A simple procedure for purifying mammalian duodenal Ca^{2+} -binding proteins on a 100 mg scale and an investigation of the stoichiometry of their high-affinity binding of Ca^{2+} ions

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Vitamin D-dependent Ca^{2+} -binding proteins were isolated on a 100 mg scale from the duodenal mucosae of pig, sheep and rabbit. Ion-exchange chromatography in two stages, which used a known property of the proteins, a charge difference with or without bound Ca^{2+} ions, was sufficient to obtain the pure proteins from several litres of heated mucosal extracts. In the preparation, treatment with a cation-exchange resin rather than with EDTA was used to obtain Ca^{2+} -free conditions when they were required. The proteins were characterized by their amino acid compositions. All three proteins contained two tightly bound Ca^{2+} ions per molecule, a property now considered to be common to the mammalian Ca^{2+} -binding proteins of this type. The labile nature of the pig Ca^{2+} -free protein was demonstrated by the formation of a form which bound only one Ca^{2+} ion. Titration of the Ca^{2+} -free binding proteins with $^{45}\text{Ca}^{2+}$ in a flow-dialysis cell, and of Ca^{2+} -free α -lactalbumin used as a control, demonstrated the binding stoichiometry and indicated that Ca^{2+} ions were bound to the mucosal proteins in dilute buffer at pH 7.5 with a K_d of the order of 10 nM.

Ca^{2+} absorption in the small intestine is stimulated by 1,25-dihydroxycholecalciferol, the hormonal metabolite of cholecalciferol (vitamin D_3) (Omdahl *et al.*, 1971), and one effect of its administration is the appearance of Ca^{2+} -binding protein in the cytoplasm of intestinal enterocytes (Wasserman *et al.*, 1978). Steady-state concentrations of Ca^{2+} -binding protein in chicks and rats correlate with rates of intestinal Ca^{2+} transport, as affected by dietary Ca^{2+} and other factors (Wasserman & Corradino, 1973; Edelstein *et al.*, 1978; Fehrer & Wasserman, 1979). In addition, embryonic-chick intestine in organ culture (Corradino *et al.*, 1976) and isolated rat epithelial cells (Freund & Bronner, 1975) responded to the addition of hormone by increased Ca^{2+} uptake and the synthesis of Ca^{2+} -binding protein. However, studies of the temporal relationship between binding-protein synthesis and Ca^{2+} absorption in vitamin D-deficient chicks (Spencer *et al.*, 1978; Morrissey *et al.*, 1978) and rats (Thomasset *et al.*, 1979; Buckley & Bronner, 1980) showed that 1,25-dihydroxycholecalciferol stimulated Ca^{2+} absorption in advance of binding-protein synthesis. A role for Ca^{2+} -binding protein in intestinal Ca^{2+} transport is clearly indicated, but its nature is uncertain. At present, more proposals favour an intracellular

function (Hamilton & Holdsworth, 1975; Spencer *et al.*, 1978; Morrissey *et al.*, 1978) than one directly connected with Ca^{2+} transport across the brush border (Corradino *et al.*, 1976; Galvanovsky *et al.*, 1981).

Although the intestinal Ca^{2+} -binding protein may function in the same way in chicks as in mammals, the avian and mammalian proteins are quite different in many respects (Wasserman *et al.*, 1978). For example, the chick protein binds four Ca^{2+} ions per molecule with high affinity, whereas the bovine (Fullmer & Wasserman, 1977; Szebenyi *et al.*, 1981) and rat (Bruns *et al.*, 1977) proteins bind two and the pig (Dorrington *et al.*, 1978) and mouse (Hosoya *et al.*, 1977) proteins bind one. We wished to prepare binding protein in substantial amounts from several mammalian species, in order to investigate their Ca^{2+} -binding stoichiometries and other properties, but found that the published procedures were difficult to apply on a large enough scale. The one procedure that has been used for large-scale purification, especially of the bovine protein, used equipment that is not available in many laboratories and it yielded protein in both native and modified forms (Wasserman *et al.*, 1978). We have devised a procedure for purifying mammalian Ca^{2+} -binding proteins which is easily applied to any

amount of mucosa. The homogeneous proteins that we obtained showed uniform binding stoichiometry for Ca^{2+} ions, but lability in the Ca^{2+} -free form may explain the various binding stoichiometries reported for different mammalian Ca^{2+} -binding proteins.

