Investigation of the forms of pig duodenal Ca^{2+} -binding protein produced by limited tryptic hydrolysis

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Vitamin D-dependent Ca^{2+} -binding protein from pig duodenum was hydrolysed with trypsin in the presence of Ca^{2+} and two products were obtained: T1, which differed from the native protein by loss of Ac-Ser-Ala-Gln-Lys from the *N*-terminus and Ile-Ser-Gln-OH from the *C*-terminus, and T2, which differed from T1 by loss of a *C*-terminal lysine. The hydrolysis inactivated one of the two high-affinity Ca^{2+} -binding sites on the native protein, and the remaining site was stable in T1 but labile in T2 when the proteins were Ca^{2+} -free. Binding studies showed that T1 had K_d values of 2.8 ± 0.1 nM, 57 ± 13 μM and 0.8 ± 0.3 μM for Ca^{2+} , Mg^{2+} and Mn^{2+} respectively, and T2 had K_d 2.2 ± 0.3 nM for Ca^{2+} . The affinity for Mn^{2+} , together with the other K_d values, identified the site on T1 as the site on the native protein previously found to have K_d 0.6 μM for Mn^{2+} , rather than one with K_d 50 μM for Mn^{2+} . In contrast with both the native protein and another form of the protein with a single Ca^{2+} -binding site, the intrinsic fluorescence of T1 and T2 was little affected by the addition of Ca^{2+} . It was concluded that the active binding site in T1 and T2, and also the site in the native protein with the higher affinity for Mn^{2+} , was probably in the *C*-terminal half of the molecule.

INTRODUCTION

1,25-Dihydroxycholecalciferol stimulates Ca^{2+} absorption, and one effect of its administration is the appearance of a Ca^{2+} -binding protein in the cytoplasm of duodenal enterocytes. A role for the protein in Ca^{2+} absorption is clearly indicated, but its nature is still uncertain (Bishop *et al.*, 1984). The proteins from mammalian species are very similar to one another in molecular mass (M_r 9000) and composition (Wasserman *et al.*, 1978; Bryant & Andrews, 1983), and the sequence of the pig protein (Hofmann *et al.*, 1979) differs in only a few residues from that of the bovine one (Fullmer & Wasserman, 1981). Structural studies (Szebenyi *et al.*, 1981), allied to sequence homology, indicate that in each case the *N*-terminal half of the molecule consists largely of a Ca^{2+} -binding loop of 14 amino acid residues, situated between two helical sections, and the *C*-terminal half of a 12-residue Ca^{2+} -binding loop, also between two helices. The two halves are joined by a linker region.

In the pig protein, both binding sites had K_d values for Ca^{2+} and Mg^{2+} of about 5 nM and 100 μM respectively, but one (A) had K_d 0.6 μM for Mn^{2+} whereas the other (B) had K_d 50 μM (Bryant & Andrews, 1984). Which of the sites was in which half of the molecules was not determined, but the results reported in the present paper indicate a solution to the problem. Trypsin attacked the Ca^{2+} -liganded pig protein at both ends of the polypeptide chain, and affected Ca^{2+} -binding in the two halves of the molecule differently.

MATERIALS AND METHODS

Materials

Vitamin D-dependent Ca^{2+} -binding protein was isolated from pig duodenal mucosa as described by Bryant

& Andrews (1983). The protein was labelled with $^{45}\text{Ca}^{2+}$ by addition of $^{45}\text{Ca}^{2+}$ (1.8 mol/mol of protein) to the Ca^{2+} -free protein. Chymotrypsin-free trypsin (type XIII), di-isopropylfluorophosphate-treated carboxypeptidase A (type I-DFP) and soya-bean trypsin inhibitor (type 1-S) were obtained from Sigma Chemical Co., Poole, Dorset, U.K., DE-52 ion-exchange DEAE-cellulose was from Whatman Biochemicals, Maidstone, Kent, U.K., micropolyamide sheets (type A1700) and 3 M-mercaptoethanesulphonic acid were from Pierce & Warriner (U.K.), Chester, Cheshire, U.K., and Amberlite CG-50 ion-exchange resin (100–200 mesh), dansyl chloride and Aristar-grade Tris were from BDH Chemicals, Poole, Dorset, U.K. Quin 2 (free acid) was obtained from Lancaster Synthesis, Morecambe, Lancs., U.K. and had the properties described by Bryant (1985), and $^{45}\text{CaCl}_2$ and $^{54}\text{MnCl}_2$ were obtained from Amersham International, Amersham, Bucks., U.K.

