67 kDa calcimedin, a new Ca^{2+} -binding protein

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A set of four proteins, termed calcimedins, are isolatable from smooth, cardiac and skeletal muscle by using a fluphenazine–Sephacryl affinity column. The calcimedins show apparent M_r values of 67 000, 35 000, 33 000 and 30 000 by SDS/polyacrylamide-gel electrophoresis. The 67 000- M_r calcimedin (67 kDa calcimedin) has now been purified to homogeneity by using DEAE-cellulose chromatography followed by Ca^{2+} -dependent binding to phenyl-Sepharose. The amino acid analysis of the 67 kDa calcimedin shows this protein does not contain trimethyl-lysine but does contain 2 mol of tryptophan/mol of protein. The 67 kDa calcimedin shows positive ellipticity in the near-u.v. range with c.d. Ca^{2+} -binding studies indicate one high-affinity Ca^{2+} -binding site with K_d 0.4 μM . The data show that the 67 kDa calcimedin is distinct from other Ca^{2+} -binding proteins described to date.

INTRODUCTION

A newly described set of Ca^{2+} -dependent fluphenazine–Sephacryl-binding proteins (Moore & Dedman, 1982a) initially isolated from smooth muscle show apparent M_r values of 67 000, 35 000, 33 000 and 30 000 when compared with rabbit skeletal-muscle myofibrillar proteins by electrophoresis on 10% SDS/polyacrylamide gels. A similar set of proteins were also isolated from rabbit longissimus dorsi muscle, but not from either rat or hamster brain tissue or from 20 mosm erythrocyte lysates.

These proteins, termed calcimedins (Moore & Dedman, 1982b), interact with several hydrophobic ligands coupled to a polysaccharide support. These include the phenothiazine-ring-coupled Affi-Gel (Bio-Rad Laboratories), W-7 (a substituted naphthalene-sulphonamide described as a calmodulin antagonist; Hidaka *et al.*, 1981) coupled to Sepharose, the Shaltiel C_6 and C_8 alkyl chains coupled to agarose (Miles Research Laboratories) and both phenyl- and octyl-Sepharose affinity resins (Sigma Chemical Co.). These proteins do not bind to a Sepharose column without proper ligand or to a calmodulin–Sephacryl affinity matrix.

Importantly, the interaction of the calcimedins with these matrices is always Ca^{2+} -dependent and hence it is likely to involve a conformational shift induced by the prior binding of micromolar Ca^{2+} . This type of interaction has been documented previously for both calmodulin (Dedman *et al.*, 1977; Seamon, 1980) and troponin C (Iida, 1979; Tanaka & Hidaka, 1981).

The 67 kDa calcimedin, unlike calmodulin, contains tryptophan residues. Microenvironment perturbations occur with Ca^{2+} titration that are consistent with a conformational change in the molecule induced by micromolar Ca^{2+} .

EXPERIMENTAL

Isolation of the 67 kDa calcimedin

Total calcimedin proteins were isolated from 350 g of chicken gizzard tissue by homogenization, in a Waring

