Investigation of the binding of Ca^{2+} , Mg^{2+} , Mn^{2+} and K^{+} to the vitamin D-dependent Ca^{2+} -binding protein from pig duodenum

D. Terence W. BRYANT and Patrick ANDREWS

National Institute for Research in Dairying, Shinfield, Reading, Berkshire RG2 9AT, U.K.

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The cation-binding properties of the vitamin D-dependent Ca^{2+} -binding protein from pig duodenum were investigated, mainly by flow dialysis. The protein bound two Ca^{2+} ions with high affinity, and Mg^{2+} , Mn^{2+} and K^{+} were all bound competitively with Ca^{2+} at both sites. The sites were distinguished by their different affinities for Mn^{2+} , the one with the higher affinity being designated A (K_d $0.61 \pm 0.02 \mu\text{M}$) and the other B (K_d $50 \pm 6 \mu\text{M}$). Competitive binding studies allied to fluorimetric titration with Mg^{2+} showed that site A bound Ca^{2+} , Mg^{2+} and K^{+} with K_d values of $4.7 \pm 0.8 \text{ nM}$, $94 \pm 18 \mu\text{M}$ and $1.6 \pm 0.3 \text{ mM}$ respectively, and site B bound the same three cations with K_d values of $6.3 \pm 1.8 \text{ nM}$, $127 \pm 38 \mu\text{M}$ and $2.1 \pm 0.6 \text{ mM}$. For the binding of these cations, therefore, there was no significant difference between the two sites. In the presence of 1 mM-Mg^{2+} and 150 mM-K^{+} , both sites bound Ca^{2+} with an apparent K_d of $0.5 \mu\text{M}$. The cation-binding properties were discussed relative to those of parvalbumin, troponin C and the vitamin D-dependent Ca^{2+} -binding protein from chick duodenum.

1,25-Dihydroxycholecalciferol stimulates Ca^{2+} absorption in the small intestine and one effect of its administration is the appearance of Ca^{2+} -binding protein in the cytoplasm of duodenal enterocytes, both in chicks and in mammals. The proteins from different mammalian species are very similar to one another in many respects, but quite different from the chick protein (Wasserman *et al.*, 1978). No structural relationship between the mammalian and chick proteins has yet been demonstrated. A role for these proteins in Ca^{2+} absorption is clearly indicated, but its nature is uncertain.

Two properties of the proteins which are presumably of major functional importance are their affinities for Ca^{2+} and the stoichiometry of the binding. The mammalian proteins are probably all alike in binding $2 \text{ mol of Ca}^{2+}/\text{mol}$, but their affinity for Ca^{2+} is uncertain. Most values for the K_d for Ca^{2+} are in the range $0.2\text{--}1 \mu\text{M}$ (Hitchman & Harrison, 1972; Bruns *et al.*, 1977; Fullmer & Wasserman, 1977, 1980; Chiba *et al.*, 1983), but we found that when the proteins from three different mammalian species were titrated with Ca^{2+} in a flow-dialysis cell the results indicated that Ca^{2+} was bound with a K_d of the order of 10 nM (Bryant & Andrews, 1983).

The direct determination of K_d values in the nanomolar region for Ca^{2+} -protein interactions is

hindered by the difficulty of avoiding contamination with Ca^{2+} . The use of EGTA or EDTA as Ca^{2+} buffers to control free Ca^{2+} concentrations avoids the difficulty, but interaction between the chelating agents and the protein may then be a source of error in the measurements (Kronman & Bratcher, 1983). Interaction between a Ca^{2+} -binding protein and EGTA can be detected if the Ca^{2+} -liganded protein is titrated with EGTA in a flow-dialysis cell (Haiech *et al.*, 1980), and by this method we found that the Ca^{2+} -binding protein from pig duodenum interacts with EGTA in the presence of Ca^{2+} (D. T. W. Bryant & P. Andrews, unpublished work). We have therefore used competition with other cations, which resulted in apparent K_d values for Ca^{2+} which were high enough to be determined satisfactorily in the absence of a chelating agent, to investigate the affinity of the pig protein for Ca^{2+} . Titration with Mn^{2+} revealed a marked difference in the properties of the two sites, and competitive binding studies with Ca^{2+} and either Mn^{2+} or Mg^{2+} produced sets of apparent K_d values for Ca^{2+} from which intrinsic binding constants were obtained.