Tryptic hydrolysis

The production of altered forms of Ca^{2+} -binding protein by limited tryptic hydrolysis was investigated by incubating the protein (0.5 mg) with trypsin (40 μg) at 20 °C in 0.1 ml of buffer (either 20 mM-imidazole, pH 6.1, or 20 mM-Tris/HCl, pH 7.5) containing 0.1 mM- CaCl_2 . Samples (10 μl) were taken at intervals, soya-bean trypsin inhibitor (20 μg) was added, and the mixture was analysed by polyacrylamide-gel electrophoresis.

The effect of tryptic hydrolysis on the stoichiometry of Ca^{2+} -binding was investigated by flow dialysis, performed as described by Bryant & Andrews (1983). The upper chamber of the flow-dialysis cell contained $^{45}\text{Ca}^{2+}$ -labelled binding protein (170 μg) in 20 mM-Tris/HCl buffer, pH 7.5 (0.5 ml), either alone, or after incubation with trypsin (50 μg) for 2 h at 20 °C, or after addition of soya-bean trypsin inhibitor (190 μg), then trypsin (50 μg), and incubation for 2 h.

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Isolation of altered forms of binding protein

The altered forms were isolated from incubations similar to the above, after 2–3 h at either pH, but containing larger amounts of Ca^{2+} -binding protein and the other reagents in proportion. After the addition of trypsin inhibitor, reaction mixtures were dialysed thoroughly against 10 mM-Tris/HCl buffer, pH 7.8, containing 0.1 mM- CaCl_2 and applied to a column (8 cm $\times$ 1 cm) of DE-52 ion-exchange cellulose equilibrated with same buffer. Proteins were eluted by stepwise increases in buffer concentration, then with a Cl^- gradient. Effluent fraction volume was 5 ml, and fractions containing protein were dialysed against deionized water and freeze-dried.

Polyacrylamide-gel electrophoresis

Disc electrophoresis was done with 7% -polyacrylamide gels at pH 8.5, and disc electrophoresis in the presence of sodium dodecyl sulphate and 2-mercaptoethanol with 5% -polyacrylamide gels, as described by Bryant & Andrews (1983). The gels were stained with Coomassie Blue G-250, and after destaining the relative amounts of dye bound to the protein bands were estimated by densitometry.

Amino acid analysis

Proteins were hydrolysed *in vacuo* in 3 M-mercaptoethanesulphonic acid for 24 h (Penke *et al.*, 1974). Amino acid analyses were obtained with a Biotronik automatic analyser type LC 5000 (Biotech Instruments, Hertford, Herts., U.K.).

Identification of N-terminal amino acid residues

N-terminal amino acid residues were determined by dansylation as described by Hartley (1970), with 1 mg (100 nmol) of protein and other quantities increased accordingly. The reaction with dansyl chloride was allowed to proceed overnight at room temperature, then the reaction mixture was dialysed against deionized water for 3 days before the protein was hydrolysed. Dansylated amino acids were identified by t.l.c. on polyamide (Woods & Wang, 1967).

Identification of C-terminal amino acid residues

The proteins (1 mg/ml) were incubated with carboxypeptidase A (15 $\mu\text{g}/\text{ml}$) in 20 mM-Tris/HCl buffer, pH 7.5, at 37 °C and samples were taken at 1, 5 and 18 h. Protein was precipitated with 10% (w/v) sulphosalicylic acid (0.5 vol.), 0.1 M-sodium citrate buffer, pH 2.2 (1 vol.) was added, the precipitate was removed by centrifugation, and 50 μl portions of the supernatant solutions were analysed for amino acids.

Fluorescence measurements

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, and that of quin 2 was excited at 339 nm and measured at 492 nm. Titrations were performed as described by Bryant (1985).

