Ca²⁺-binding protein from human kidney

Purification and properties

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A Ca²⁺-binding protein (CaBP) from human kidney was purified by two different procedures. The first involved heat-precipitation of a kidney cytosol fraction followed by gel filtration and chromatofocusing. This resulted in a 200-fold increase in the specific Ca²⁺-binding activity with a yield of 10%. A specific antibody was raised against the purified CaBP, as demonstrated by one precipitate in crossed immunoelectrophoresis of a kidney cytosol fraction. The antibody was coupled to Sepharose 4B and CaBP was then purified by immunoadsorbent chromatography. Applying this technique, a 500-fold purification of CaBP with a yield of 50% was obtained. Both preparations appeared homogeneous in crossed immunoelectrophoresis against a polyvalent antiserum and migrated as a single band corresponding to a mol.wt. of 26000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In gel filtration under non-denaturing conditions CaBP was eluted corresponding to a mol.wt. of 28000. The association constant for the high-affinity Ca²⁺-binding sites of CaBP was estimated by gel filtration to be $0.1 \times 10^6 \text{ M}^{-1}$, and the protein displayed Ca²⁺-dependent electrophoretic mobility, with more rapid anodic migration in the presence of EDTA. The protein eluted at a position corresponding to a pI of 4.5 in chromatofocusing. Immunochemical experiments with the specific antibody showed no cross-reaction between renal and intestinal CaBP.

The intestine and kidney are two of the major target organs for the hormonally active vitamin D metabolite 1,25(OH)₂D₃, which, analogously to steroid hormones, binds to specific intracellular receptors (Haussler & Norman, 1969; Stumpf *et al.*, 1980) and stimulates the biosynthesis of mRNA species that code for vitamin D-regulated proteins. The soluble CaBP, first discovered in chick intestine (Wasserman & Taylor, 1966), is one of these proteins. Based on several physiological experiments (Taylor & Wasserman, 1969; Christakos & Norman, 1980) it has been proposed that CaBP is involved in the vitamin D-dependent intestinal Ca²⁺ transport, but the exact function of this protein is still unknown. In several species, including man, the same or similar vitamin D-

dependent CaBPs have been found in other organs, such as the kidney (Piazolo *et al.*, 1971; Hermsdorf & Bronner, 1975). Histochemical studies on rat, rabbit and human (Roth *et al.*, 1982; Taylor *et al.*, 1982) have demonstrated that CaBP in the kidney is localized to the cells in tubular regions of the distal nephron in which a selective Ca²⁺ transport is known to occur (Lassiter *et al.*, 1963; Taylor *et al.*, 1982). The possible role of CaBP in renal Ca²⁺ reabsorption is also supported by the reduced CaBP level found in kidney tissue from patients with juvenile diabetes (Orci *et al.*, 1982), a disease often associated with abnormal Ca²⁺ metabolism including hypercalciuria (Heath *et al.*, 1979).

Biochemical information available on vitamin D-dependent CaBP from human kidney and intestine is limited. Mammalian intestinal CaBP generally has a mol.wt. in the range 9000–12000 (Norman *et al.*, 1982), but human intestinal CaBP has variously been reported to have mol.wts. of 11000 (Davie, 1981), 20000 (Alpers *et al.*, 1972) and 28000 (Davie, 1981). A human renal CaBP has

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Abbreviations used: CaBP, Ca²⁺-binding protein; 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; SDS, sodium dodecyl sulphate.

been isolated by gel filtration and ion-exchange chromatography (Piazolo *et al.*, 1971; Morrissey & Rath, 1974). The mol.wt. was estimated to be in the range 25 000–28 000, and Piazolo *et al.* (1971) judged the purified CaBP to be homogeneous when tested by polyacrylamide-gel electrophoresis, although the purification factor was surprisingly low. A specific antibody was raised in rabbits (Piazolo *et al.*, 1975), but no immunological characterization of the purified CaBP was reported.

With the aim of developing a quantitative method for studying the significance of the vitamin D-dependent CaBP in normal individuals and in patients with disturbed Ca^{2+} metabolism, human renal CaBP was purified and a specific antibody was obtained.

