Purification and spectral studies on the Ca^{2+} -binding properties of 67 kDa calcimedlin

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The 67 kDa calcimedlin, isolated by using a phenyl-Sepharose affinity column followed by DEAE-cellulose and gel-filtration chromatographies, was homogeneous by the criterion of SDS/polyacrylamide-gel electrophoresis. In non-SDS gels, the protein moved faster in the presence of EDTA, suggesting that Ca^{2+} binding affects its mobility in a manner similar to other Ca^{2+} -binding proteins such as calmodulin and S-100 proteins. The 67 kDa protein underwent a conformational change upon binding Ca^{2+} , as revealed by u.v. difference spectroscopy and near-u.v. c.d. measurements. Tryptophan and tyrosine residues were perturbed upon Ca^{2+} binding, moving to a more non-polar environment in the presence of Ca^{2+} . Upon excitation of the protein at 280 nm, the fluorescence emission maximum was centered around 325 nm, suggesting that the tryptophan residues are located in a fairly hydrophobic region. Ca^{2+} addition did not induce a significant change in the intrinsic protein fluorescence intensity at 325 nm. Addition of Ca^{2+} to the 67 kDa protein labelled with 2-*p*-toluidinylnaphthalene-6-sulphone (TNS) resulted in a 25% increase in fluorescence intensity, accompanied by a blue shift of the emission maximum from 442 to 432 nm. Hence, the probe in the presence of Ca^{2+} moves to a more non-polar microenvironment, like calmodulin and other Ca^{2+} -binding proteins. Fluorescence titration with Ca^{2+} using TNS-labelled protein revealed one class of binding site, with a K_d value of 2×10^{-5} M.

INTRODUCTION

In recent years, Moore & Dedman (1982) have isolated a new set of four Ca^{2+} -binding proteins from smooth muscle with apparent molecular masses of 67000, 35000, 33000 and 30000 on SDS/polyacrylamide gels. These proteins were termed calcimedlins, and function in a molecular fashion similarly to calmodulin. Calcimedlins, like calmodulin, interact with several hydrophobic ligands coupled to a polysaccharide matrix. These supports include the phenothiazine-ring-coupled Affi-Gel, W-7 (Hidaka *et al.*, 1981) coupled to a Sepharose, and both phenyl- and octyl-Sepharose affinity resins. Calcimedlins do not bind to a Sepharose column without proper ligand or to a calmodulin-Sepharose affinity matrix. They are not as widely distributed in tissues as calmodulin, but are more abundant in muscle tissue homogenates than is calmodulin. They differ from calmodulin in molecular mass, pI, DEAE-cellulose binding properties and heat-stability. However, like calmodulin, calcimedlins also become hydrophobic upon binding Ca^{2+} . Calcimedlins have been shown to stimulate the Ca^{2+} -ATPase activity of isolated liver microsomal fractions, and this activation could be inhibited by micromolar concentrations of calmodulin antagonist (Moore *et al.*, 1984).

Calcimedlins could conceivably be involved in mediating the calcium signal, in addition to calmodulin and troponin-C. Intracellular calcium signals probably follow several independent pathways, and the concentrations of various calcium mediators vary from tissue to tissue, depending on the physiological demands.

To date, no one has characterized these proteins in physico-chemical detail. In this work we have studied the Ca^{2+} -binding properties of 67 kDa protein isolated from chicken gizzard, using a combination of spectroscopic techniques.

MATERIALS AND METHODS

The isolation procedure used in our laboratory is a modification of the method developed in Dedman's laboratory (Matthew *et al.*, 1987). Chicken gizzard (700 g) was homogenized in a Waring blender in 2 litres of buffer, consisting of 40 mM-Tris, pH 7.5, 80 mM-NaCl, 2 mM-EDTA, 0.05% NaN_3 , 1 mM-phenylmethane-sulphonyl fluoride and 0.5 ml of aprotinin. The homogenate was centrifuged in a Beckman J2-21 centrifuge at 10000 rev./min for 75 min. Calcimedlins in the supernatant were precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to 90% saturation. Precipitated protein was dissolved in 40 mM-Tris (pH 7.5)/80 mM-NaCl and centrifuged at 14000 rev./min for 45 min. Free Ca^{2+} concentration in the supernatant was adjusted to 2 mM. The supernatant in the presence of Ca^{2+} was applied to a phenyl-Sepharose column equilibrated with 80 mM-NaCl/2 mM- CaCl_2 /40 mM-Tris, pH 7.5. Calcimedlins along with calmodulin were eluted from this column with 4 mM-EGTA. Eluted proteins were next applied to a DEAE-cellulose column in the presence of 20 mM-imidazole (pH 6.2) and 1 mM-EDTA. Ca^{2+} -binding proteins were eluted with a linear NaCl gradient. The peak corresponding to 67 kDa calcimedlin was pooled and applied to an Ultrogel AcA44 (LKB) column equilibrated with 50 mM-Tris, pH 7.5,