Materials and methods

Pig and sheep duodena were obtained from the local abattoir, transported to the laboratory in ice and processed immediately. Duodena from adult New Zealand White rabbits, maintained on a balanced pelleted diet, were also used.

DE-52 ion-exchange DEAE-cellulose was obtained from Whatman Biochemicals, Maidstone, Kent, U.K., Chelex 100 ion-exchange resin (200–400 mesh) from Bio-Rad Laboratories, Watford, Herts., U.K., and Amberlite CG-50 ion-exchange resin (100–200 mesh) from BDH Chemicals, Poole, Dorset, U.K. Fines were removed from the Amberlite resin before use. Phenylmethanesulphonyl fluoride was obtained from Sigma Chemical Co., Poole, Dorset, U.K., 3M-mercaptoethanesulphonic acid from Pierce & Warriner (U.K.), Chester, Cheshire, U.K., and $^{45}\text{CaCl}_2$ from Amersham International, Amersham, Bucks., U.K. Tris was GPR grade (BDH).

Bovine α -lactalbumin was isolated from cow's milk as described by Barman (1970) and its molarity in solution determined spectrophotometrically with $A_{1\text{cm},280}^{1\%}$ 20 (Kronman & Andreotti, 1964) and a molecular weight of 14 500. The Ca^{2+} -free form was prepared by treatment with 0.1 M-EDTA/EGTA as described by Blum *et al.* (1977).

Ca^{2+} analysis

Analyses were performed with an IL551 atomic-absorption spectrophotometer (Instrumentation Laboratory, Wilmington, MA, U.S.A.). Buffer solutions (10 ml) were evaporated to dryness with an i.r. lamp. The residue was then ashed and dissolved in dilute HCl (1 ml) containing SrCl_2 as an interference suppressor for analysis. Protein solutions were treated similarly, or contained 2 mM-EDTA and 7 mM- LaCl_3 as interference suppressors and were analysed directly. With either method, samples were analysed both with and without known amounts of Ca^{2+} added as internal standards. Similar results were obtained by both methods.

Low- Ca^{2+} buffers

Dilute Tris buffers prepared in distilled water contained approx. $3\ \mu\text{M}$ - Ca^{2+} . Their Ca^{2+} content was decreased to $1\ \mu\text{M}$ by passage through columns (50 cm $\times$ 2.5 cm) of Amberlite CG-50 ion-exchange resin, previously washed with HCl and equilibrated with the appropriate buffer. They were then filtered through cellulose nitrate membrane filters, porosity $0.45\ \mu\text{m}$, to remove resin particles.

Chelex assay for Ca^{2+} -binding activity

A procedure with $^{45}\text{Ca}^{2+}$ and Chelex 100 ion-exchange resin (Briggs & Fleischman, 1965; Wasserman *et al.*, 1968) was used as a guide to the relative amounts of Ca^{2+} -binding protein present in tissue extracts and column effluent fractions. Chelex 100 resin was equilibrated with the same buffer as that in which the protein was dissolved, and used as a suspension (50%, v/v) in buffer. The assay mixture (1.4 ml) contained 0.7 ml of Chelex suspension, 0.2 ml of $^{45}\text{CaCl}_2$ solution (0.1 mg of Ca^{2+} ; 2×10^5 c.p.m.), protein and buffer. The mixture was shaken for 15 min at room temperature, then centrifuged at 2000 g for 5 min, and 0.75 ml was taken for $^{45}\text{Ca}^{2+}$ counting. Radioactivity in the supernatant, after subtraction of a blank without protein, was taken as a measure of binding activity.