Materials and methods

The buffer used in all experiments was 20 mM-Tris/HCl , pH 7.5, prepared from deionized water

and Aristar-grade Tris (BDH Chemicals, Poole, Dorset, U.K.). It contained $<0.1 \mu\text{M}$ - Ca^{2+} , as determined by atomic-absorption analysis. Other chemicals were AnalR grade. $^{45}\text{CaCl}_2$ was obtained from Amersham International, Amersham, Bucks., U.K., and $^{54}\text{MnCl}_2$ from New England Nuclear, Southampton, Hants., U.K.

Ca^{2+} -binding protein containing 2.0 mol of Ca^{2+} /mol of protein was isolated from pig duodenal mucosa as described by Bryant & Andrews (1983). Its molarity in solution was determined spectrophotometrically by using $A_{1\text{cm},280}^{1\%}$ 1.9 and an M_r of 8900. The ' Ca^{2+} -free' form was prepared by passing a solution of the protein (8 mg) in buffer (1 ml) through a column (15 cm $\times$ 1 cm) of Amberlite CG-50 ion-exchange resin (BDH Chemicals) equilibrated with the same buffer. The protein so obtained contained 0.2 mol of Ca^{2+} /mol.

Flow dialysis

The Ca^{2+} -free protein was titrated with $^{45}\text{Ca}^{2+}$ or $^{54}\text{Mn}^{2+}$ in an apparatus similar to that described by Feldmann (1978), except that the upper chamber was enlarged to hold 2 ml of protein solution. Membrane discs were cut from Visking dialysis tubing and soaked in deionized water before use. Buffer was pumped through the spiral at a rate of 1 ml/min, and six 1 ml fractions were collected after each addition (5 μl or 10 μl) of $^{45}\text{Ca}^{2+}$ or $^{54}\text{Mn}^{2+}$ to the upper chamber. The equilibrium concentration of $^{45}\text{Ca}^{2+}$ or $^{54}\text{Mn}^{2+}$ in the buffer flow was obtained by averaging measurements on the fourth, fifth and sixth fractions. Corrections were applied for the loss of radioactive cation from the upper chamber (less than 5% of the total during an experiment). In each experiment, the relationship between free Ca^{2+} or free Mn^{2+} concentration in the upper chamber and in the buffer flow was determined from a blank titration with protein absent.

In competitive binding experiments, both the protein solution and the buffer flow contained the competing cation(s).

A titration in the flow-dialysis cell gave values for free and protein-bound cation concentration which were used to construct a Scatchard plot. Results which gave non-linear Scatchard plots were analysed by computer to obtain the best estimates on a least-squares basis for the K_d values and concentrations of two independent sites, assuming a log-normal structure for the bound/free ratio.

Fluorescence titration

Fluorescence measurements were obtained with an Hitachi-Perkin-Elmer model MPF-3L fluorescence spectrometer. 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 MgCl_2 solution was added in 5 μl portions. The fluorescence change at saturation with Mg^{2+} was determined from a double-reciprocal plot of the results, which were then used to denote degree of saturation on the assumption that the fluorescence change was proportional to the amount of Mg^{2+} bound.

Results

The sequence of results and calculations used to determine the cation-binding properties of the pig duodenal protein is shown in Scheme 1.

K_d values for Mn^{2+}

The results obtained when the protein was titrated with Mn^{2+} showed that the Mn^{2+} was bound at two sites with different affinities (Fig. 1). The site with the higher affinity for Mn^{2+} was designated A and the other B, and three determinations gave $K_d^{\text{Mn}^{2+}}$ $0.61 \pm 0.02 \mu\text{M}$ for site A and $K_d^{\text{Mn}^{2+}}$ $50 \pm 6 \mu\text{M}$ for site B.

Apparent K_d values for Ca^{2+} in the presence of Mn^{2+}

When the protein was titrated with Ca^{2+} in the presence of Mn^{2+} , the results indicated that there was competition between Ca^{2+} and Mn^{2+} at both sites (Fig. 2a). Analysis of the results gave two sets of values for apparent $K_d^{\text{Ca}^{2+}}$ (Fig. 2b). The values obtained for the binding-site concentrations indicated that the residual Ca^{2+} in the protein was located mainly in the site which bound Ca^{2+} with the higher affinity in the presence of Mn^{2+} .