Abbreviation used: TNS, 2-*p*-toluidinylnaphthalene-6-sulphonate.

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containing 200 mM-NaCl, 2 mM-EDTA and 0.05% NaN_3 . The first major peak obtained corresponded to 67 kDa, and the isolated protein was homogeneous when tested by SDS/polyacrylamide-gel electrophoresis.

Standard polyacrylamide-gel electrophoresis was performed at pH 8.6 with Tris/glycine buffer as described by Schaub & Perry (1969). SDS/polyacrylamide-gel electrophoresis and molecular-mass determinations were carried out as described in one of our previous papers (Mani *et al.*, 1982). Amino acid analyses were performed on a Durrum D-500 amino acid analyser (Dionex Corp.) as described by Leung *et al.* (1986). The thiol groups in 67 kDa calcimedins were titrated by the method of Ellman (1959). The number of thiol groups modified/molecule of 67 kDa was calculated, by assuming an absorption coefficient of $13\,600\text{ M}^{-1}\cdot\text{cm}^{-1}$ for the liberated 5-nitro-2-thiobenzoate. Tryptophan was determined spectrophotometrically by the method of Bencze & Schmid (1957). Protein concentrations were determined by the Bradford method (1976). Chicken gizzard calmodulin was used in generating the standard curve. The A_{278} was measured for a protein sample of known concentration, and from this $A_{1\text{ cm}, 278}^{1\%} = 6.0$ was established for 67 kDa protein. For spectral work reported in this paper we used 6.0 as the absorption coefficient for this protein.

U.v. absorption and u.v. difference spectra were recorded on a Perkin-Elmer Lambda 5 spectrophotometer over the wavelength range 320–250 nm with 1 cm-path-length cells. The spectra were measured at 25 °C with a Lauda thermoregulator connected to the sample compartment. To generate a u.v. difference spectrum, Ca^{2+} was added to the sample cell and an equal volume of Chelex-100 treated water was added to the reference cell. Corrections were made for dilutions before the spectrum was plotted.

C.d. measurements were made on a Jasco J500C instrument fitted with a DP-500N data processor as described previously (Mani & Kay, 1983). A mean residue weight of 115 was used in calculating the molar ellipticity.

Fluorescence spectra were obtained with a Perkin-Elmer model MPF-44 spectrofluorimeter, and all measurements were made at 20 °C. The instrument was operated in a ratio mode. The A_{278} of the sample was less than 0.1. The protein was initially dissolved in 0.1 M-Tris/HCl buffer, pH 7.5, containing 4 mM-EDTA, and thereafter was subjected to exhaustive dialysis with at least four changes against solvent that had been passed through a Chelex-100 column.

Ca^{2+} -binding assays were carried out as described for S-100b protein (Mani & Kay, 1986), by using Centricon 10 micro-concentrators from Amicon as ultrafiltration devices to perform rapid-flow dialysis as a means of monitoring Ca^{2+} binding to 67 kDa protein (Blatt *et al.*, 1968). The 67 kDa protein was incubated for 15 min with excess Ca^{2+} , and was then centrifuged in a Beckman model L centrifuge at 4000 rev./min for 30 min. Free Ca^{2+} in the supernatant (in this case, the solution centrifuged across the dialysis membrane) was analysed by using the metallochromic indicator Arsenazo III (Sigma) as described by Thomas (1982). The 67 kDa apoprotein (~ 50 nmol) was placed in a prewashed Centricon 10 micro-concentrator along with a known amount of excess Ca^{2+} (~ 10 -fold molar excess), incubated for 15 min, and the solution was centrifuged. The supernatant was assayed for Ca^{2+} . From the amounts of free Ca^{2+} found in the supernatant along with the total Ca^{2+} added and the amount of protein used, it was possible to calculate the amount of Ca^{2+} bound to the protein.