Materials and methods

Materials

Four human kidneys that were unacceptable for transplantation owing to vessel abnormalities were used in the present study. They were removed immediately after death. A piece of normal jejunum was obtained from a patient having an intestinal bypass operation performed because of morbid obesity. Tissues were stored at -20°C until used. Chymotrypsin, bovine serum albumin, ovalbumin and myoglobin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., lysozyme was from Seikagaku Fine Chemicals, Tokyo, Japan, agarose (type HSA) from Litex, Copenhagen, Denmark, and Sephadex G-100, Sephadex G-75, Sepharose 4B, Protein A–Sepharose CL-4B, Polybuffer Exchanger 94, Polybuffer 74 and CNBr-activated Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. Complete Freund's adjuvant was a product of Statens Seruminstitut, Copenhagen, Denmark, and Chelex 100 and Bio-Rad Protein Concentrate Dye were obtained from Bio-Rad, Richmond, CA, U.S.A. $^{45}\text{CaCl}_2$ (sp. radioactivity 0.25–1.0 Ci/mol) was delivered by Amersham International, Amersham, Bucks., U.K. All other chemicals were of analytical grade, and the water was purified by a Milli-Q Reagent Grade water system (Millipore, Bedford, MA, U.S.A.).

Assays

Protein concentration was estimated by the absorbance at 280 nm in eluted fractions. For more precise determination, protein concentration was determined by Bio-Rad Protein Assay with bovine serum albumin as standard.

CaBP activity eluted from the chromatographic columns was assayed either by the Chelex $^{45}\text{Ca}^{2+}$ assay (Wasserman *et al.*, 1968) or by adding $^{45}\text{CaCl}_2$ to the protein solution before chromato-

graphy and analysing radioactivity in the eluted fractions. The Chelex assay was performed at room temperature. Chelex 100 was washed extensively with 0.05 M-Tris/HCl, pH 8.0, and an 80% (w/w) slurry of the resin was used. Protein solution or buffer alone (0.25 ml), 0.05 ml of $^{45}\text{CaCl}_2$ (0.3 μCi) and 0.05 ml of Chelex 100 slurry were placed in 3 ml polypropylene tubes. The tubes were capped and the contents were mixed by slowly rotating the test-tube racks for 15 min. After centrifugation at 1500 g for 10 min, radioactivity was measured in 0.2 ml of the supernatant in a Packard Tri-Carb 300 (Packard, Downers Grove, IL, U.S.A.), with 5 ml of Instagel II (Packard) as scintillation fluid. CaBP activity was expressed as the percentage radioactivity in 0.2 ml of supernatant solution in relation to radioactivity in 0.2 ml of a solution containing $^{45}\text{CaCl}_2$ and buffer alone made up to the same total volume.

Purification procedures

All preparative steps were performed at 4°C unless otherwise stated.

Preparation of a kidney cytosol fraction. About 100 g of kidney tissue was homogenized in three times its own weight of 0.05 M-Tris/HCl, pH 8.0, or in the same buffer containing 0.15 M-NaCl, by using a Kenwood mixer for 3×60 s. The homogenate was then centrifuged at 50 000 g for 45 min and the clear supernatant was used for further purification of CaBP.

Procedure I: heat precipitation, gel filtration and chromatofocusing. The kidney cytosol fraction was heated to 60°C and kept at this temperature for 15 min and then cooled on ice. The heat-precipitated proteins were removed by centrifugation and the supernatant was then concentrated to 90 ml by ultrafiltration in a cell fitted with a YM 5 membrane (Amicon, Lexington, MA, U.S.A.). Of the heat-treated and concentrated supernatant 30 ml was subsequently applied to a Sephadex G-100 column (5 cm $\times$ 80 cm) equilibrated in, and eluted with, 0.05 M-Tris/HCl, pH 8.0, at a flow rate of 3 ml/cm² per h. Fractions from three gel-filtration experiments containing more than 0.5% CaBP activity were pooled and concentrated by ultrafiltration to 10 ml and then dialysed against 0.02 M-imidazole buffer, pH 7.4, and applied to a column (0.9 cm $\times$ 30 cm) of Polybuffer Exchanger 94. The column was equilibrated in the imidazole buffer and elution was performed with Polybuffer 74 at a flow rate of 16 ml/cm² per h, producing a descending pH gradient from 7 to 4. Fractions (5 ml) collected were monitored for pH and then 2.0 ml of 2 M-Tris/HCl buffer, pH 8.0, was added.