Calculation of K_d values for Ca^{2+}

For the binding of Ca^{2+} to a protein in the presence of a competing cation X, the apparent $K_d^{\text{Ca}^{2+}}$ is related to $K_d^{\text{Ca}^{2+}}$ by

$$\text{apparent } K_d^{\text{Ca}^{2+}} = K_d^{\text{Ca}^{2+}} \left(1 + \frac{[\text{X}]}{K_d^{\text{X}}} \right) \quad (1)$$

where $[\text{X}]$ is the concentration of free X and K_d^{X} is its dissociation constant. Eqn. (1) shows that a graph of apparent $K_d^{\text{Ca}^{2+}}$ against $[\text{X}]$ is linear, with an ordinate intercept equal to $K_d^{\text{Ca}^{2+}}$ and a slope of $K_d^{\text{Ca}^{2+}}/K_d^{\text{X}}$.

In the experiments with Mn^{2+} as the competing cation (Fig. 2) the Mn^{2+} concentrations were high enough to use total Mn^{2+} concentration instead of free Mn^{2+} concentration in eqn. (1) without appreciable error. Graphs of the two sets of apparent $K_d^{\text{Ca}^{2+}}$ values against total Mn^{2+} concentration were linear (Fig. 2b) and had ordinate intercepts of $54 \pm 66 \text{ nM}$ and $4.1 \pm 2.5 \mu\text{M}$, and slopes of $(1.27 \pm 0.34) \times 10^{-4}$ and $(7.68 \pm 1.26) \times 10^{-3}$, for Ca^{2+} bound with higher and lower

Scheme 1. Determination of cation-binding properties of Ca^{2+} -binding protein from pig duodenal mucosa. Details of the experiments and the calculations are given in the text. The sites with higher and lower affinities for Mn^{2+} are designated A and B respectively.

affinity respectively. The margin of error on the ordinate intercepts was too large for them to be reliable values for $K_d^{\text{Ca}^{2+}}$, but the slopes ($K_d^{\text{Ca}^{2+}}/K_d^{\text{Mn}^{2+}}$) were determined more accurately. Calculation then gave two pairs of values for $K_d^{\text{Ca}^{2+}}$ (Scheme 1) because the results did not show which slope applied to which site, although the sites themselves were characterized by their different affinities for Mn^{2+} .

The question as to which pair of values for $K_d^{\text{Ca}^{2+}}$ was correct was resolved by experiments in which competition between Ca^{2+} and Mg^{2+} was investigated, as follows.

Determination of values for $K_d^{\text{Mg}^{2+}}$

When the protein was titrated with Ca^{2+} in the presence of Mg^{2+} , the results indicated that there was competition between the two cations at both sites and that, at each Mg^{2+} concentration, the Ca^{2+} was bound at both sites with the same apparent K_d (Fig. 3a). The Mg^{2+} concentrations were high enough to use total Mg^{2+} instead of free Mg^{2+} concentration in the calculations, and the plot of apparent $K_d^{\text{Ca}^{2+}}$ values against Mg^{2+} concentration was linear as required by eqn. (1), with a slope ($K_d^{\text{Ca}^{2+}}/K_d^{\text{Mg}^{2+}}$) of $(4.99 \pm 0.44) \times 10^{-5}$ (Fig.

Fig. 1. Binding of Mn^{2+} to pig duodenal Ca^{2+} -binding protein

The experimental points (○) were obtained by titrating the protein ($11.3 \mu\text{M}$) with $^{54}\text{Mn}^{2+}$ in a flow-dialysis cell. The curve is the calculated result if the protein binds Mn^{2+} at one site with K_d $0.63 \mu\text{M}$ and at the other site with K_d $44 \mu\text{M}$. The results are for one experiment.

Fig. 2. Binding of Ca^{2+} to pig duodenal Ca^{2+} -binding protein in the presence of Mn^{2+} . (a) The protein ($12.6 \mu\text{M}$) was titrated with $^{45}\text{Ca}^{2+}$ in a flow-dialysis cell in the presence of different concentrations of Mn^{2+} . Experimental points, Mn^{2+} concentrations and the pairs of values for apparent $K_d^{\text{Ca}^{2+}}$ used to calculate the corresponding curves are respectively: (○), 1 mM, 0.19 μM and 13.7 μM ; (●), 2 mM, 0.23 μM and 19.0 μM ; (□), 3 mM, 0.45 μM and 25.9 μM . (b) Graph of values for apparent $K_d^{\text{Ca}^{2+}}$ obtained from flow-dialysis titrations against total Mn^{2+} concentration.