RESULTS

Ca^{2+} -binding proteins including calcimedins and calmodulin were eluted from the phenyl-Sepharose column in the presence of EDTA. Calcimedins were resolved from calmodulin on a DEAE-cellulose column by using a linear NaCl gradient to 0.25 M. Under these conditions 67 kDa calcimedin was eluted around 0.2 M-

Fig. 1. Elution profile of 67 kDa calcimedin from a DEAE-cellulose column

Solvent system used was 20 mM-imidazole (pH 6.2)/1 mM-EDTA containing 1000 ml of a linear NaCl gradient from 0 to 0.25 M. Proteins were eluted at a flow rate of 35 ml/h and 10 ml fractions were collected.

Fig. 2. Electrophoresis of 67 kDa calcimedlin (a) in 15%-polyacrylamide gel plus SDS, and (b and c) in 12.5%-polyacrylamide gels in Tris/glycine buffer (pH 8.6) in 1 mM-EDTA (b) and 1 mM- Ca^{2+} (c)

NaCl, as indicated in Fig. 1. Isolated protein was essentially homogeneous, and the main contaminants were a low-molecular-mass protein and calcimedlins of molecular masses around 30 kDa. Fractions corresponding to 67 kDa protein, i.e. peak IV from the DEAE-cellulose column, was next applied to an Ultrogel AcA44 column (LKB). Proteins were eluted at a flow rate of 5 ml/h, and under these conditions the proteins were resolved into two peaks. The first major peak obtained corresponded to 67 kDa calcimedlin. When the protein was run in SDS/15%-polyacrylamide gels, it migrated at 67 kDa and was homogeneous (Fig. 2). The gels were calibrated with standard proteins by using the Sigma gel kit protein samples. The mobility of 67 kDa protein in non-SDS gels is shown in Fig. 2. The protein moved faster in the presence of EDTA. The observed decrease in mobility in the presence of Ca^{2+} could be due to a decrease in negative charge on the protein resulting from binding Ca^{2+} . In this respect, 67 kDa calcimedlin behaves similarly to calmodulin and brain S-100b protein, but differently from muscle troponin-C.

A comparison of the amino acid analysis of our preparation with that of the 67 kDa calcimedlin prepared by Moore (1986) from chicken gizzard is summarized in Table 1. A good agreement exists between the two, the major difference being in the contents of histidine, arginine and valine. We carried out acid hydrolysis for 24, 48 and 72 h, and the values reported here for valine and isoleucine correspond to the time-extrapolated values, whereas Moore (1986) had performed hydrolysis for 24 h only. However, when we compared our analysis with the published sequences of calelectrin and the lymphocyte p68 Ca^{2+} -binding protein, considerable deviation was noticed for the following amino acids: Gly, Val, Met, Ile, Arg, Cys and Trp. Hence it appears that

Table 1. Amino acid composition of 67 kDa protein

References: *Moore (1986); †Südhof *et al.* (1988); ‡Crompton *et al.* (1988).

Amino acid	Composition (mol of residue/100 mol of total residues)			
	This study	67 kDa calcimedlin*	Calelectrin†	p68 CaBP‡
Ala	8.5	9.8	8.6	8.6
Arg	4.5	2.3	6.2	6.7
Asx	10.4	9.5	10.7	10.5
Cys	—	—	0.9	0.9
Glx	13.5	15.7	12.0	11.9
Gly	10.9	11.7	6.7	6.7
His	2.0	4.3	1.9	1.9
Ile	4.1	2.8	6.8	7.0
Leu	8.5	6.1	9.8	9.8
Lys	7.6	9.0	7.9	7.7
Met	1.7	1.8	3.3	3.1
Phe	2.9	3.7	3.7	3.7
Pro	3.1	2.9	2.2	2.2
Ser	7.3	8.7	5.8	5.9
Thr	4.4	4.6	5.8	5.6
Trp	0.7	0.8	0.3	0.3
Tyr	2.7	2.6	3.4	3.4
Val	7.0	3.8	3.9	3.9