Procedure II: chromatography on anti-CaBP–Sepharose 4B. Anti-CaBP–Sepharose 4B was prepared by coupling purified specific antibody

against CaBP to CNBr-activated Sepharose, with 0.2M-sodium citrate, pH 6.5, as coupling buffer. The immunoglobulin concentration of the anti-CaBP solution to be coupled was 3.9 mg/ml and the ratio between the volumes of the protein solution and the packed Sepharose 4B was 1:1. Based on the absorbance at 280 nm before and after coupling, the amount coupled was estimated to be 95% of the initial amount. Of the kidney cytosol fraction 40 ml was applied (7 ml/cm² per h) to a column (1.0 cm × 20 cm) packed with anti-CaBP-Sepharose 4B equilibrated in the homogenizing buffer [0.05 M-Tris/HCl (pH 8.0)/0.15 M-NaCl]. The column was washed with 175 ml of the equilibration buffer, and CaBP was eluted at room temperature with 60 ml of 0.1 M-glycine, pH 3.0 (immunoabsorbent eluate). The eluate was immediately neutralized by adding 0.125 ml of 2 M-Tris solution to each fraction (5 ml).

Preparation of intestinal tissue

Mucosa from jejunum was homogenized in four times its own weight of 0.05 M-Tris/HCl, pH 8.0, in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 50000g for 45 min, and the supernatant (intestinal cytosol fraction) was used for immunological experiments.

Immunological methods

Preparation of antiserum. An antiserum against purified CaBP was produced in rabbits by immunization with purified CaBP and a polyvalent antiserum was produced with a partly purified CaBP preparation (gel-filtration fraction). A solution containing about 1 mg of antigen/ml was mixed with equal volumes of complete Freund's adjuvant. Over a period of 6 weeks, rabbits were injected intracutaneously with 200 µl of the antigen solution at 2-week intervals. At 1 week after the fourth injection the rabbits were bled for 40 ml. The immunization was continued with a booster injection every fourth week, and 40 ml of blood was collected 1 week after each injection. The immunoglobulins were isolated from the antisera by chromatography on Protein A-Sepharose (Hjelm *et al.*, 1972). The purified antisera were stored at 4°C in 0.15 M-NaCl containing 0.02% NaN₃.

Immuno-electrophoresis. Crossed immuno-electrophoresis and rocket immuno-electrophoresis (Lauréll, 1972) were performed on 1.5 mm-thick agarose gels (1% agarose in 0.02 M-barbital buffer, pH 8.6) cast on glass plates (1.5 mm × 100 mm × 100 mm). For charge-shift purposes 1 mM-CaCl₂ or 1 mM-EDTA was added to the buffer. In crossed immuno-electrophoresis the first dimension was run for 1 h at 8 V/cm and the second dimension for 18 h at 2 V/cm. The plates were stained with Coomassie Brilliant Blue R250.

Immunodiffusion. Double immunodiffusion (Ouchterlony, 1967) was performed on 1.5 mm-thick agarose gels (1% agarose in 0.02 M-veronal buffer, pH 8.6) cast on glass plates. The immunodiffusion was carried out in a moist chamber at room temperature for 48 h.

