PURIFICATION OF A 240 000 M_r CALMODULIN-BINDING PROTEIN FROM A MICROSOMAL FRACTION OF BRAIN

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1. Introduction

Although calmodulin is generally regarded as a Ca^{2+}-dependent activator of a variety of enzymes, the same protein has been shown to associate with cellular proteins apparently devoid of enzyme activities. Thus, 85 000- [1], 76 000- [2], 70 000- [3], 5000- [4] and 22 000- [5] M_r calmodulin-binding proteins have been purified from brain tissue.

While the proteins listed as above were obtained from the supernatant fraction of the tissue homogenate, our work had shown calmodulin-binding protein(s) associated with the particulate fraction [6,7]. Although this protein distributes in a variety of mammalian tissues, its highest concentration was found in brain, followed by adrenal gland [7]. Thus, the particulate fraction derived from 1 g cerebrum was able to bind ~80 μg calmodulin in a Ca^{2+}-dependent fashion [7], which accounts for ~20% of the total amount (400 μg/g tissue [6]) of the EGTA-extractable calmodulin in the brain. This amount is much greater than those reported for the soluble calmodulin-binding proteins in the brain [1]. Upon subcellular fractionation of a brain homogenate, distribution of the activity of the particulate calmodulin-binding protein paralleled that of synaptic membranes and this protein has therefore been implicated in the regulation of synaptic function [7]. It therefore seemed of considerable importance to purify this particulate binding protein from the brain and clarify its nature. Here we have purified a 240 000 M_r protein from the bovine brain as a major calmodulin-binding protein in a microsomal fraction that contains most of the disrupted synaptic membranes and vesicles.

Abbreviations: EGTA, ethyleneglycol bis-(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; M_r, relative molecular mass

2. Materials and methods

2.1. *Materials*

Sepharose 4B and cyanogen-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals. Calmodulin–Sepharose 4B was prepared as in [8]. Calmodulin was purified from bovine brain as in [9]. [^{3}H]Calmodulin was prepared as in [7]. Its specific activity was 500 mCi/mmol.

2.2. *Purification of calmodulin-binding protein from particulate fraction of brain*

The following procedure was carried out at 4°C unless specified otherwise. Fresh bovine cerebral cortices (400 g), freed of coagulated blood and meninges, were homogenized in a Waring Blender with 9 vol. (3.6 liters) of medium I consisting of 5 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 0.5 mM EGTA, 0.25 mM phenylmethyl sulfonylfluoride, 0.1 mM diisopropyl fluorophosphate and pepstatin A (0.05 μg/ml). Unbroken cells and cell debris were removed by a centrifugation at 900 × *g* for 10 min. The supernatant was then centrifuged for 30 min at 7000 × *g*. The resulting supernatant was concentrated over Amicon PM-30 membrane. The concentrate was then centrifuged at 105 000 × *g* for 1 h. The pellet was homogenized with 1 liter medium I then centrifuged as above. The resulting pellet (microsomal fraction), containing 1280 mg protein, was homogenized with 20 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol, 100 mM KCl, 1 mM EGTA, 0.25 mM phenylmethyl