Determinations of K_d values for Ca^{2+} and Mg^{2+} and the stoichiometry of Ca^{2+} -binding

In order to obtain the Ca^{2+} -free proteins, solutions of either T1 or T2 (5 mg) in 20 mM-Tris/HCl buffer, pH 7.5,

Fig. 1. Polyacrylamide gel electrophoresis of pig duodenal Ca^{2+} -binding protein and the products of its limited tryptic hydrolysis

(a) The gels show: A, native protein (NP) and soya-bean trypsin inhibitor (SBTI); B and C, mixtures obtained after tryptic hydrolysis of native protein in the presence of Ca^{2+} for 1 h at pH 6.1 and pH 7.5 respectively, followed by addition of soya-bean trypsin inhibitor. O, origin; T1, T2, main products; P, peptides. (b) and (c) Time course of product formation during tryptic hydrolysis at pH 6.1 and pH 7.5 respectively. $\circ$, Unchanged native protein and trypsin-trypsin-inhibitor complex (not separated); $\bullet$, T1; $\blacktriangle$, T2. Further details are given in the text.

(2.5 ml) were passed through a column (9 cm $\times$ 1 cm) of Amberlite CG-50 ion-exchange resin equilibrated with the same buffer.

K_d values for Ca^{2+} -T1 and Ca^{2+} -T2 complexes and the stoichiometry of the bound Ca^{2+} were determined by

titrating mixtures of quin 2 and either Ca²⁺-free T1 or Ca²⁺-free T2 with Ca²⁺ and measuring the fluorescence of the quin 2 (Bryant, 1985). Apparent K_d values for Ca²⁺ in the presence of Mg²⁺ were determined for T1 in the same way, and used to obtain the K_d for Mg²⁺ by application of the equation:

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

where $[\text{Mg}^{2+}]$ is the concentration of free Mg²⁺ and $K_d^{\text{Ca}^{2+}}$ and $K_d^{\text{Mg}^{2+}}$ are the dissociation constants for Ca²⁺ and Mg²⁺ respectively binding to T1. Total Mg²⁺ concentration instead of free Mg²⁺ concentration was used in the calculations.

Determination of K_d for Mn²⁺

K_d for the Mn²⁺-T1 complex was determined by titrating Ca²⁺-free T1 with ⁵⁴Mn²⁺ in a flow-dialysis cell (Bryant & Andrews, 1984).

RESULTS

Tryptic hydrolysis of Ca²⁺-binding protein and its effect on the stoichiometry of Ca²⁺ binding

Electrophoresis showed that two major products were formed, together with peptides, when the Ca²⁺-liganded binding protein was incubated with trypsin (Fig. 1a), whereas when the Ca²⁺-free protein was used only rapidly migrating peptides were detected. The two major products were designated T1 and T2 respectively in order of increasing electrophoretic mobility. The time-course

Fig. 2. Chromatography of products of limited tryptic hydrolysis of pig duodenal Ca²⁺-binding protein on DE-52 DEAE-cellulose

See the text for details. Peak 1 was eluted with the column equilibration buffer, and further elution was with buffers containing higher Tris concentrations but otherwise similar: A, 15 mM; B, 20 mM; C, 25 mM; D, 30 mM. At E, a gradient of Cl⁻ in buffer D was applied. Fractions were pooled as indicated by the bars, —, A_{230} ; ---, [Cl⁻].

Table 1. Amino acid composition of native Ca²⁺-binding protein and the products of its limited tryptic hydrolysis

The values were obtained as whole numbers as described in the text.

	Composition (no. of residues/molecule)		
	Native protein	T1	T2
Asp	7	7	7
Thr	1	1	1
Ser	7	5	5
Glu	17	15	15
Pro	3	3	3
Gly	4	4	4
Ala	5	4	4
Cys	0	0	0
Val	3	3	3
Met	0	0	0
Ile	3	2	2
Leu	10	10	10
Tyr	1	1	1
Phe	5	5	5
His	0	0	0
Lys	10	9	8
Arg	1	1	1
Trp	0	0	0

of their formation (Figs. 1b and 1c) showed that, at pH 6.1, T1 was formed from the native protein in good yield before much T2 appeared. At pH 7.5, after the native protein had disappeared, the yield of T2 increased while that of T1 decreased. The results indicate that T1 was formed directly from the native protein whereas T2 was formed mainly, if not entirely, by hydrolysis of T1. When T1 was isolated as described below and the Ca²⁺-liganded form incubated with trypsin at pH 7.5 it was converted into T2, the amount of which then remained unchanged.