Immunoblotting. Immunoblotting was performed essentially by the method of Burnette (1981). Proteins were transferred from polyacrylamide slab gels to nitrocellulose paper sheets by electrophoresis. The paper was washed in 0.05 M-Tris/0.15 M-NaCl/0.005 M-NaN₃ (pH 10.2), containing 2% (w/v) Tween 20 (Bjerrum *et al.*, 1983) and then incubated for 16 h in primary antibody diluted 1:100 in the same buffer containing 0.05% Tween 20. After washing, the paper sheets were reacted for 2 h with peroxidase-conjugated second antibody (Dako, Copenhagen, Denmark) diluted 1:1000. Peroxidase activity was localized by staining with 3-amino-9-ethylcarbazole.

Preparation of a specific antibody against CaBP. When crossed immuno-electrophoresis of kidney cytosol fraction was performed against the antibody raised against the purified CaBP, a very high and thin precipitate was conspicuous in addition to the precipitate representing CaBP. To obtain a specific antibody the antiserum was purified by immuno-adsorption. The fraction containing proteins eluted at a position corresponding to a pI of 5.0 from the chromatofocusing experiments was coupled to CNBr-activated Sepharose 4B by using 0.1 M-Na₂CO₃, pH 8.3, as coupling buffer. The solution to be coupled contained 0.35 mg of protein/ml and the ratio between the volumes of protein solution and the packed Sepharose was 1:1. Based on the absorbance at 280 nm before and after coupling, the amount coupled was estimated to be 95% of the initial amount. The immuno-adsorption of the antibody against the purified CaBP was performed in 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.15 M-NaCl. The fractions collected (specific antibody) were pooled and then dialysed against 0.15 M-NaCl containing 0.02% NaN₃ or against 0.2 M-sodium citrate, pH 6.5.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis on 10% (w/v) polyacrylamide slabs was performed as described by Laemmli (1970). Before electrophoresis samples were boiled for 5 min in the presence of 1% SDS and 2.5% (v/v) 2-mercaptoethanol. Polyacrylamide-gel electrophoresis on 15% (w/v) polyacrylamide slabs for immunoblotting experiments were performed by the method of Davis (1964).

Estimation of the association constant for the Ca²⁺-binding sites of CaBP

The association constant of Ca²⁺ and CaBP

was estimated from gel-filtration experiments performed on a column (0.9 cm × 50 cm) of Sephadex G-75 equilibrated in, and eluted with, 0.05 M-Tris/HCl buffer, pH 8.0, containing 70 000 c.p.m. of $^{45}\text{CaCl}_2$ /ml. Purified CaBP (100 µg; purification procedure I) equilibrated with Tris/HCl buffer was applied to the column and radioactivity in the collected fractions was determined. The protein concentration was determined by using the Bio-Rad protein assay. The Ca^{2+} concentration in the Tris/HCl buffer was determined by atomic-absorption spectrophotometry (model 103; Perkin-Elmer, Norwalk, CT, U.S.A.) before adding $^{45}\text{CaCl}_2$. Assuming the number of high-affinity Ca^{2+} -binding sites to be four per molecule of CaBP, the association constant ($K_{\text{ass.}}$) of the Ca^{2+} -binding sites was calculated from the following expression: $K_{\text{ass.}} = [\text{CaBP}] / \{4 \times [\text{BP}][\text{Ca}^{2+}]\}$, where [CaBP] is the concentration of protein with bound calcium and $[\text{Ca}^{2+}]$ and [BP] are the concentrations of free Ca^{2+} and protein not associated with Ca^{2+} respectively. The concentration of CaBP in each fraction comprising the CaBP peak was calculated from the $^{45}\text{Ca}^{2+}$ radioactivity. BP concentrations were computed from the difference between the total protein concentration and CaBP concentration. The association constant was determined for each fraction and a mean value ($\pm$ S.D.) was obtained.