blender, in 700 ml of buffer composed of 75 mM-NaCl/40 mM-Tris/HCl buffer, pH 7.3, containing 0.1% NaN_3 , 2 mM-EDTA, 0.1 mM-phenylmethanesulphonyl fluoride and 1 mg each of the proteinase inhibitors aprotinin, leupeptin and pepstatin (Sigma Chemical Co.). All procedures were done at 4 °C. The homogenate was centrifuged at 7000 rev./min for 20 min in a JS 7.5 rotor (Beckman). The supernatants were again recovered and filtered through several layers of cheesecloth. An additional 1 mg of leupeptin was added and the fluid was made 1 mM with respect to CaCl_2 (final concentration above the EDTA concentration). The extract was further clarified by centrifugation at 14000 rev./min in the JA 20 rotor followed by filtration through glass-wool. The clarified extract was then chromatographed on a fluphenazine–Sephacryl matrix (1.5 cm $\times$ 6.5 cm) previously equilibrated with 75 mM-NaCl/40 mM-Tris/HCl buffer, pH 7.3, containing 0.1% NaN_3 and 1 mM- CaCl_2 . When the A_{280} of the effluent reached 0.01 or less, the column was washed with 0.5 M-NaCl/40 mM-Tris/HCl buffer, pH 7.3, containing 0.1% NaN_3 and 1 mM- CaCl_2 . The A_{280} was again allowed to return to baseline. The NaCl concentration was lowered to 75 mM, and Ca^{2+} -dependent binding proteins were eluted with 1 mM-EGTA in 75 mM-NaCl/40 mM-Tris/HCl buffer, pH 7.3, containing 0.1% NaN_3 . The protein peak was pooled and dialysed against 10 mM-imidazole/1 mM-EDTA buffer, pH 6.4 (three changes over 4 h), to lower the NaCl content for binding to a DEAE-cellulose column (1.5 cm $\times$ 25 cm) equilibrated with 10 mM-imidazole/10 mM-NaCl/1 mM-EDTA buffer, pH 6.4. The calcimedin proteins were eluted by a 10–120 mM-NaCl gradient in 10 mM-imidazole/1 mM-EDTA buffer, pH 6.4. Calmodulin was separated from the calcimedins at this step, since it is not eluted until NaCl concentrations above 0.25 M are reached. The 67 kDa calcimedin peak (approximate NaCl concentration 80 mM) was pooled, concentrated by Ca^{2+} -dependent binding to a phenyl-Sepharose column (0.7 cm $\times$ 8 cm) and dialysed against the EGTA/NaCl/Tris buffer. Any low- M_r contaminants were removed by chromatography

Abbreviation used: W-7, *N*-(6-aminoethyl)-5-chloronaphthalene-1-sulphonamide.

on a Sephadex G-200 column in the same buffer. If a buffer change was required, it was done at this step (the protein was dialysed against the new buffer followed by chromatography in the new buffer).

Preparation of affinity column

Fluphenazine-Sepharose was prepared by the method described by Charbonneau & Cormier (1979). Fluphenazine dihydrochloride was generously given by E. R. Squibb and Sons (Princeton, NJ, U.S.A.). Maximal coupling was determined to be 50% on the basis of the A_{485} value. The protein-binding capacity of the fluphenazine-Sepharose column is 5.2 mg of protein/ml of conjugated resin and was determined with purified chicken gizzard calmodulin.

The calmodulin-Sepharose affinity matrix was prepared by suspending 1.5 g of CNBr-activated Sepharose 4B (Pharmacia) in 10 ml of a purified chicken gizzard calmodulin solution (Dedman *et al.*, 1977) (3 mg/ml in 0.1 M-NaCl/0.1 M-NaHCO₃ buffer, pH 8.5). The suspension was gently mixed by inversion at 4 °C for 16 h, washed thoroughly and resuspended in 10 ml of this same buffer. Then 2 ml of 2-aminoethanol was added to block any remaining binding sites. Mixing was continued for an additional 5 h. The coupled resin was then washed with the NaHCO₃ buffer, with 0.2 M-glycine buffer, pH 2.5, and finally with 75 mM-NaCl/40 mM-Tris/HCl buffer, pH 7.3, containing 0.1% NaN₃ and 1 mM-CaCl₂. Protein concentrations were determined by the Bradford (1976) method and showed coupling was greater than 95%.

