PURIFICATION OF A 155 000 M_r CALMODULIN-BINDING PROTEIN FROM A MICROSOMAL FRACTION OF BRAIN

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1. Introduction

Release of transmitter substances and secretion of hormones are triggered by the intracellular concentration of Ca^{2+} [1]. It is attractive to postulate that this action of Ca^{2+} is mediated by calmodulin, an intracellular Ca^{2+}-receptive protein. Calmodulin-binding protein(s) associated with the particulate fraction have been shown in brain tissue [2]. Although this particle-associated binding activity is distributed in a variety of mammalian tissues, its highest activity was found in brain, followed by adrenal gland [3]. Moreover, subcellular fractionation of a brain homogenate showed that this particulate calmodulin-binding activity is mainly localized in a fraction where synaptic membranes and vesicles are concentrated [3]. Thus, this binding protein(s) has been implicated in synaptic function. Subsequently, we have purified a 240 000 M_r calmodulin-binding protein from a brain microsomal fraction rich in synaptic membranes and vesicles [4]. The 240 000 M_r protein accounted for 3.0% of the total protein in this fraction.

During the purification of the 240 000 M_r protein, we noticed the presence of another calmodulin-binding protein, which slightly preceded the 240 000 M_r protein upon DEAE-cellulose column chromatography: a portion of its peak was eluted from the DEAE-cellulose column with 75 mM KCl as represented by the second calmodulin-binding peak in fig.1A of [4], the remainder being eluted from the column with 100 mM KCl together with the 240 000 M_r protein. Here, we report the purification of this calmodulin-binding protein whose M_r was estimated to be 155 000 upon SDS–polyacrylamide gel electrophoresis. The concentration of the 155 000 M_r protein in brain was almost comparable to that of 240 000 M_r protein on a molar basis and the sum of both proteins accounted for ~2/3rds of the total calmodulin-binding activity found in the particulate fraction of a brain homogenate.

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2. Materials and methods

2.1. *Materials*

Calmodulin and [^{3}H]calmodulin were prepared as in [5] and [3], respectively. Calmodulin–Sepharose 4B was prepared as in [6]. Neurofilaments were prepared from bovine brain as in [7].

2.2. *Purification of 155 000* M_r *protein from microsomal fraction of brain*

Calmodulin-binding proteins (155 000 and 240 000 M_r) were purified from the urea extract of the acetone powder of the microsomal fraction of bovine brain by successive column chromatographies using DEAE-cellulose, calmodulin–Sepharose, Sepharose 4B, and rechromatography on calmodulin–Sepharose. Throughout the purification, the calmodulin-binding activity was monitored by a binding assay using [^{3}H]calmodulin. Formation of a protein–[^{3}H]calmodulin complex was detected and quantitated by polyacrylamide gel electrophoresis as in [4]. For the samples from the second calmodulin–Sepharose column (final purification stage), urea was not included in the electrophoresis system. For all other samples, 8 M urea was present in the electrophoresis system (details in [8]). The preparation of the acetone powder from the microsomal fraction, extraction of

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proteins from the acetone powder with a medium containing 1 mM EGTA and 6 M urea, and subsequent procedures for column chromatography were essentially the same as in [4] except that, for elution from DEAE-cellulose, the stepwise increase in [KCl] skipped from 50–100 mM (no wash at 75 mM KCl) so that a mixture of 240 000 and 155 000 M_r binding proteins was eluted with 100 mM KCl. The active peak was collected and chromatographed on calmodulin–Sepharose as in [4]. The active peak thus obtained was then subjected to gel filtration on Sepharose 4B. It was on this Sepharose 4B column that both proteins were separated from each other (fig.1). For 240 000 and 155 000 M_r proteins, peaks 1 (tubes 51–56) and 2 (tubes 62–65), respectively, were separately collected. In some preparations, where peak 2 still contained a trace of 240 000 M_r protein, complete removal of the 240 000 M_r protein from the peak 2 was achieved by DEAE-cellulose column chromatography of peak 2. The column was eluted with 40 mM Tris–HCl (pH 7.5), 15 mM 2-mercaptoethanol, 1 mM EGTA, 6 M urea and increasing concentrations (50, 75 and 100 mM) of KCl. The 155 000 M_r protein eluted from the column with 75 mM KCl was completely free of the 240 000 M_r protein. The active peaks thus obtained were dialyzed against a buffer consisting of 20 mM Tris–HCL (pH 7.5), 5 mM 2-mercaptoethanol, 100 mM KCl,