Polyacrylamide-gel electrophoresis

Disc electrophoresis was done with 7%-polyacrylamide gels prepared in 25 mM-Tris/phosphate buffer, pH 8.5, and a similar buffer (50 mM) in the electrode compartments. A constant current of 2.5 mA/tube was applied for 45 min. When electrophoresis was complete, gels were fixed in 40% (w/v) trichloroacetic acid and stained for protein with Coomassie Blue G-250. Electrophoresis in the presence of sodium dodecyl sulphate was done with 5%-acrylamide gels prepared in 0.05 M-sodium phosphate buffer, pH 8.0, containing 5 mM-2-mercaptoethanol and 0.1% (w/v) sodium dodecyl sulphate. The same buffer mixture was used as the electrode-compartment buffer, and with an increased concentration of sodium dodecyl sulphate (3%, w/v) to dissolve the proteins.

Amino acid analysis

Proteins were hydrolysed under N_2 in 3 M-mercaptoethanesulphonic acid for 24 h (Penke *et al.*, 1974). Amino acid analyses were obtained with an LKB automatic analyser type 4102 (LKB Instruments, Croydon, Surrey, U.K.).

Flow dialysis

The stoichiometry of high-affinity binding of Ca^{2+} to Ca^{2+} -free proteins was determined by a modification of the flow-dialysis technique of Colowick & Womack (1969). Protein was titrated with $^{45}\text{Ca}^{2+}$ of suitable specific radioactivity in a flow-dialysis cell, and the concentration of free $^{45}\text{Ca}^{2+}$ in the protein solution was estimated from its equilibrium concentration in the diffusate. The relationship between free Ca^{2+} concentration in the dialysis cell and in the diffusate was determined for each experiment from a blank titration with protein absent. The apparatus used was similar to that described by Feldmann (1978), except that the upper chamber was enlarged to hold 2 ml of solution.

Membrane discs were cut from Visking dialysis tubing and soaked in deionized water before use. Then 20 mM-Tris/HCl buffer, pH 7.5, was pumped through the spiral at a rate of 1 ml/min, and six 1 ml fractions were collected after each addition of $^{45}\text{Ca}^{2+}$ to the upper chamber. The equilibrium concentration of $^{45}\text{Ca}^{2+}$ in the buffer flow was reached in the third fraction, and its value obtained by averaging $^{45}\text{Ca}^{2+}$ measurements on the next three fractions. It was about 0.5% of the free $^{45}\text{Ca}^{2+}$ concentration in the upper chamber. Protein was dissolved in 20 mM-Tris/HCl buffer, pH 7.5, and $^{45}\text{Ca}^{2+}$ solution was added in 5 μl portions.

Fluorescence titrations

Fluorescence measurements were obtained with an Hitachi-Perkin-Elmer model MPF-3L fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, Bucks., U.K.). Protein fluorescence was excited at 275 nm and measured at 306 nm, with 10 nm slits for both monochromators. Titrations were performed in a 1 cm-square cell containing 2 ml of protein solution at 25°C, and CaCl_2 solution was added in 5 μl portions.

Purification of duodenal Ca^{2+} -binding proteins

The purification procedure is summarized in the flow diagram in Scheme 1. The steps were performed at 5°C, except where stated, and Tris/HCl buffers, pH 7.5, were used throughout.

Preparation and heat treatment of mucosal extracts. Mucosa was obtained from lengths of small intestine proximal to the stomach: pig, 3 m;

sheep, 2 m; rabbit, 1 m. The intestinal contents were rinsed out with 50 mM buffer, then the gut was slit lengthways and the mucosal layer scraped off with the edge of a microscope slide. Mucosa was homogenized with a Silverson homogenizer run at full speed in the same buffer (3 vol.) containing 30 μM -phenylmethanesulphonyl fluoride, and the homogenates were centrifuged at 35 000 g for 30 min. The supernatants were then heated at 65°C for 20 min, and centrifuged again to remove denatured protein. Chelex assays indicated that heating caused little or no change in the Ca^{2+} -binding activity of the extracts.