Amino acid analysis

Amino acid analyses were performed on a Dionex-500 high-pressure ion-exchange amino acid analyser by Dr. Robert McGregor at the Analytical Center, University of Texas Medical School, Houston, TX, U.S.A. Three samples of the 67 kDa calcimedlin between 0.5 and 0.8 mg each were dialysed extensively against distilled water, freeze-dried and hydrolysed under N₂ at 110 °C for 24 h in 6 M-HCl containing 0.2% (w/v) phenol. The values obtained were averaged and expressed as mol/100 mol of residues. Tryptophan was determined by the colorimetric assay described by Basha & Roberts (1977). Two samples of 0.8 and 0.9 mg of 67 kDa calcimedlin were hydrolysed in 5 mM-KOH and the tryptophan contents were determined by complex-formation with dye. The colours developed in the hydrolysates were compared with a standard curve generated with authentic L-tryptophan. Samples of pure amino acid as well as weighed quantities of bovine serum albumin and transferrin were also hydrolysed and determined at the same time to allow for correction due to possible losses encountered in the procedures. Over 90% of the expected tryptophan in these standard samples was found with this method.

Buffer preparation for cation titration

A 0.1 M-KCl/40 mM-Mops/KOH buffer, pH 7.0, was freed of Ca²⁺ by passage through a column of 50 g of Chelex (K⁺ form) (Sigma Chemical Co.). Atomic absorption of the buffer showed it contained less than 0.1 μM-calcium. A stock of 0.5 M-EGTA solution was prepared from weighed oven-dried chemical dissolved in Chelex-treated distilled water and adjusted to pH 7.0 with KOH. Atomic absorption of this solution also

showed it contained less than 0.1 μM-calcium. A 10 mM dilution of an Orion Ca²⁺ standard with the KCl/Mops buffer was used for the titrations.

Near-u.v. c.d.

Near-u.v. c.d. spectroscopy was performed on a Cary 60 instrument in the laboratory of Dr. Kenneth Breslauer, Rutgers University, New Brunswick, NJ, U.S.A. A 1 cm rectangular cell was used at a constant temperature of 25 °C. N₂ flow was 1.1 m³/h (40 ft³/h) at 100 kPa (15 lbf/in²) gauge pressure. The region between 250 and 300 nm was scanned after each addition. Volume changes were minimized by using a 10 mM concentration of CaCl₂; each step resulted in a volume change of less than 0.4%. However, measurements were corrected for actual volumes. The noise level was no more than 5% of the signal at wavelengths above 240 nm, although was considerably greater than 5% below 220 nm. The molar-absorptivity calculation used the estimated M_r for the 67 kDa calcimedlin by SDS/polyacrylamide-gel electrophoresis. The final calcium concentration in the protein solution was also determined by atomic absorption. The change in the molar absorptivity was determined by using the 0.0 and 52 μM-CaCl₂ curves.

Ca²⁺-binding studies

Ca²⁺ binding was measured by using the gel-filtration method described by Hummel & Dreyer (1962) (see also Akers, 1973). ⁴⁵CaCl₂ (27.1 Ci/g; New England Nuclear) was diluted with non-radiolabelled CaCl₂ at a ratio of 1:1000. Solutions ranging from 0.1 μM to 20 μM free Ca²⁺ were prepared from stock solutions of 1 mM-CaCl₂ in 0.1 M-KCl/40 mM-Mops/KOH buffer, pH 7.0, and 1 mM-EGTA in 0.1 M-KCl/40 mM-Mops/KOH buffer, pH 7.0 (Moore & Dedman, 1982a). The pH of the mixtures did not vary more than 0.02 pH unit. Purified 67 kDa calcimedlin was dialysed extensively against the above buffer containing 10 μM-EGTA followed by dialysis against buffer without EGTA. The protein was then chromatographed on a Sephadex G-200 column in the EGTA-free buffer. Peak tubes were pooled and 0.5 ml samples containing 74 μg of 67 kDa calcimedlin/ml were placed into separate dialysis sacs. Each sample was equilibrated for 2 h at 4 °C against 0.1 μM-, 0.5 μM-, 1.0 μM-, 10 μM- and 20 μM-CaCl₂ (1:1000 ⁴⁵CaCl₂) solutions buffered with EGTA in 0.1 M-KCl/50 mM-Mops/KOH buffer, pH 7.0, containing 0.1 M-MgCl₂. Each sample was then chromatographed on a Sephadex G-25 column (8 ml) pre-saturated at the same Ca²⁺ concentration as the sample. Fractions (0.25 ml) were collected and 50 μl portions were taken for liquid-scintillation counting of radioactivity. The amount of ⁴⁵Ca²⁺ bound was determined by measuring the area of the peak of radioactivity eluted from the column above the saturating baseline.