When binding protein labelled with ⁴⁵Ca²⁺ was placed in the upper chamber of a flow-dialysis cell the equilibrium activity in the buffer flow was 300 c.p.m., but when labelled protein which had been incubated with trypsin for 2 h was placed in the chamber instead the activity in the buffer rose to 29000 c.p.m. in 2 min and then declined slowly as activity was lost from the chamber. After the addition of 100 mM-CaCl₂ (20 μ l) to displace protein-bound ⁴⁵Ca²⁺, the activity rose to 64000 c.p.m., then declined slowly. When the cell contained labelled protein which had been incubated with trypsin in the presence of trypsin inhibitor, the equilibrium activity in the buffer flow was again 300 c.p.m., but it rose to 64000 in 2 min after the addition of a large excess of Ca²⁺. The results indicate that about half the ⁴⁵Ca²⁺ bound to native protein was released by tryptic hydrolysis.

Isolation of T1 and T2

Fractionation of the protein mixture obtained by incubating Ca²⁺-liganded binding protein (48 mg) with trypsin at pH 6.1, followed by addition of excess soya-bean trypsin inhibitor, is shown in Fig. 2. Electrophoresis of material from the pooled fractions indicated that T1 (peak 2; 16 mg) and T2 (peak 3;

Table 2. Amino acid residues liberated by treatment with carboxypeptidase A from native Ca^{2+} -binding protein and the products of its limited tryptic hydrolysis

The values are averages from two experiments. Further details are given in the text.

Incubation time (h) . . .	Amino acids liberated by carboxypeptidase A (mol/mol of protein)		
	1	5	18
Native protein	Gln, 0.3; Ser, Ile, 0.1	Gln, 0.6; Ser, 0.3; Ile, 0.3	Gln, 0.8; Ser, 0.4; Ile, 0.3; Lys, Val, Leu, < 0.1
T1	Lys, 0.9; Val, 0.6; Leu, 0.4; Gln, 0.3; others, < 0.1	Lys, 1.3; Val, 0.8; Leu, 0.7; Gln, Phe, Glu, 0.5; others, < 0.2	Lys, 2.0; Val, 1.3; Leu, 1.2; Gln, 1.1; Phe, Glu, 1.0; Ser, 0.5; others, < 0.5
T2	Lys, 0.5; Val, Leu, 0.4; Gln, 0.3; others, < 0.1	Lys, 0.7; Val, 0.6; Leu, Gln, Phe, 0.5; others, < 0.3	Lys, 1.1; Val, 0.9; Leu, Gln, Phe, Glu, 0.8; Ser, 0.5; others, < 0.5

5.5 mg) were obtained free from other proteins. Unchanged binding protein (peak 1) and trypsin-trypsin-inhibitor complex (peak 4) have similar electrophoretic mobilities, but they were widely separated by chromatography. Excess trypsin inhibitor was eluted at 180 mM- Cl^- .

Products T1 and T2 gave single bands on electrophoresis in the presence of sodium dodecyl sulphate, with migration rates similar to that of the native protein.

Amino acid composition

The native binding protein, T1 and T2 were each hydrolysed and analysed four times. The native protein does not contain cysteine (see Fig. 5) and no precautions were taken to stabilize cysteine in the hydrolyses. Each protein contained tyrosine and arginine, and calculations were based on the assumption that, like the native protein, T1 and T2 contained one residue of each. The results were averaged and then rounded-off to the nearest whole number for each amino acid (Table 1). The analysis of the native protein agreed with the composition based on sequence (Fig. 5), except that three residues of proline were found instead of four. The reason for the difference is not known, but since three residues of proline were also found in T1 and T2 it was concluded that no proline was lost in their formation. The analyses showed that formation of T1 from the native protein involved the loss of two serine residues, two glutamine or glutamic acid, one alanine, one isoleucine and one lysine, and formation of T2 from T1 involved the loss of one lysine residue.

Values for $A_{1\text{cm}}^{1\%}$ obtained when amino acid analyses were used as quantitative measurements of protein concentration were 2.2 for T1 and 3.3 for T2.