First DEAE-cellulose chromatography. The binding proteins in the heated extracts were first chromatographed in their Ca^{2+} -liganded forms. The heated extracts were thoroughly dialysed against 20 mM buffer and applied to columns of DE-52 DEAE-cellulose equilibrated with the same buffer. Non-adsorbed protein was eluted with the equilibration buffer. The pig (Fig. 1a) and rabbit proteins were eluted with 50 mM buffer, whereas the sheep protein was retained more strongly and was eluted with a Cl^- gradient in the same buffer and emerged at a Cl^- concentration of approx. 75 mM (Fig. 1b). Some heterogeneity was observed in the Chelex-assay elution profile in each case, and only the main peak was used in the next step. Columns of DE-52 DEAE-cellulose measuring 25 cm $\times$ 4.6 cm gave satisfactory results with 350–500 ml of heated dialysed extract, and 60 cm $\times$ 4.6 cm columns gave similar results with 1 litre.

Second DEAE-cellulose chromatography. Pooled

Scheme 1. Flow diagram for the purification of mammalian duodenal Ca^{2+} -binding proteins on a 100 mg scale. Experimental details for each step are given in the text. For the yields of Ca^{2+} -binding protein from the mucosa of different species, see Table 1.

Fig. 1. Chromatography of heated dialysed extracts of duodenal mucosae on DE-52 DEAE-cellulose

The columns (25 cm $\times$ 4.6 cm) were equilibrated with 20 mM-Tris/HCl buffer, pH 7.5. Fraction volumes were 25 ml. The samples applied were 350 ml of heated dialysed extract from (a) pig mucosa or (b) sheep mucosa. —, A_{280} ; O, $^{45}\text{Ca}^{2+}$ -binding activity measured by Chelex assay; ----, Cl^- gradient.

fractions containing Ca^{2+} -binding protein from the first ion-exchange chromatography were passed through an Amberlite CG-50 column (15 cm $\times$ 2.5 cm; equilibrated with 50 mM buffer) to remove Ca^{2+} ions. Fractions containing the sheep protein were first diluted 2-fold to lower the Cl^- content. Then the protein solutions were applied to columns (20 cm $\times$ 2.5 cm) of DE-52 DEAE-cellulose equilibrated with low- Ca^{2+} 50 mM buffer. The columns were washed with low- Ca^{2+} 70 mM buffer until no protein appeared in the effluent, with low- Ca^{2+} 50 mM buffer (100 ml), and with 50 mM buffer containing 1 mM- CaCl_2 to elute Ca^{2+} -binding protein (Fig. 2). After a major peak of Ca^{2+} -binding-protein had appeared in the eluate, 0.3 M-NaCl was applied to each column and elution continued. No more Ca^{2+} -binding activity was detected when the pig (Fig. 2a) or sheep proteins were being chromatographed, but some was eluted after the main peak of rabbit Ca^{2+} -binding protein (Fig. 2b). Electrophoresis showed that the second Ca^{2+} -binding peak contained several proteins, but the binding protein(s) in the peak have not yet been characterized.

Fractions containing Ca^{2+} -binding protein were dialysed against 1 mM- NH_3 until salt-free, and the solutions were freeze-dried. The products were stored at 5°C.

Fig. 2. Purification of Ca^{2+} -binding proteins on DE-52 DEAE-cellulose

The columns (20 cm $\times$ 2.5 cm) were equilibrated with 50 mM-Tris/HCl buffer, pH 7.5. Fraction volumes were 10 ml. The samples applied were Ca^{2+} -binding proteins from (a) pig mucosa or (b) rabbit mucosa, partly purified by ion-exchange chromatography (Fig. 1). Elution was with Tris/HCl buffers, pH 7.5, as follows: A, 70 mM buffer; B, 50 mM buffer; C, 50 mM buffer containing 1 mM- CaCl_2 ; D, 50 mM buffer containing 0.3 M-KCl. —, A_{280} ; O, $^{45}\text{Ca}^{2+}$ -binding activity measured by Chelex assay.