Analytical procedures

Protein concentrations were determined with the Pierce BCA Protein Assay Reagent (Pierce Chemical Co.). Denaturing SDS/polyacrylamide-gel electrophoresis in slab gels followed the procedure of Laemmli (1970), with a 10% or 12.5% running gel and 4.5% stacking gel. Gels were stained with 0.025% Coomassie Brilliant Blue R-250 in 25% (v/v) propan-2-ol/10% (v/v) acetic acid. Gels were destained with 10% (v/v) propan-2-ol in 7.5% (v/v) acetic acid.

Fig. 1. SDS/polyacrylamide-gel electrophoresis of calmodulin and calcimedins from chicken heart

Lane 1, 0.4 M-NaCl-eluted protein from DEAE-cellulose; 3 μ g of protein loaded. Lane 2, 0.1 M-NaCl-step-elution peak from DEAE-cellulose; 14 μ g of protein. Lane 3, 80 M-NaCl-gradient-elution peak from DEAE-cellulose re-bound and eluted from phenyl-Sepharose; 2.8 μ g of protein.

RESULTS

The calcimedins, together with calmodulin, are isolated from tissue homogenates by Ca^{2+} -dependent binding to fluphenazine-Sepharose (Moore & Dedman, 1982a). All four calcimedins bind DEAE-cellulose at NaCl concentrations lower than 30 mM and are eluted with 0.1 M-NaCl. Calmodulin (Fig. 1, lane 1) and the calcimedins (Fig. 1, lane 2) are completely separated from each other. In contrast, if a linear NaCl gradient is used, each calcimedlin can be isolated separately from the other calcimedins (see Fig. 2). The four proteins are eluted in the order: 33 kDa protein (50 mM), 30 kDa protein (70 mM), 67 kDa protein (80 mM) and 35 kDa protein (0.10 M). Again, all four calcimedins are eluted before calmodulin. Each calcimedlin can then be re-bound to the fluphenazine-Sepharose matrix by addition of 1 mM- CaCl_2 . (When phenyl-Sepharose was substituted for the fluphenazine-Sepharose column, the 33 kDa calcimedlin did not bind whereas the other three calcimedins did.) Thus the 67 kDa calcimedlin was purified by Ca^{2+} -dependent binding to the fluphenazine-Sepharose matrix followed by NaCl elution from

Fig. 2. SDS/polyacrylamide-gel electrophoresis of selected fractions across the NaCl-gradient-eluted DEAE-cellulose column

Lanes 1-6, peak fractions corresponding to NaCl concentrations of 50 mM, 60 mM, 70 mM, 75 mM, 80 mM and 90 mM. Lane 7, standard lane containing the total calcimedins and calmodulin, for comparison.

DEAE-cellulose. Fractions from the DEAE-cellulose column were electrophoresed and those containing the 67 kDa calcimedlin were pooled. The purified protein was concentrated by binding to and elution from the phenyl-Sepharose column (Fig. 1, lane 3).

The amino acid analysis for the 67 kDa calcimedlin is shown in Table 1 and represents an average of three determinations. Notable differences between the calcimedlin and calmodulin are the presence of tryptophan, which is not found in calmodulin, and the lack of trimethyl-lysine. This modified residue has been found in all calmodulins with two exceptions: that from *Dictyostelium* (Bazari & Clarke, 1981) and that from octopus (Molla *et al.*, 1981).