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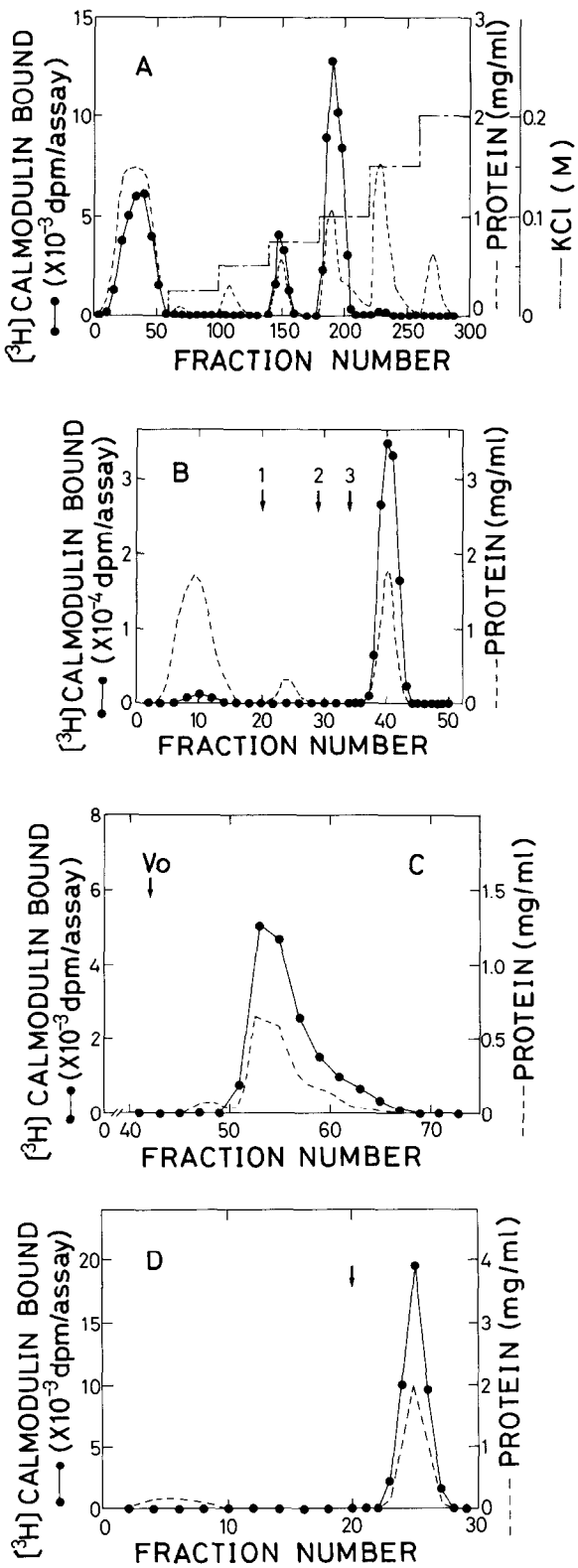

sulfonylfluoride and Triton X-100 at 2% final conc. The mixture was kept in ice for 2 h with gentle stirring and then centrifuged at 105 000 × *g* for 1 h. The pellet was homogenized with 3 liters ice-cold acetone. The resulting precipitates were collected by filtration through a filter paper then quickly dried at room temperature to ~2 g powder. The acetone powder thus prepared was homogenized in a Waring Blender with 400 ml medium II consisting of 40 mM Tris–HCl (pH 7.5), 15 mM 2-mercaptoethanol, 1 mM EGTA and 6 M urea. The suspension was stirred gently overnight and then centrifuged at 105 000 × *g* for 1 h. The supernatant fluid was applied to a DEAE-cellulose column (2.5 × 20 cm) pre-equilibrated with medium II. The column was washed with 4-bed vol. medium II, then eluted with medium II plus increasing concentrations of KCl (4 bed vol. each of 25, 50, 75, 100, 150 and 200 mM). Three active peaks were eluted from the column (fig.1A). The first peak was myelin basic protein. The third peak (tube 188 through 203) was collected, made 2 mM in Ca^{2+} by addition of 1 M $CaCl_2$ then applied to a column (2.5 × 8 cm) of calmodulin-coupled Sepharose 4B pre-equilibrated with medium III consisting of 40 mM Tris–HCl (pH 7.5), 15 mM 2-mercaptoethanol, 2 mM $CaCl_2$ and 6 M urea. Fig.1B illustrates its elution profile. The column was first eluted with medium III and, at arrow 1, with medium III plus 300 mM NaCl, then, at arrow 2, again with medium III. At arrow 3, the column was eluted with medium III containing 5 mM EGTA in place of $CaCl_2$. An active peak (tubes 38–42) was concentrated over Amicon PM-10 membrane and applied to a column (1.5 × 90 cm) of Sepharose 4B pre-equilibrated with medium II. The column was then eluted with medium II (fig.1C). An active peak (tubes 51–56) was collected and dialyzed overnight against a medium IV consisting of 20 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 100 mM KCl, and 0.2 mM $CaCl_2$ with 3 changes of the medium