Results

Yields

The weights of Ca^{2+} -binding proteins which were obtained in different preparations are given in Table 1. Estimates based on Chelex assays indicated recoveries of 40–50% from the mucosal extracts. Yields of the pig protein are similar to those given by Hitchman *et al.* (1973), which were obtained on a much smaller scale.

Properties of duodenal Ca^{2+} -binding proteins

Electrophoretic behaviour. The purified proteins gave single bands on polyacrylamide-gel electrophoresis, whether run in the absence of added Ca^{2+} , in the presence of 1 mM- CaCl_2 or in the presence of 1 mM-EDTA. The amounts of protein used (25 μg) were enough for 1% of protein impurity, if staining

Table 1. *Preparation of Ca^{2+} -binding proteins from mammalian duodenal mucosae*

The proteins were purified as described in the text.

Species	Mucosa used		Ca^{2+} -binding protein obtained	
	No. of animals	Wt. (g)	Wt. (mg)	Yield (mg/g of mucosa)
Pig	8	980	80	0.08
	10	2030	290	0.14
Sheep	12	1800	72	0.04
Rabbit	8	91	95	1.0

with equal intensity, to be detected. In each case migration rate was unaffected by the presence of added Ca^{2+} , indicating that the endogenous Ca^{2+} in the proteins was bound with high affinity, but the rates were faster in the presence of EDTA (Hitchman & Harrison, 1972). Whatever the conditions, the rabbit protein migrated slower than the pig protein, whereas the sheep protein, like the bovine protein (Fullmer & Wasserman, 1975), migrated faster.

The purified proteins also gave single bands on electrophoresis in the presence of sodium dodecyl sulphate. All three proteins migrated at the same rate, indicating that they had molecular weights of about 8800, similar to that of the pig protein (Hofmann *et al.*, 1979).

Effect of storage. After 6 months storage at 5°C , the freeze-dried Ca^{2+} -liganded proteins still showed single bands on electrophoresis, but after storage for the same time in solution at -10°C they showed in addition two faint faster-moving bands. The Ca^{2+} -free proteins in solution, also stored at -10°C , changed similarly but more rapidly. Such changes in storage are well documented for the bovine protein (Fullmer & Wasserman, 1981).

Amino acid composition. Amino acid analyses of the purified proteins are given in Table 2.

Specific absorption coefficients. The values for $A_{1\text{cm},280}^{1\%}$ obtained when amino acid analyses were used as quantitative measurements of protein concentration were 1.9, 1.9 and 2.3 for the pig, sheep and rabbit proteins respectively. Dorrington *et al.* (1974) reported a value of 1.6 for the pig protein.

Ca^{2+} content after dialysis. The proteins (1 mg/ml) were dissolved in Ca^{2+} -free 10 mM-Tris/HCl buffer, pH 7.5, and dialysed against low- Ca^{2+} distilled water, with several changes, for 48 h. Protein concentration in the bags was then determined from absorbance measurements, and the solutions inside and outside the bags were analysed for Ca^{2+} by atomic absorption. The concentration outside (approx. $1\ \mu\text{M}$) was subtracted from the concentration inside to give the concentration of bound Ca^{2+} . Results for the duodenal proteins and for bovine α -lactalbumin, which was used as a control, are given in Table 3.

Table 2. *Amino acid composition of mammalian duodenal Ca^{2+} -binding proteins*

(a) By analysis, present work; (b) from the sequence (Hofmann *et al.*, 1979); (c) from the sequence of the minor A component (Fullmer & Wasserman, 1981).