C.d. can be used to detect conformational changes since c.d. spectra are highly dependent on the environment of the tryptophan, tyrosine, phenylalanine and cystine residues. K^+ and Ca^{2+} do not have intrinsic near-u.v.

Table 1. Amino acid analysis of 67 kDa calcimedin

The analysis was performed as indicated in the Experimental section. Abbreviation: N.D., not determined.

Amino acid	Composition (mol of residue/100 mol of total residues)
Asx	9.5
Thr	4.6
Ser	8.7
Glx	15.7
Pro	2.9
Gly	11.7
Ala	9.8
Val	3.8
Met	1.8
Ile	2.8
Leu	6.1
Tyr	2.6
Phe	3.7
Trp*	0.8
Lys	9.0
Me ₃ Lys	0.0
His	4.3
Arg	2.3
Cys	N.D.

* Determined by the method of Basha & Roberts (1977).

absorption; binding of these cations as detected by near-u.v. spectra can be interpreted to result from conformational changes in the molecule and/or changes in the signal of aromatic residues that are located at the binding site (Strickland, 1974). The near-u.v. spectra between 250 and 300 nm for the 67 kDa calcimedin is shown in Fig. 3. The protein shows a predominantly positive molar absorptivity, unlike that previously reported for calmodulin (Crouch & Klee, 1980), troponin C (Kay *et al.*, 1974) and S-100a protein (Mani & Kay, 1983). Ca²⁺ titration was monitored by scanning from 300 to 600 nm after each addition (2 μ l of a 10 mM-CaCl₂ stock solution). The difference spectra calculated between 0.0 and 52 μ M-CaCl₂ is shown in the upper portion of the Figure. Ca²⁺ addition affected both tyrosine and tryptophan groups.

Ca²⁺ binding was determined by the gel-filtration method of Hummel & Dreyer (1962) since the calcimedins are relatively low-abundance proteins (less than 1% of the total protein; the concentration of the 67 kDa calcimedin in heart is approx. 50 mg per kg). Binding studies with ⁴⁵Ca²⁺ showed that the 67 kDa calcimedin bound Ca²⁺ with a stoichiometry of approx. 1:1 with a *K_d* of 0.4 μ M (Fig. 4). The data indicate a single high-affinity Ca²⁺-binding site.

DISCUSSION

The calcimedins are a group of proteins, originally isolated from smooth muscle, which appear to have a hydrophobic binding domain exposed by the presence of Ca²⁺. The binding to a variety of ligands is always Ca²⁺-dependent and is unaffected by a wide range of ionic strength and pH conditions, although binding can be precluded by heat treatment for 5 min at 90 °C

Fig. 3. Near-u.v. c.d. of 67 kDa calcimedin

(b) —, Curve in the presence of 0.0 CaCl₂; ·····, curve in the presence of 26.1 μ M-CaCl₂; ----, curve in the presence of 52.4 μ M-CaCl₂. (a) Difference spectrum obtained between the — and ---- curves in (b).

Fig. 4. Ca²⁺-binding activity of the 67 kDa calcimedin

The reciprocal of the free Ca²⁺ concentration is plotted versus the molar ratio of the 67 kDa calcimedin to bound Ca²⁺.

(Moore & Dedman, 1982a). We have suggested that this Ca^{2+} -induced hydrophobic site was a significant feature of the mode of action of these proteins based on their ability to stimulate hepatic microsomal Ca^{2+} -dependent ATPase activity and to inhibit calmodulin activation of cyclic nucleotide phosphodiesterase (Moore *et al.*, 1984). The data in the present paper not only show that 67 kDa calcimedin is significantly different from calmodulin but also suggest this calcimedin does have a Ca^{2+} -induced conformational change that may be related to its physiological role.