Fig.1. Purification of 240 000 M_r calmodulin-binding protein by a combination of column chromatographies. For detailed explanation see section 2. Throughout the purification, the calmodulin-binding activity was monitored by the binding assay using polyacrylamide gel electrophoresis (section 2.2). (A) DEAE-cellulose; (B) calmodulin-coupled Sepharose 4B; (C) Sepharose 4B; (D) calmodulin-coupled Sepharose 4B (second run). Fractions of 10 ml (A), 3 ml (B), 1.8 ml (C) or 1 ml (D) each were collected. The first peak from the DEAE-cellulose column (A) was represented by myelin basic protein.

during the dialysis. The dialyzed solution was clarified by centrifugation for 30 min at 200 000 × *g* and applied to a column (15 ml resin bed) of calmodulin–Sepharose 4B pre-equilibrated with medium IV. The column was eluted with the same medium, then at the arrow indicated in fig.1D, the eluting medium switched to medium IV containing 1 mM EGTA in place of $CaCl_2$. The calmodulin-binding protein was eluted from the column with EGTA.

2.3. *Analytical methods*

The calmodulin-binding activity of the particulate material was determined as in [7]. For the solubilized material, either polyacrylamide gel electrophoresis [10–12] or gel filtration [13] was employed. In short, samples to be assayed were incubated with [^{3}H]calmodulin in the presence of Ca^{2+}, then bound and unbound [^{3}H]calmodulins were separated either by electrophoresis on 10% polyacrylamide gels in 122 mM glycine–20 mM Tris (pH 8.3) buffer as in [14] or by gel filtration using Sepharose G-100. Then the radioactivity of the bound [^{3}H]calmodulin was determined. Each experiment was accompanied by a control assay where Ca^{2+} in the medium was replaced by EGTA, and the control value subtracted from the corresponding experimental value. Calmodulin and protein concentrations were determined as in [15].

3. Results and discussion

Here, a microsomal fraction from the brain containing most of the disrupted synaptic membranes and vesicles, as monitored by an electron-microscope, was used as the source of the calmodulin-binding protein. First, we examined for the solubilization of the binding activity. Among the agents tested, only 6 M urea or 6 M guanidine–HCl was able to solubilize it. Treatments with Triton X-100 (2%), Lubrol PX (2%) and KI (0.6 M) failed to solubilize it. KCl at 0.6 M was only slightly effective: with 0.6 M KCl, <5% of the activity solubilized with 6 M urea was solubilized. We then solubilized the binding activity with 6 M urea and purified it to apparent homogeneity upon polyacrylamide gel electrophoresis by successive column chromatographies as illustrated in fig.1. Fig.2 summarizes the purification. The purification method is reproducible in repeated runs and the yield of the protein was ~12 mg from 400 g brain. We found that the binding activity was hardly separated from a 50 000–60 000 M_r protein present in abundance in brain tissue by means of column chromatographies*. Therefore, the purification procedure finally adopted includes the treatment of the microsomal fraction

* The 50 000–60 000 M_r protein (or its possible aggregate form) was eluted from the DEAE-cellulose and Sepharose columns immediately after and before, respectively, the 240 000 M_r protein. It copurified with the 240 000 M_r protein from the calmodulin affinity columns.

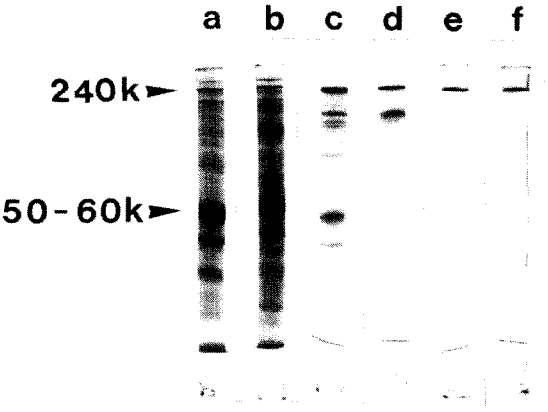

Fig.2. SDS–polyacrylamide gel electrophoresis of the purification steps of 240 000 M_r protein. SDS–polyacrylamide gel electrophoresis was carried out in 7.5% gels in a buffer system [16] in the presence of 0.1% SDS. The gels were then stained for proteins with Coomassie brilliant blue. (a) Microsomal fraction (starting material); (b) urea extract of (a); (c) after DEAE-cellulose column (fig.1A); (d) after calmodulin–Sepharose column (fig.1B); (e) after Sepharose 4B column (fig.1C); (f) after the second calmodulin–Sepharose column run (fig.1D). M_r-values were calibrated by filamin (255 000), spectrin (band 1 = 240 000), skeletal muscle myosin heavy chain (200 000), β'-subunit of RNA polymerase (165 000), β-subunit of RNA polymerase (155 000), phosphorylase *b* (94 000), bovine serum albumin (68 000) and actin (43 000)