Composition (no. of residues/molecule)						
Species	...	Pig		Rabbit	Sheep	Bovine
		(a)	(b)	(a)	(a)	(c)
Asp	7-8	7	8	6	6	
Thr	1	1	2-3	2	2	
Ser	6	7	4	6	6	
Glu	17-18	17	14-15	17-18	17	
Pro	4	4	4	4	4	
Gly	4-5	4	4-5	5	5	
Ala	5	5	5	3	2	
Cys	0	0	0	0	0	
Val	3	3	2	3	3	
Met	0	0	0	0	0	
Ile	3	3	4	2	2	
Leu	10-11	10	11	11-12	12	
Tyr	1	1	1	1	1	
Phe	5	5	5	4-5	5	
His	0	0	0	0	0	
Lys	9-10	10	12	9-10	10	
Arg	1	1	0	0	0	
Trp	0	0	0	0	0	
Total residues	76-81	78	76-79	73-77	75	

Table 3. *Stoichiometry of Ca^{2+} bound with high affinity to mammalian Ca^{2+} -binding proteins*

Molarities of the duodenal proteins were calculated with a molecular weight of 8800. Results are the mean of two determinations.

Species	Protein	Ca^{2+} bound after dialysis (mol/mol of protein)	Ca^{2+} bound in flow-dialysis cell (mol/mol of protein)
Pig	Duodenal protein	1.9	2.0
	Altered form of duodenal protein	—	1.0
Sheep	Duodenal protein	1.8	1.5
Rabbit	Duodenal protein	2.1	2.0
Cow	α -Lactalbumin	1.1	1.0

Preparation of Ca^{2+} -free duodenal proteins. The Ca^{2+} -liganded proteins (15 mg) were dissolved in 2 ml of 10 mM-Tris/HCl buffer, pH 7.5, and the solutions allowed to percolate through columns of Amberlite CG-50 ion-exchange resin equilibrated with the same buffer.

A column measuring 40 cm $\times$ 1.5 cm was used in initial experiments with the pig protein. Absorption measurements and Ca^{2+} determinations indicated protein recoveries of $>90\%$ and 0.1–0.16 mol of

Fig. 3. Ca^{2+} titrations in a flow-dialysis cell

See the text for details. The cell contained: (a) no protein; (b) Ca^{2+} -free native pig Ca^{2+} -binding protein ($14.0\mu\text{M}$); (c) Ca^{2+} -free altered pig Ca^{2+} -binding protein ($10.7\mu\text{M}$); (d) Ca^{2+} -free bovine α -lactalbumin ($14.0\mu\text{M}$). Open circles are experimental values, the solid line in (b)–(d) is the calculated result for K_d 10 nM and the broken line in (b) is for K_d $0.2\mu\text{M}$.

Ca^{2+} /mol of protein respectively after two passes through the column, but the protein so obtained bound only 1 mol of Ca^{2+} /mol with high affinity in flow dialysis (Fig. 3c; see below). On electrophoresis it still showed a single band and was indistinguishable from the native protein.

A shorter resin column ($15\text{ cm} \times 1\text{ cm}$) was used in subsequent experiments, and then both pig and rabbit proteins were obtained nearly free of Ca^{2+} (0.1 mol/mol of protein) and able to bind 2 mol of Ca^{2+} /mol with high affinity in flow dialysis.

High-affinity Ca^{2+} binding measured by flow dialysis. For blank titrations, graphs of ${}^{45}\text{Ca}^{2+}$ concentration in the buffer flow against ${}^{45}\text{Ca}^{2+}$ concentration in the upper chamber were linear and

Fig. 4. Effect of Ca^{2+} on the intrinsic fluorescence of native and altered forms of pig duodenal Ca^{2+} -binding protein

The proteins were dissolved in 20 mM-Tris/HCl buffer, pH 7.5. Excitation was at 275 nm. and emission at 306 nm. O, Ca^{2+} -free native protein ($16.6\mu\text{M}$); Δ , Ca^{2+} -free altered protein ($7.0\mu\text{M}$).

passed through the origin, both when the ${}^{45}\text{Ca}^{2+}$ concentrations in the upper chamber were below $0.1\mu\text{M}$ (Fig. 3a) and when they were in a range up to $10\mu\text{M}$. Different membrane discs gave graphs with different slopes, possibly owing to differences in tension when the discs were clamped in the cell. Therefore an initial blank titration was performed with each membrane disc and the apparatus was then washed free of ${}^{45}\text{Ca}^{2+}$ before protein solution was placed in the upper chamber.