Results and discussion

Purification

Table 1 shows typical results of the two purification procedures. Procedure I yielded about 2 mg of purified CaBP per 100 g of kidney tissue. This represents approx. 10% of the total Ca^{2+} -binding activity present in the starting material. The specific Ca^{2+} -binding activity was increased 220-fold as judged by Chelex assay. A heating procedure could be included in the purification procedure, because many Ca^{2+} -binding proteins are heat-stable (Gleason & Lankford, 1981). Heating of the kidney cytosol fraction to 60°C for 15 min resulted in precipitation of more than 50% of the proteins, with little or no loss of Ca^{2+} -binding activity. To establish the molecular-weight distribution of the heat-stable proteins, gel filtration on Sephadex G-100 was performed. Two peaks with Ca^{2+} -binding activity were eluted (Fig. 1). The first appeared with the void volume, indicating a mol.wt. of 150 000 or higher. This fraction was not further investigated. The second peak (mol.wt. 28 000) comprised 30–40% of the Ca^{2+} -binding activity eluted. In some experiments the distribution of Ca^{2+} -binding activity was measured by adding $^{45}\text{CaCl}_2$ to the protein solu-

Table 1. Purification of human renal CaBP

Method	Fraction	Protein amount (mg)	CaBP activity			CaBP immunoreactivity		
			Specific activity (CaBP activity/mg)	Recovery (%)	Purification factor (fold)	Specific activity (mm rocket height/mg)	Recovery (%)	Purification factor (fold)
Procedure I	Kidney cytosol fraction	3850	0.18	100	1			
	Heat-precipitated fraction	1475	0.51	108	3			
	Gel-filtration fraction	66	4.1	39	22			
	Chromatofocusing fraction	1.8	47	12	261			
Procedure II	Kidney cytosol fraction	582	0.18	100	1	0.61	100	1
	Immunoabsorbent eluate	0.6	40	22	222	302	51	495

Values are for 100 g (procedure I) or 10 g (procedure II) of tissue. CaBP activity was determined by the Chelex assay. CaBP immunoreactivity was measured by rocket immunoelectrophoresis. For details see the Materials and methods section.

tion applied to the Sephadex G-100 column. A similar pattern of Ca²⁺-binding activities emerged.

Vitamin D-dependent CaBPs are generally acidic proteins with low pI and high negative net charge at neutral pH (Fullmer & Wasserman, 1975). Thus they bind efficiently to anion-exchangers at this pH, and this property was used in the final purification step. Partly purified CaBP from the gel filtration experiments (28 000-mol. wt. fraction) bound completely to a column of Polybuffer Exchanger 94. A descending pH gradient was formed with Polybuffer 74, and several protein peaks were eluted. CaBP emerged as a symmetrical peak corresponding to a pI of 4.5 (Fig. 2).

Of the applied Ca²⁺-binding activity 30–50% was recovered, and only negligible amounts of protein and CaBP could be eluted from the column with 1 M-NaCl. The pI of 4.5 is similar to values reported for other renal and intestinal CaBPs from

the rat and chicken (Ingersoll & Wasserman, 1971; Delorme *et al.*, 1982). Chromatofocusing has been used as a tool for studying variations in pI induced by binding of Ca²⁺ to CaBP (Delorme *et al.*, 1982). When chromatofocusing was performed with 1 mM-CaCl₂ in the buffers we observed no change in the apparent pI. It is possible that the negative net charge of the protein was not significantly changed by the presence of 1 mM-CaCl₂ under these experimental conditions. Another possible explanation could be that apparently only the Ca²⁺-free form of CaBP was absorbed to the Polybuffer Exchanger. This is supported by the observation that no Ca²⁺ (measured by atomic-absorption spectrophotometry) was associated with the substantial amounts of CaBP eluted at pH 4.5. The Ca²⁺ present in the gel-filtration fraction applied to the column was detected in the fractions eluted before the start of the pH gradient, indicating that Ca²⁺ dissociates from CaBP when the protein binds to the column material.

The antiserum used for the preparation of the anti-CaBP-immunoabsorbent was monospecific, as demonstrated by the presence of only one precipitate in crossed immunoelectrophoresis (Fig. 3). Table 1 summarizes the results obtained in the purification of CaBP with the immunoabsorbent technique (procedure II). About 1 mg of CaBP, corresponding to about 10 g of kidney tissue, was adsorbed to the anti-CaBP-Sepharose. Elution was performed with 0.2 M-glycine, pH 3.0, and most of the CaBP appeared in a volume corresponding to 1.5 bed vol. Specific Ca²⁺-binding activity was increased 220-fold with a yield of 20%. When CaBP was assayed specifically by rocket immunoelectrophoresis, purification was estimated as 500-fold with a yield of 50%. This indicates that CaBP

Fig. 1. Chromatography of the heat-precipitated fraction on Sephadex G-100

For experimental details see the text. The bar indicates fractions pooled. ○, Protein concn.; ●, CaBP activity.