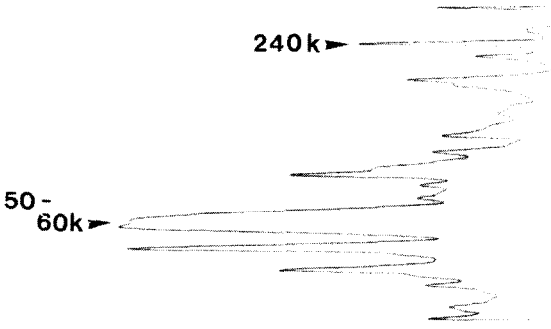

Fig.3. Densitometric scanning of a stained gel on which a sample of microsomal fraction was electrophoresed in the presence of 0.1% SDS (see fig.2a).

with acetone at the sacrifice of the binding protein yield since this treatment removes most of the 50 000–60 000 M_r protein from the preparation. The M_r-value of this protein, as determined by SDS–polyacrylamide gel electrophoresis, was 240 000 (fig.2). A densitometric scanning of a SDS–polyacrylamide gel on which a sample of the microsomal fraction was electrophoresed showed this protein to be 3.0% of the total proteins (fig.3). A separate fractionation study gave 12.3 mg for total protein of the microsomal fraction derived from 1 g brain. With this value, the amount of the 240 000 M_r protein was calculated to be 370 mg/kg brain.

One must be careful before identifying a protein as a calmodulin-binding protein. In [17] it was reported that the interaction of calmodulin with 'calmodulin-binding proteins' of basic nature is non-specific and of questionable physiological significance. The 240 000 M_r protein reported here is an acidic protein as judged from its elution profile from a DEAE-cellulose column. Another problem is that some proteins behave like calmodulin-binding proteins upon affinity chromatography using calmodulin–Sepharose. Here, a 50 000–60 000 M_r protein present in abundance in brain tissue was retained by an affinity column in the presence of Ca^{2+} and eluted from it with EGTA. Therefore, this 50 000–60 000 M_r protein would have been designated as a calmodulin-binding protein if precautions were not taken (see below). We found that the purified 50 000–60 000 M_r protein does not bind calmodulin at all in our assay system. In addition, precipitates were formed when the 50 000–60 000 M_r protein was centrifuged at 200 000 × g for 30 min in the presence of Ca^{2+} (K. S., unpublished) suggesting that the retention of this protein on the column may be, at least in part, due to the precipitate (possibly aggregate) formation of the protein in the presence of Ca^{2+}. The Ca^{2+}-dependent binding of calmodulin to the 240 000 M_r protein was confirmed by 3 different methods: polyacrylamide gel electrophoresis (section 2); Sephadex G-100 gel filtration column chromatography (section 2); calmodulin–Sepharose affinity chromatography (fig.1B,1D). Complex formation between the 240 000 M_r protein and calmodulin was seen on these systems either in the presence or absence of 6 M urea.

The 240 000 M_r protein accounted for most of the calmodulin-binding activity in the microsomal fraction. (The first peak appearing in the elution profile from the DEAE-cellulose column is myelin basic protein.) Upon subcellular fractionation of a brain homogenate, we found that the 240 000 M_r protein is exclusively localized in the microsomal fraction (K. S., S. K., unpublished). Although this protein was obtained from the particulate fraction, it is not considered to be an intrinsic membrane protein because it was not solubilized from the particulate fraction with non-ionic detergents. Instead, it was solubilized with 6 M urea or guanidine–HCl. There is a possibility that this protein associates with the structures underlying the membranes. A similar calmodulin-binding protein has been extracted from human erythrocyte ghosts with 6 M urea and identified as spectrin [10,11], a main constituent of the cytoskeletal network underlying the erythrocyte membrane [18,19].

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