our protein resembles more closely the 67 kDa calcimedlin isolated by Moore (1986) from the same source. The number of thiol groups in 67 kDa protein was determined by the method of Ellman (1959). The rate of reaction of the 67 kDa protein with 5,5'-dithiobis-(2-nitrobenzoic acid) in 50 mM-Tris, pH 7.5, containing either 0.5 mM- Ca^{2+} or 1 mM-EDTA was slow, and only 0.5 thiol group reacted per molecule of protein. However, when the reaction was carried out in the presence of 6 M-urea/50 mM-Tris, pH 7.5, 1.8 thiol groups reacted per molecule of protein. This would suggest that, out of two thiol groups in the protein, one is more reactive than the other, or, alternatively, both the thiol groups become more reactive in the presence of 6 M-urea. The u.v. absorption spectrum of 67 kDa calcimedlin is characterized by the presence of a shoulder around 290 nm corresponding to tryptophan. The fine structure that one usually associates with Ca^{2+} -binding proteins such as calmodulin, S-100b and troponin-C in the 250–270 nm region is not noticeable, and this is because the tyrosine/phenylalanine ratio is nearly 1 for 67 kDa protein, whereas for the other above-mentioned Ca^{2+} -binding proteins this ratio is high, e.g. for S-100b it is 7.

U.v. difference spectroscopy

The possible effect of Ca^{2+} on the tryptophan, tyrosine and phenylalanine aromatic groups was investigated by using u.v. difference spectroscopy. Fig. 3 shows the difference spectrum of 67 kDa protein when Ca^{2+} was added to the sample cell, compared with non-treated protein in the reference cell. The difference peak at 292 nm is characteristic of a red shift of the tryptophan absorption band. The dominant difference peaks at 287 and 280 nm arise from the perturbation of one or more tyrosine residues. The sign of the tryptophan and tyrosine

Fig. 3. U.v. difference spectrum of 67 kDa calcimedin in 0.1 M-Tris (pH 7.5)/0.5 mM- CaCl_2

The units are expressed as differences in molar absorption, $\Delta\epsilon$. The protein concentration was 0.66 mg/ml.

difference peak (i.e. a 'red shift') suggests that the chromophores are in a less polar environment in the presence of Ca^{2+} (Donovan, 1969). Alternatively, the perturbations may be due to local charge effects as a result of Ca^{2+} binding. The binding of Ca^{2+} by carboxylate groups would decrease the free negative charge on the latter, which might result in changes in the geometry of the binding site and re-orientation of the phenolic group. Similar observations have been noted for skeletal- and cardiac-muscle troponin-C in our laboratory (Hincke *et al.*, 1978).

Near-u.v. c.d. studies

The aromatic c.d. spectrum of 67 kDa protein is shown in Fig. 4. The positive band at 292 nm is due to tryptophan, and the two bands at 284 and 279 nm can be attributed to tyrosine residues. The spectrum shows that the tryptophan residue at 292 nm and the tyrosine residues in the 280 nm region are perturbed upon Ca^{2+} addition. When we added 1 mM- Mg^{2+} to 67 kDa apoprotein, there was no noticeable change in the c.d. spectrum, suggesting that Mg^{2+} has no significant effect on the protein conformation, or alternatively that Mg^{2+} simply does not bind to this protein at this concentration. In the far-u.v. c.d. region also, Mg^{2+} had no significant effect on the secondary structure of the protein. In another series of experiments, Mg^{2+} was added initially to the apoprotein, and subsequently Ca^{2+} was added. Ca^{2+} in the presence of Mg^{2+} could still induce the same conformational change, implying that Ca^{2+} can bind to 67 kDa calcimedin in the presence of Mg^{2+} .

Far-u.v. c.d. results

Fig. 5 represents a typical far-u.v. c.d. spectrum of the protein in the absence and in the presence of Ca^{2+} . Addition of Ca^{2+} induced only a small change in the overall secondary structure of the protein molecule: the $[\theta]_{222}$ value decreased by only 10%. Mg^{2+} addition, on the other hand, produced no significant change in the $[\theta]_{222}$ value, suggesting that the observed effect with Ca^{2+} , though small, is specific for this metal ion.