When the Ca^{2+} -free binding proteins were titrated with ${}^{45}\text{Ca}^{2+}$, the concentration of free ${}^{45}\text{Ca}^{2+}$ in the protein solutions was extremely low until the proteins were nearly saturated, and when saturation of the high-affinity binding was reached it increased linearly in parallel with the corresponding blank titration (Figs. 3b and 3c). Similar behaviour was observed with Ca^{2+} -free bovine α -lactalbumin (Fig. 3d), which binds one Ca^{2+} ion with high affinity (Permyakov *et al.*, 1981).

The stoichiometry of high-affinity binding for each protein was clearly indicated and, except for the altered form of the pig protein (Fig. 3c), the results agreed well with the Ca^{2+} analyses of the proteins before they were converted into Ca^{2+} -free forms (Table 3). The ${}^{45}\text{Ca}^{2+}$ concentrations in the buffer flow indicated K_d values of the order of 10 nM for the high-affinity binding of Ca^{2+} to the proteins.

Fluorescence titrations. Both the native Ca^{2+} -free pig duodenal protein and the altered form had fluorescence spectra typical of proteins containing tyrosine but no tryptophan, with excitation and

emission maxima of 276 nm and 306 nm respectively. The fluorescence intensity was similar for the two proteins, and increased when they were titrated with Ca^{2+} by 50% for the native protein and 32% for the altered protein. The titration curves showed sharp end-points (Fig. 4) at 1.3 and 0.93 mol of Ca^{2+} /mol of protein for the native and altered proteins respectively.

Discussion

In considering methods for purifying substantial amounts of duodenal Ca^{2+} -binding proteins, we were guided by the report that up to 6 mg of binding protein can be prepared from 60 g of pig duodenal mucosa (Hitchman *et al.*, 1973). We assumed that the preparation of 100 mg of binding protein would involve the extraction of at least 1 kg of mucosa and the processing of several litres of extract. As shown in Table 1, this was a useful guide as far as the larger species were concerned, but the yield of protein from rabbit mucosa was considerably higher. Since mucosal extracts contain proteolytic enzymes, which can decrease the yield of native binding protein by the formation of modified forms during purification (Wasserman *et al.*, 1978; Ueng & Bronner, 1979), we extracted the mucosae with buffer containing a proteinase inhibitor in order to minimize the problem.

Numerous techniques have been used in purifying the mammalian Ca^{2+} -binding proteins (Drescher & DeLuca, 1971; Hitchman & Harrison, 1972; Hitchman *et al.*, 1973; Bruns *et al.*, 1977; Wasserman *et al.*, 1978; Gleason & Lankford, 1981; Delorme *et al.*, 1982), but we found that only two techniques, which both worked well on a large scale, were sufficient for our purpose. Heat treatment (Hitchman & Harrison, 1972) precipitated much unwanted protein from mucosal extracts with little or no loss of binding activity, as measured by the Chelex assay, and was easily applied to large volumes of extract because the heating time was not critical if the temperature was kept below 70°C. Fractionation of the soluble proteins from this stage by ion-exchange chromatography gave homogeneous binding protein. We used a modified form of the method introduced by Hitchman *et al.* (1973), which takes advantage of a charge difference on the binding proteins with or without bound Ca^{2+} ions. The mammalian proteins have such a high affinity for Ca^{2+} ions that the ions remain protein-bound during gel filtration (Hitchman & Harrison, 1972), electrophoresis, dialysis and ion-exchange chromatography. Therefore, instead of chromatographing the proteins in the presence of excess Ca^{2+} in order to maintain them in the Ca^{2+} -liganded form (Hitchman *et al.*, 1973), we used conditions in which only proteins with a very high affinity for Ca^{2+} ions chromatographed in the liganded form. Ca^{2+} -free