Fig. 2. Chromatofocusing on Polybuffer Exchanger 94 of the pooled gel-filtration fractions

For experimental details see the text. The bar indicates fractions pooled. ●, Protein concn.; ○, CaBP activity.

Fig. 3. Crossed immunoelectrophoresis of kidney cytosol fraction (120 µg) against the specific anti-CaBP antibody. The first dimension was run for 1 h at 8 V/cm and the second dimension was run for 18 h at 2 V/cm. The concentration of immunoglobulin G was 50 µg/cm².

constitutes about 0.2% of the protein present in a kidney cytosol fraction, corresponding to 2 µg of CaBP per mg of protein. This value is comparable with the value of 3 µg of CaBP per mg of protein found in kidney tissue from the chick (Christakos *et al.*, 1979).

The specific Ca²⁺-binding activities of CaBP isolated by the two procedures were similar. This indicates that CaBP tolerated the heating procedure as well as exposure to low pH without losing its Ca²⁺-binding activity.

Homogeneity

Polyacrylamide-gel electrophoresis of the two purified CaBP preparations in the presence of SDS displayed one band (Fig. 4), indicating a high degree of purity.

CaBP was also homogeneous when tested by crossed immunoelectrophoresis against the polyvalent antiserum, as demonstrated by the presence of only one precipitate (Fig. 5). Electrophoresis of a kidney cytosol fraction against the same antibody normally gave rise to four or five precipitates (Fig. 6).

In gel-filtration experiments on Sephadex G-75 the Ca²⁺-binding activity was eluted concomitant with a symmetrical protein peak (Fig. 7). Small amounts of immunoreactive CaBP were detected before the main peak, possibly owing to some aggregation of the protein.

Molecular weight

In gel-filtration experiments on a Sephadex G-75 column calibrated with three molecular-weight standards in the range 14000–44000, CaBP was eluted in a position corresponding to a mol.wt. of 28000 (Fig. 7). The molecular weight of the fully denatured CaBP was estimated to be 26000 by SDS/polyacrylamide-gel electrophoresis (Fig. 4).

Fig. 4. Polyacrylamide-gel electrophoresis in the presence of SDS.

Purified CaBP (7 µg) was applied and molecular-weight markers (ovalbumin, 44000; chymotrypsin, 25000; myoglobin, 17000; lysozyme, 13000) were run in parallel.

Fig. 5. Crossed immunoelectrophoresis of 3 µg of purified CaBP against the polyvalent antiserum.

The first dimension was run for 1 h at 8 V/cm and the second dimension was run for 18 h at 2 V/cm. The concentration of immunoglobulin G was 105 µg/cm².

The molecular weights of CaBP obtained by the two methods are thus similar. The result also confirms that human renal CaBP belongs to the class of vitamin D-dependent CaBP characteristically present in birds and some mammalian tissues (Norman *et al.*, 1982).

Estimation of the association constant for the Ca²⁺-binding sites of CaBP

Gel filtration has been used as a method to measure the binding of small molecules and ions to macromolecules (Hummel & Dreyer, 1962; Coleman, 1972), and it is thus possible with this technique to estimate $K_{ass.}$ for CaBP and Ca²⁺.

Fig. 6. Crossed immunoelectrophoresis patterns showing Ca²⁺-dependent electrophoretic mobility of CaBP. Kidney cytosol (120 μ g) fraction was electrophoresed against the polyvalent antiserum in the presence of either 1 mM-CaCl₂ (a) or 1 mM-EDTA (b). The first dimension was run for 1 h at 8 V/cm and the second dimension was run for 18 h at 2 V/cm. The concentration of immunoglobulin G was 110 μ g/cm². Precipitate representing CaBP is indicated by arrows.

