooke *et al.*, 1979a,b; Haddad, 1982) for its disruption clearly indicated a high-affinity association between the plasma and cellular proteins. The precise role for this interesting relationship is not currently understood, but an actin-scavenger function has been suggested, possibly complementing the behaviour of plasma gelsolin in removing microfilaments from plasma (Van Baelen *et al.*, 1980; Korn, 1982).

We have recently become aware of a similar methodological approach in the isolation of an actin-binding protein from rabbit and calf plasma (Vandekerckhove & Sandoval, 1982). The reported features of this protein almost certainly indicate that it was the DBP in these plasmas, although this property was not directly assessed. Estimates of their lower recovery of this protein (<20%) might be attributable to use of a ligand/DBP ratio of less than 1.0.

The presently described technique appears to have several advantages over previously reported techniques for the purification of the plasma DBP (Haddad & Walgate, 1976a; Bouillon *et al.*, 1976, 1978, 1980; Imawari *et al.*, 1976; Botham *et al.*, 1976; Haddad, 1980; Haddad *et al.*, 1981a,b). In comparison with previously used methods, these steps are simple, efficient, inexpensive and rapid. Furthermore, they appear to be applicable to any DBP which possesses the feature of actin binding. To date, all such proteins examined have had this feature (Haddad & Birge, 1975; Cooke *et al.*, 1979a,b; Van Baelen *et al.*, 1980; Haddad *et al.*, 1981b; Haddad, 1982). Although mammalian and avian blood contain large amounts of DBP (Haddad & Walgate, 1976; Bouillon *et al.*, 1976, 1978, 1980; Imawari *et al.*, 1976; Botham *et al.*, 1976; Haddad, 1980; Haddad *et al.*, 1981b), blood from other species such as fish (Nahm *et al.*, 1979) have very small quantities. The techniques described herein may prove particularly valuable for the isolation of DBP from the blood of species with very small amounts of this protein. Finally, it seems possible that serial actin-affinity supports could prove useful in the rapid and selective removal of vitamin D sterols from plasma, and our preliminary experiments along these lines are supportive in this regard (results not shown).

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