conditions for the second chromatographic step were then produced by treating proteins and buffers with a cation-exchange resin rather than by adding EDTA or EGTA, to avoid the need to remove chelating agent from the product. At the last stage, any binding protein lacking an affinity for Ca^{2+} was left on the column when the binding protein was eluted by conversion into the Ca^{2+} -liganded form.

The proteins that we obtained were characterized by their amino acid analyses (Table 2). The sheep protein was very similar in composition to the bovine protein, and the protein from pig mucosa had essentially the same composition as that studied by Hofmann *et al.* (1979), who concluded that it bound only one Ca^{2+} ion with high affinity. The binding studies (Table 3) indicated that the three proteins which we obtained all bound two Ca^{2+} ions with high affinity when in native form. Since the presence of two Ca^{2+} -binding sites on the bovine protein is now well established (Szebenyi *et al.*, 1981), we conclude that this property is characteristic of the mammalian proteins in general.

Although the native pig protein bound two Ca^{2+} ions, changes in its intrinsic fluorescence on the addition of Ca^{2+} reached an end-point with only 1.3 mol of Ca^{2+} /mol of protein (Fig. 4). The presence of a Ca^{2+} ion at one site evidently affects the tyrosine chromophore more than does a Ca^{2+} ion at the other site, a property consistent with a structure similar to that of the bovine protein (Szebenyi *et al.*, 1981). This may explain why Dorrington *et al.* (1978) underestimated the Ca^{2+} -binding capacity of the pig protein. Their determination was based on spectroscopic measurements, which in some experiments reached an end-point similar to that of our fluorimetric titration. The ease with which the pig protein and to some extent the sheep protein lost binding capacity when Ca^{2+} was removed provides another explanation for differing estimates of the binding stoichiometry of the mammalian proteins, and indicates also that they are best stored in the Ca^{2+} form. The altered pig protein binds 1 mol of Ca^{2+} /mol and appears to be a form of the protein which has lost the ability to bind Ca^{2+} at one site, rather than a mixture of native and inactive proteins. Its intrinsic fluorescence was related to the amount of Ca^{2+} bound in a manner different from that of the native protein (Fig. 4). Hake parvalbumin, which in native form binds two Ca^{2+} ions with high affinity and is closely related in structure to the mammalian Ca^{2+} -binding proteins, also loses up to half of its binding capacity when in the Ca^{2+} -free form (Haiech *et al.*, 1979).

The flow-cell experiments (Fig. 3) indicated that the native and modified mammalian proteins had K_d values for Ca^{2+} of the order of 10 nM, rather than the previously reported values of 0.2–0.3 μM (Hitchman & Harrison, 1972; Bruns *et al.*, 1977), 1 μM

(Wasserman *et al.*, 1978) or $2.5\mu\text{M}$ (Hosoya *et al.*, 1977). When the flow-cell was tested with α -lactalbumin, which binds one Ca^{2+} ion with a K_d of about 3 nM (Permyakov *et al.*, 1981), the results again indicated a K_d of the order of 10 nM . The flow-cell method therefore overestimated the K_d for α -lactalbumin, and may also have done so for the mammalian proteins. The retention of Ca^{2+} by the mammalian proteins during dialysis, chromatography and electrophoresis indicates a K_d considerably lower than $1\mu\text{M}$, and results quoted by Szebenyi *et al.* (1981) for the bovine protein are consistent with at least one site having a K_d of 10 nM or less, because it retains Ca^{2+} in the presence of an equimolar concentration of EDTA. The binding of Ca^{2+} ions by the mammalian proteins must form at least part of their function, and their properties in this respect seem to deserve further investigation.

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