Fig. 3. Binding of Ca^{2+} to products T1 and T2 from limited tryptic hydrolysis of pig duodenal Ca^{2+} -binding protein

(a) Experimental points for the binding of Ca^{2+} to T1 (○) and T2 (●) in the absence of a competing cation, and (△) to T1 in the presence of 100 mM- Mg^{2+} . The left-hand curve was drawn with a K_d for Ca^{2+} of 2.61 nM and the right-hand one with an apparent K_d for Ca^{2+} of 5.02 μM . (b) Graph of values for apparent K_d for Ca^{2+} binding to T1 in the presence of Mg^{2+} , against total Mg^{2+} concentration.

Fig. 4. Binding of Mn^{2+} to product T1 from limited tryptic hydrolysis of pig duodenal Ca^{2+} -binding protein

The experimental points (○) are for one experiment and the curve was drawn with a K_d of $0.85 \mu\text{M}$.

Terminal amino acid residues

The results of the *N*-terminal amino acid determinations were: native protein, none detected; T1, serine; T2, serine.

The results of the *C*-terminal amino acid determinations are shown in Table 2. Both T1 and T2 were hydrolysed

more rapidly and to a greater extent than was the native protein, and the liberated amino acids showed that they differed from the native protein at the *C*-terminal end. The mixtures of amino acids liberated from T1 and T2 were similar, except that the proportion of lysine was greater from T1 than T2. To some extent the yields of amino acids from T1 and T2 at the larger incubation times were irregular, as compared with their sequences. This suggests that different peptide bonds in molecules of the same protein were hydrolysed at different rates.

K_d values for Ca^{2+} and Mg^{2+}

Measurements of Ca^{2+} bound to T1 and T2 in the absence of a competing cation, determined by the method with quin 2, are shown in Fig. 3(a). Three determinations with each protein gave mean K_d values for Ca^{2+} of $2.8 \pm 0.1 \text{ nM}$ and $2.2 \pm 0.3 \text{ nM}$ for T1 and T2 respectively. The results also showed that T1 and T2 bound 1.0 and 0.8 mol of Ca^{2+} /mol respectively when the titrations were performed with the freshly-prepared Ca^{2+} -free forms, but whereas Ca^{2+} -free T1 retained its binding activity on storage, Ca^{2+} -free T2 lost its binding activity in less than 2 days.

When T1 was titrated with Ca^{2+} in the presence of Mg^{2+} there was competition between the two cations (Fig. 3a) and apparent K_d values for Ca^{2+} were obtained. These values gave a linear graph (Fig. 3b) when plotted according to eqn. (1), the slope of which $[(4.89 \pm 0.26) \times 10^{-5}]$, combined with the K_d for Ca^{2+} , gave a K_d for Mg^{2+} of $57 \pm 13 \mu\text{M}$.

K_d value for Mn^{2+} binding to T1

A typical result for the binding of Mn^{2+} to T1 is shown in Fig. 4. Three determinations gave a K_d for Mn^{2+} of $0.8 \pm 0.3 \mu\text{M}$.

Fig. 5. Product formation by limited tryptic hydrolysis of pig duodenal Ca^{2+} -binding protein

The amino acid sequence of the native protein was taken from Hofmann *et al.* (1979). Possible points of attack by trypsin are indicated by vertical arrows and the sequences of the tryptic products T1 and T2 by horizontal arrows.

Effect of Ca^{2+} on the intrinsic fluorescence of T1 and T2

Both T1 and T2 had fluorescence spectra typical of proteins containing tyrosine and no tryptophan, but whereas the fluorescence of the native protein increased by 50% when it was saturated with Ca^{2+} (Bryant & Andrews, 1983), that of Ca^{2+} -free T1 increased by only 6% and that of freshly-prepared Ca^{2+} -free T2 showed no change in the presence of excess Ca^{2+} .

DISCUSSION

The Ca^{2+} -binding protein from pig duodenum contains many trypsin-sensitive bonds (Hofmann *et al.*, 1979) but electrophoresis in the presence of sodium dodecyl sulphate indicated that both T1 and T2, the products of limited tryptic hydrolysis, contained most of the polypeptide chain intact. Since the amino acid sequence of the native protein is known, the sequence of both products is readily deduced from the results of amino acid analyses. As shown in Fig. 5, the seven-residue difference (two serine, two glutamine, one alanine, one lysine and one isoleucine) between native protein and T1 is only accounted for by the loss of a small tryptic peptide from each end of the native polypeptide chain, Ac-Ser-Ala-Gln-Lys from the *N*-terminus and Ile-Ser-Gln-OH from the *C*-terminus. Therefore T1 consists of residues 5–75 of the native protein. Similarly, the only difference in composition between T1 and T2 is one lysine residue, therefore T2 consists of residues 5–74 of the native protein. The results are consistent with the determinations of terminal amino acid residues, which showed that changes occurred at both ends of the polypeptide chain, and with the sequential formation of T1 and T2 from the native protein.