Fig. 3. Binding of Ca^{2+} to pig duodenal Ca^{2+} -binding protein in the presence of Mg^{2+} . (a) The protein ($11.3\text{--}14.0 \mu\text{M}$ in different experiments) was titrated with Ca^{2+} in a flow-dialysis cell in the presence of different concentrations of Mg^{2+} . Experimental points, Mg^{2+} concentrations and the values for apparent $K_d^{\text{Ca}^{2+}}$ used to calculate the corresponding curves are respectively: (○), 10 mM, 0.64 μM ; (●), 25 mM, 1.36 μM ; (□), 50 mM, 2.26 μM ; (■), 75 mM, 3.10 μM ; (△), 100 mM, 5.30 μM . (b) Graph of values for apparent $K_d^{\text{Ca}^{2+}}$ obtained from flow-dialysis titrations against total Mg^{2+} concentration.

3b). Calculation then gave a $K_d^{\text{Mg}^{2+}}$ value corresponding to each of the four $K_d^{\text{Ca}^{2+}}$ values (Scheme 1).

The correct pair of $K_d^{\text{Mg}^{2+}}$ values was selected by comparing calculated results with results obtained experimentally when the protein was titrated

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

The open circles are experimental points and the curves are calculated results for $K_d^{\text{Mg}^{2+}}$ values of (a) $1.6 \mu\text{M}$; (b) $94 \mu\text{M}$ at one site and $127 \mu\text{M}$ at the other; (c) 7.7mM .

fluorimetrically with Mg^{2+} . Saturation with Mg^{2+} increased the intrinsic fluorescence by 17%, but the relationship between fluorescence change and Mg^{2+} binding at either site is uncertain because the single tyrosine chromophore is asymmetrically situated relative to the two sites (Hofmann *et al.*, 1979). However, if the fluorescence change is associated at site A with $K_d^{\text{Mg}^{2+}} 1.6 \mu\text{M}$ or site B with $K_d^{\text{Mg}^{2+}} 7.7 \text{mM}$, or partly with one and partly the other, the calculated results differ widely from the experimental results (Fig. 4). On the other hand, if the fluorescence change is associated with binding at site A with $K_d^{\text{Mn}^{2+}} 94 \mu\text{M}$ and site B with $K_d^{\text{Mg}^{2+}} 127 \mu\text{M}$, the calculated and experimental results agree.

Determination of values for $K_d^{\text{K}^{+}}$

In the presence of 1mM-MgCl_2 and 150mM-KCl , apparent $K_d^{\text{Ca}^{2+}}$ was $0.5 \mu\text{M}$ for both sites. Substitution for $K_d^{\text{Ca}^{2+}}$ and $K_d^{\text{Mg}^{2+}}$ in the expression

$$\text{apparent } K_d^{\text{Ca}^{2+}} = K_d^{\text{Ca}^{2+}} \left(1 + \frac{[\text{Mg}^{2+}]}{K_d^{\text{Mg}^{2+}}} + \frac{[\text{K}^{+}]}{K_d^{\text{K}^{+}}} \right) \quad (2)$$

and the use of total Mg^{2+} and K^{+} concentrations for $[\text{Mg}^{2+}]$ and $[\text{K}^{+}]$ respectively gave $K_d^{\text{K}^{+}}$ values of $1.6 \pm 0.3 \text{mM}$ for site A and $2.1 \pm 0.6 \text{mM}$ for site B.

Discussion

The results confirm our earlier observations (Bryant & Andrews, 1983) and show that the affinity of the protein for Ca^{2+} is much greater than the reported K_d values of $0.2\text{--}0.3 \mu\text{M}$ (Hitchman & Harrison, 1972) and $0.56 \mu\text{M}$ (Chiba *et al.*, 1983) for the same protein indicated. The validity of these values is uncertain, however, because the first was determined in an assay with Chelex 100 resin, which is unsuitable for the purpose (Waisman & Rasmussen, 1983), and the second by equilibrium dialysis with no control of free Ca^{2+} concentrations at the nanomolar level. K_d values of $0.3 \mu\text{M}$ for the rat protein (Bruns *et al.*, 1977) and $1 \mu\text{M}$ for the bovine protein (Fullmer & Wasserman, 1977) are uncertain for the same reasons, and interaction with EGTA or the nature of the protein might account for the difference between our result for native pig protein and the K_d of $0.23 \mu\text{M}$ obtained for a modified form of the bovine protein by equilibrium dialysis in a Ca^{2+} -EGTA buffer (Fullmer & Wasserman, 1980). The pig, sheep and rabbit duodenal proteins all have a very high affinity for Ca^{2+} (Bryant & Andrews, 1983), and, since the pig and bovine proteins are very similar in primary structure (Hofmann *et al.*, 1979; Fullmer & Wasserman, 1981), it seems likely that the cation-binding properties of the pig protein (Table 1) are typical of those of the mammalian proteins in general.