Fig. 4. Aromatic c.d. spectra of 67 kDa protein in (----) 0.1 M-Tris (pH 7.5) and in (—) 0.1 M-Tris (pH 7.5) containing 0.5 mM- CaCl_2

Fig. 5. Far-u.v. c.d. spectra of 67 kDa protein in (—) 0.1 M-Tris (pH 7.5) and in (----) 0.5 M-Tris (pH 7.5) containing 0.5 mM- CaCl_2

Fluorescence spectroscopy

The 67 kDa calcimedin, which has both tryptophan and tyrosine residues, has its emission maximum centred around 325 nm when the protein is excited at 280 nm. The emission peak at 325 nm is probably due to tryptophan, and from the observed position of the emission maximum one can infer that the tryptophan residue(s) is partly buried in the interior of the protein molecule. Ca^{2+} addition to apoprotein resulted in only a 5% decrease in fluorescence intensity at 325 nm, and for this reason we were not able to carry out a Ca^{2+} titration to estimate K_d .

Fig. 6. Change in relative TNS fluorescence intensity at 435 nm as a function of Ca^{2+} concentration for 67 kDa protein in 0.1 M-Tris, pH 7.5 at 20 °C

Excitation wavelength was 345 nm. The concentration of the protein was 1.5 μ M.

Ca^{2+} -binding proteins that are members of the E-F hand family upon binding Ca^{2+} undergo a conformational change, and this process results in exposing a hydrophobic domain (Moore *et al.*, 1984). The fact that 67 kDa protein in the tissue homogenate binds to a phenyl-Sepharose column in the presence of Ca^{2+} suggests that this protein also exposes a hydrophobic region when it binds Ca^{2+} . For this reason we used a hydrophobic fluorescent probe, TNS (2-*p*-toluidinylnaphthalene-6-sulphonate), which fluoresces weakly in polar solvents, but its intensity becomes enhanced in apolar solvents or when bound to a hydrophobic pocket on the protein (McClure & Edelman, 1966). Addition of Ca^{2+} to TNS-labelled protein resulted in a 25 % enhancement in fluorescence intensity, and this was accompanied by a blue shift of the emission maximum, implying that the probe in the presence of Ca^{2+} occupies a more hydrophobic environment. The increase in relative fluorescence intensity at 435 nm as a function of Ca^{2+} concentration is plotted in Fig. 6. From this plot a K_d value of $2 \times 10^{-5} \pm 0.5 \times 10^{-5}$ M was obtained for Ca^{2+} , and this corresponds to the Ca^{2+} concentration required to produce 50 % of the observed increase in fluorescence intensity. The Ca^{2+} -binding assay, as described in the Materials and methods section, revealed the binding of 4 mol of Ca^{2+} /mol of 67 kDa protein at pH 7.5, and this finding is in agreement with the observation by Matthew *et al.* (1986).

DISCUSSION

A set of four Ca^{2+} -binding proteins from smooth muscle, termed calcimedins, bind hydrophobic-matrix resins in a Ca^{2+} -dependent manner (Moore & Dedman,

1982), and this property was used in the isolation scheme developed in our laboratory. The original isolation procedure (Matthew *et al.*, 1987), which involved batchwise treatment with DEAE-cellulose before a phenyl-Sepharose column, yielded very little protein in our hands, and for this reason we subjected the muscle homogenate after initial centrifugation to precipitation with 90 %-satd. $(NH_4)_2SO_4$. Proteins precipitated by 90 %-satd. $(NH_4)_2SO_4$ were subsequently applied to a phenyl-Sepharose column in the presence of Ca^{2+} . By the procedure outlined in the Materials and methods section, the yield obtained was considerably better, and we often isolated from 350 g of chicken gizzard nearly 250 mg of calcimedins along with calmodulin after the first phenyl-Sepharose column.