The residues removed by trypsin were too far along the polypeptide chain from both the Ca^{2+} -binding sites, which probably consist of residues 17–30 and 57–68 respectively, to indicate which site was most likely to be affected in its Ca^{2+} -binding properties. However, since removal of the *C*-terminal lysine residue from T1 resulted in a marked change in the stability of the remaining binding site in the Ca^{2+} -free protein, it seems likely that this site was *C*-terminal also. Addition of Ca^{2+} had little or no effect on the intrinsic fluorescence of T1 and T2, whereas that of the native protein was increased by 50% and that of another form of the protein with a single binding site by 32% (Bryant & Andrews, 1983). The chromophore, Tyr-16, is immediately adjacent to the *N*-terminal binding site, so if the relative magnitudes of the fluorescence changes indicate the proximity of bound Ca^{2+} to the chromophore, the conclusion again is that the active binding site in T1 and T2 is *C*-terminal. Although the binding sites on the native protein were not distinguishable by their K_d values for Ca^{2+} and Mg^{2+} , one (A) had K_d 0.6 μM whereas the other (B) had K_d 50 μM for Mn^{2+} (Bryant & Andrews, 1984). The three K_d values for T1 (K_d 2.8 $\pm$ 0.1 nM, 57 $\pm$ 13 μM and 0.8 $\pm$ 0.3 μM for Ca^{2+} , Mg^{2+} and Mn^{2+} respectively) are not appreciably different for those for site A and on that account we think that site A is in the *C*-terminal half of the molecule.

N.m.r. studies with the pig protein have shown that a well-defined structure exists even in the absence of Ca^{2+} , but structural stability is enhanced when Ca^{2+} is present (Shelling *et al.*, 1983). Proteolysis is a sensitive

probe of protein structure (Rupley, 1967), and the resistance of the Ca^{2+} -liganded pig protein to tryptic hydrolysis, as compared with the extensive proteolysis of the Ca^{2+} -free forms, is consistent with the Ca^{2+} -liganded proteins having much the more compact and rigid structure. Even Ca^{2+} bound in one half of the molecule in T2 still made the rest of the molecule resistant to trypsin, possibly through interhelix contacts such as those found in the bovine protein (Szebenyi *et al.*, 1981).

The bovine protein suffered only limited tryptic hydrolysis in the presence of Ca^{2+} and yielded two products, like the pig protein, but according to Fullmer *et al.* (1975) the binding of Ca^{2+} was unaffected. The structures of the products from the bovine protein were not determined, but on the basis of similar electrophoretic mobilities they were thought to be the same as two altered forms of the bovine protein ('minor A' and 'minor B') which were formed during purification and storage by hydrolysis at two adjacent lysine residues near the *N*-terminal end and loss of three and four amino acid residues respectively (Fullmer & Wasserman, 1977). The sequences of minor A and minor B were otherwise identical with that of the native protein (Fullmer & Wasserman, 1980), and identical with that of the native pig protein at the *C*-terminal end for nearly 30 residues, apart from two differences in amidation (Fullmer & Wasserman, 1981). It seems unlikely therefore that they arose by tryptic action, or are identical with the products of limited tryptic hydrolysis. If the native pig and bovine proteins have the same high affinity and stoichiometry for Ca^{2+} , as seems probable, then the report that minor A binds 2 mol of Ca^{2+} /mol with a K_d of 0.23 μM (Fullmer & Wasserman, 1980) indicates an alteration of binding properties in its formation which is different from that resulting from tryptic hydrolysis of the pig protein. Altered forms of the pig protein also arise on storage, but their formation is unaffected by the presence of soya-bean trypsin inhibitor or a variety of other proteinase inhibitors (S. Critch & P. Andrews, unpublished work). Their nature remains to be determined.

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