Sequence homology suggests that the binding sites in the pig protein are structurally identical with those in the bovine protein, and X-ray analysis has shown that, in the bovine protein, the N-terminal site is a Ca^{2+} -binding loop of 14 amino acid residues, whereas the C-terminal site is a loop of 12 residues, in each case with α -helix at each end (Szebenyi *et al.*, 1981). A structural difference between the two sites on the pig protein is presumably the cause of their different affinities for Mn^{2+} , although there was no significant difference in their affinities for Ca^{2+} , Mg^{2+} or K^{+} . The C-terminal site may be the one with the lower affinity for Mn^{2+} , because it is very similar to the two sites on parvalbumin, and the affinity of these sites for Mn^{2+} , if any, is very much less than their affinity for Ca^{2+} (Cavé *et al.*, 1979).

The mammalian duodenal proteins have major structural features in common with parvalbumin and troponin C (Barker & Dayhoff, 1979), and the binding sites on these have been well characterized (Table 1). On the basis of whether or not they could bind Mg^{2+} competitively with Ca^{2+} under physiological conditions, they have been classified as either $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites or as Ca^{2+} -specific sites respectively. The two types are believed to have different functions, because the latter can be occupied more rapidly by Ca^{2+} when its concen-

Table 1. K_d values for cation-binding to sites on Ca^{2+} -binding proteins

Key to references: a, present work; b, Haiech *et al.* (1979); c, Cavé *et al.* (1979); d, Potter & Gergely (1975); e, Bredderman & Wasserman (1974). Abbreviation: n.a., information not available.

Protein	Site	Nature of site	K_d				Ref.
			Ca^{2+} (nM)	Mg^{2+} (μM)	Mn^{2+} (μM)	K^+ (mM)	
Pig duodenal Ca^{2+} -binding protein	A	$\text{Ca}^{2+}/\text{Mg}^{2+}$	5 ± 1	90 ± 20	0.61 ± 0.02	1.6 ± 0.2	a
	B	$\text{Ca}^{2+}/\text{Mg}^{2+}$	6 ± 2	130 ± 40	50 ± 6	2.1 ± 0.6	a
α -Parvalbumins	Both	$\text{Ca}^{2+}/\text{Mg}^{2+}$	7	~ 20	n.a.	> 10	b
β -Parvalbumins	Both	$\text{Ca}^{2+}/\text{Mg}^{2+}$	3	~ 30	Not bound	> 10	b,c
Troponin C in troponin	Two C-terminal	$\text{Ca}^{2+}/\text{Mg}^{2+}$	2	20	n.a.	n.a.	d
	Two N-terminal	Ca^{2+} -specific	200	5000	n.a.	n.a.	d
Chick duodenal Ca^{2+} -binding protein	All four	Ca^{2+} -specific	500	3600	43	n.a.	e

tration increases than can the former. Ca^{2+} -specific sites may be characteristic of sensor molecules, which respond very quickly to increased Ca^{2+} , and $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites may be characteristic of suppressor molecules, which act more slowly to remove it (Demaille, 1982; Potter & Johnson, 1982). On the basis of previous determinations of their affinity for Ca^{2+} , the sites on mammalian duodenal proteins have been described as Ca^{2+} -specific (Demaille, 1982), but our results indicate that they are $\text{Ca}^{2+}/\text{Mg}^{2+}$ sites instead. In contrast, the vitamin D-dependent Ca^{2+} -binding protein from chick duodenum has affinities for Ca^{2+} and Mg^{2+} which are very similar to those of the Ca^{2+} -specific sites on troponin C (Table 1). The fact that the mammalian proteins retain Ca^{2+} during chromatography and electrophoresis in the absence of added Ca^{2+} (Bryant & Andrews, 1983), whereas the chick protein loses it (S. Critch & P. Andrews, unpublished work), confirms the difference between them. The binding constants for the chick protein indicate that it is capable of more rapid responses to changes in Ca^{2+} concentration than are the mammalian proteins. This difference seems unlikely to be important in duodenal enterocytes, where changes in Ca^{2+} concentration owing to changes in Ca^{2+} absorption occur relatively slowly and the function of both the mammalian and chick proteins is presumably related to the magnitude of the changes rather than to the rate at which they occur. On the other hand, if the chick protein could act as a sensor molecule rather than as a suppressor molecule, this may be why proteins of this type occur in brain cells in both chicks and mammals, whereas proteins of the mammalian type do not.

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