The cellular function of the calcimedins is not understood. It has been suggested that 67 kDa calcimedins may be important in membrane events associated with the inflammatory response (Morse & Moore, 1986). Since higher contents of calcimedins are found in a secretory organ such as the liver, it may be involved in the secretory process. It is possible that all the four calcimedins have a specialized cellular function, providing a discrete discrimination of the intracellular Ca^{2+} signal. The 67 kDa calcimedins interact with cyclic nucleotide phosphodiesterase (Moore *et al.*, 1984), and by doing so is able to inhibit calmodulin activation of cyclic nucleotide phosphodiesterase, and this interaction probably involves unmasking of a certain hydrophobic region in the protein molecule in the presence of Ca^{2+} . This is thought to be the mechanism by which calmodulin interacts with its target enzymes, and this is borne out by the fact that 67 kDa calcimedins, like calmodulin, can bind to a phenyl-Sepharose column in the presence of Ca^{2+} . Calcimedins can be eluted from the phenyl-Sepharose column only with EGTA. Even the purified 67 kDa calcimedins could bind to a phenyl-Sepharose column in the presence of 1 M-NaCl, 40 mM-Tris, pH 7.5, and 2 mM- Ca^{2+} . Ca^{2+} -binding proteins belonging to the calmodulin family generally undergo a conformational change in response to physiological demands. Changes in Ca^{2+} concentration result in altered conformation of calmodulin, S-100 proteins and vitamin D-dependent Ca^{2+} -binding protein. Similarly, 67 kDa calcimedins also undergoes a conformational change in the presence of Ca^{2+} , as revealed by the spectroscopic techniques applied in the present study.

Südhof *et al.* (1988) have sequenced a protein of 67 kDa referred to as calelectrin and, as mentioned in the Results section, our protein preparation appears to be different from their 67 kDa calelectrin on the basis of its amino acid composition. The 67 kDa protein isolated from chicken gizzard by Moore (1986) resembles our protein preparation. According to Morse & Moore (1986), the 67 kDa calcimedins isolated from chicken gizzard is distinct from p67 calelectrin and lymphocyte 68 kDa Ca^{2+} -binding protein, since antibodies raised to the smooth-muscle 67 kDa calcimedins failed to recognize the two above-mentioned proteins.

Moore (1986) reported a K_d value of 0.4 μ M for Ca^{2+} for the native 67 kDa calcimedins, and our fluorescence studies with the TNS-labelled protein gave a K_d value of 2×10^{-5} M. Moore (1986) had used the Hummel & Dreyer (1962) method for binding studies, and this method has a limitation, i.e. the Ca^{2+} bound is generally very small compared with the total Ca^{2+} used, and this was cited as

a possible reason for the observed discrepancy between spectroscopic results and this method for Protein II from liver (Shadle *et al.*, 1985). This argument is also cited in the work of Kuznicki & Filipek (1987) with their 10.5 kDa Ca^{2+} binding protein from Ehrlich ascites-tumour cells. Of course, use of a non-covalent hydrophobic probe like TNS also has limitations. For example, in this instance one assumes that the presence of the probe will have no significant effect on the ability of the protein to bind Ca^{2+} . In our opinion it is a valid assumption, since K_d values obtained in the past with TN-C, calmodulin and S-100 proteins all agree with the published values in the literature from other laboratories obtained by either similar spectroscopic approaches or equilibrium dialysis. The Ca^{2+} -binding assay indicated that the protein binds 4 mol of Ca^{2+} /mol of protein, and this finding is in agreement with the observation by Matthew *et al.* (1986) for their 67 kDa calcimedlin isolated from chicken gizzard. The fact that the fluorescence titration with Ca^{2+} indicated only one class of binding site in the presence of the probe suggested that all four Ca^{2+} -binding sites have the same affinity for Ca^{2+} . Alternatively, we may also be monitoring only one or more Ca^{2+} -binding sites that are located close to the TNS-binding site, and as a consequence the observed K_d value for Ca^{2+} corresponds only to those sites that are in the near vicinity of the probe.

As a first step in understanding the Ca^{2+} -binding properties of 67 kDa calcimedlin, we initiated this work with a careful characterization of the protein by spectroscopic methods. The ability of this protein to expose a hydrophobic site in the presence of Ca^{2+} , as evidenced by the observed conformational changes, further strengthens the idea that most Ca^{2+} -binding proteins are able to perform their physiological functions by exposing a hydrophobic site as a result of Ca^{2+} binding. It is now becoming evident that this type of conformational change is not just confined to the calmodulin family of proteins.

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