Fig. 7. Chromatography of purified CaBP on Sephadex G-75

CaBP (100 μ g) was applied in 300 μ l of buffer (column size 0.9 cm $\times$ 50 cm). The column was equilibrated in, and eluted with, 0.05 M-Tris/HCl, pH 8.0, containing 70 000 c.p.m. of ⁴⁵CaCl₂/ml. The column was calibrated with molecular-weight markers (ovalbumin, 44 000; chymotrypsin, 25 000; lysozyme, 13 000). Δ , Protein concentration; $\circ$, CaBP concentration; $\bullet$, ⁴⁵Ca²⁺ radioactivity.

under the conditions of buffer and temperature chosen for the experiments. $K_{\text{ass.}}$ was determined for each fraction containing the CaBP (Fig. 7) eluted from the gel-filtration column, and a mean value of $0.12 \times 10^6 \text{ M}^{-1}$, with an s.d. of $0.01 \times 10^6 \text{ M}^{-1}$, was found. Human renal CaBP and the chicken intestinal CaBP appear to be structurally related, having the same molecular weight and immunological determinants in common (Roth *et al.*, 1982). The calculation of $K_{\text{ass.}}$ was therefore based on the assumption that human renal CaBP binds 4 g-atoms of Ca²⁺ per mol, as has been

reported for the chicken CaBP (Bredderman & Wasserman, 1974). Vitamin D-dependent CaBPs generally have a $K_{\text{ass.}}$ for Ca²⁺ of about 10^6 M^{-1} . The $K_{\text{ass.}}$ value for human renal CaBP determined by the gel-filtration method thus was of about the same magnitude as $K_{\text{ass.}}$ values for other CaBPs with high-affinity Ca²⁺-binding sites.

Immunological properties

Crossed immunoelectrophoresis of a kidney cytosol fraction against the polyvalent antisera normally gave rise to five or six precipitates (Fig.

Fig. 8. Double immunodiffusion of purified renal CaBP and human intestinal cytosol fraction against anti-CaBP antibody

Purified CaBP (0.5 µg) and intestinal cytosol fraction (45 µg of protein) were applied in wells K and I. Specific antisera (10 µl; 40 µg of immunoglobulin G) were applied in well AS. The immunodiffusion was carried out for 48 h in a moist chamber at room temperature.

6). By adding 1 mM-CaCl₂ or 1 mM-EDTA to the gel and buffer it was possible to identify a precipitate displaying Ca²⁺-dependent electrophoretic mobility, suggesting it to be CaBP. In the presence of EDTA CaBP exhibited higher electrophoretic mobility, indicating that the removal of Ca²⁺ from the complex increased the negative net charge of the protein. The effect was evident in both dimensions of the electrophoresis. The protein migrated faster in the first dimension, and the precipitate formed in the second dimension was higher and not as distinct as the precipitate seen when the electrophoresis was performed in 1 mM-CaCl₂ (Figs. 6a and 6b). The purified CaBP also displayed Ca²⁺-dependent electrophoretic mobility when electrophoresed under the same conditions.

Human renal CaBP and an intestinal cytosol fraction containing a CaBP with a mol.wt. of about 15000 (gel-filtration experiments on Sephadex G-50; results not shown) were tested for immunological cross-reaction. The experiments were performed by applying immunodiffusion, fused rocket immunoelectrophoresis and immunoblotting. The result of immunodiffusion experiments is shown in Fig. 8. Only one precipitate between renal CaBP and the specific antibody was found without any spurs. This indicates that human renal and intestinal CaBP are immunochemically different when tested with this antibody. The result was

confirmed by the fused rocket immunoelectrophoresis and immunoblotting experiments.

By using the specific antibody and immunoabsorbent technique larger amounts of CaBP can be isolated for more detailed biochemical characterization. Furthermore it is feasible to use the specific antibody for quantitative and histochemical studies of human renal CaBP in biopsy material.

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