Previous experiments with calmodulin (Crouch & Klee, 1980), troponin C (Kay *et al.*, 1974) and the S-100a protein (Mani & Kay, 1983) indicate that all these proteins exhibit altered phenylalanine and tyrosine absorptivity with Ca^{2+} addition. These changes are related to the conformational shift that occurs in response to Ca^{2+} binding. The 67 kDa calcimedin also shows perturbations for these residues as well as the tryptophan residues. The inability to detect dimer (or other oligomer) formation in the presence of Ca^{2+} (Moore *et al.*, 1984), together with the differences in cleavage fragments obtained with the serine proteinase from myofibrils (Sohar *et al.*, 1986), suggests that the c.d. changes probably result from the conformational change.

Südhof *et al.* (1984) reported that several proteins could be isolated by Ca^{2+} -induced binding to cellular particulate material coupled with Ca^{2+} -dependent binding to phenyl-Sepharose. The isolated proteins have now been identified as being identical with those isolated by Geisow and co-workers (Geisow & Burgoyne, 1982; Geisow *et al.*, 1984) by reactivity of antibody to calelectrin. Although the estimates of isoelectric point (5.3) and Stokes radius (3.7 nm) determined for the 67 kDa calcimedin are similar, identity is not supported by the amino acid composition reported here, which is appreciably different from those of the calelectrins. The protein isolated from brain tissue (calelectrin source) does not cross-react with antibody directed to the smooth-muscle 67 kDa calcimedin (Rhoads *et al.*, 1985). In addition, no evidence of the generation of a 35 kDa fragment has been detected by proteolysis of the 67 kDa calcimedin by trypsin, chymotrypsin or V8 proteinase nor by the myofibrillar serine proteinase (results not shown).

Another protein of similar M_r (68000) has been isolated from lymphocytes (Owens & Crumpton, 1984). Although this protein was not shown to contain either proline or tryptophan, the amino acid composition is otherwise similar. In addition, both the lymphocyte protein and the 67 kDa calcimedin show one high-affinity Ca^{2+} -binding site with similar K_d values ($0.4 \mu\text{M}$ compared with $1.0 \mu\text{M}$). The 67 kDa calcimedin has not as yet been localized in lymphocytes, but it does appear to be associated with the limiting membrane in macrophages (Moore & Morse, 1986, and unpublished work).

The isolation procedure for the calcimedins incorporates proteinase inhibitors for metallo-, serine and thiol enzymes of the cathepsin type. Since the Ca^{2+} -activated proteinase (calpain) does not bind leupeptin except in the presence of Ca^{2+} , a second quantity is added at the time the Ca^{2+} concentration is raised. Despite the addition of these several proteinase inhibitors, the failure of the 33 kDa calcimedin to bind the smaller phenyl ring structure while retaining the ability to bind the fluphenazine-Sepharose matrix suggests an alteration in

the binding domain could be due to limited proteolysis during the isolation procedures. The 35 kDa calcimedin can be converted into the 33 kDa protein by V8 proteinase digestion. Incorporation of pepstatin into the isolation scheme results in diminished amounts of the 33 kDa protein (Fig. 1, lane 2).

A protein of M_r approx. 67000 has been isolated from heart mitochondria by using a calmodulin-Sepharose affinity column (Pardue & Dedman, 1984). Neither the 67 kDa calcimedin nor its antibody appear to have any effect on mitochondrial ATPase or transport activities (P. B. Moore, unpublished work). It is unlikely, therefore, that these proteins are related. Additional support for this belief is that the calcimedins do not bind to a calmodulin-Sepharose affinity matrix.

The 67 kDa calcimedin exhibits a hydrophobic binding domain that is responsive to the prevailing Ca^{2+} concentration. This type of interaction has been well documented for several other Ca^{2+} -mediatory proteins. The Ca^{2+} -activated neutral proteinase has now been shown to respond to the Ca^{2+} concentration by a conformational change (Suzuki *et al.*, 1981). Thus this mode of action may be a more universal method used to regulate the physiological role of several proteins in addition to those belonging to the calmodulin family of proteins.

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