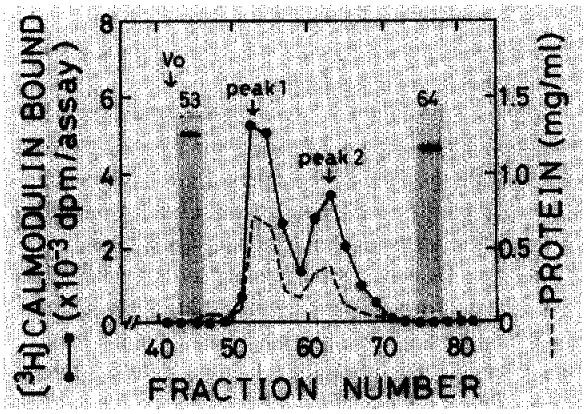

Fig.1. Sepharose 4B column chromatography. The material released from calmodulin–Sepharose with EGTA was applied to a Sepharose 4B column (1.5 × 90 cm) pre-equilibrated with 40 mM Tris–HCl (pH 7.5), 15 mM 2-mercaptoethanol, 1 mM EGTA and 6 M urea. The column was eluted with the same medium. Proteins of M_r 240 000 (peak 1) and 155 000 (peak 2) were separately eluted from the column. Samples from tubes 53 (peak 1) and 64 (peak 2) were electrophoresed on SDS–polyacrylamide gels.

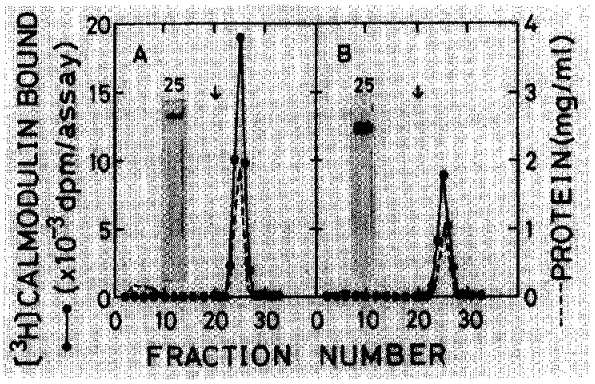

Fig.2. Rechromatography on calmodulin–Sepharose: peak 1 (A) and DEAE-rechromatographed peak 2 (B) (see text) from the Sepharose 4B column were chromatographed separately. The calmodulin–Sepharose column was eluted with 20 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 100 mM KCl and 0.2 mM $CaCl_2$. At (→), 1 mM EGTA replaced $CaCl_2$ in the buffer. The active peaks thus released from the column contained either 240 000 (A) or 155 000 (B) M_r proteins. Protein contents of each peak were analyzed by SDS–polyacrylamide gel electrophoresis.

and 0.2 mM $CaCl_2$ (no urea) with 3 changes of the dialysis buffer. The dialyzed solutions were clarified by centrifugation for 30 min at 200 000 × g and chromatographed separately on calmodulin–Sepharose as in [4]. Their elution profiles are illustrated in fig.2, and 240 000 and 155 000 M_r proteins thus obtained were homogeneous upon SDS–polyacrylamide gel electrophoresis. About 10 mg and 4 mg of the 240 000 and 155 000 M_r proteins, respectively, were obtained from 400 g brain. It must be noted that 6 M urea was included in the buffers throughout the purification except for the rechromatography on calmodulin–Sepharose where urea was omitted.

2.3. *Analytical methods*

The calmodulin-binding activity of the solubilized proteins was determined as in [4]. SDS–polyacrylamide gel electrophoresis was carried out in 7.5% gels in the buffer system of [9] in the presence of 0.1% SDS. The gels were stained for proteins with Coomassie brilliant blue. Calmodulin and protein concentrations were determined as in [10].

3. Results and discussion

The calmodulin-binding protein purified from the microsomal fraction had a M_r-value of 155 000 upon SDS–polyacrylamide gel electrophoresis since it

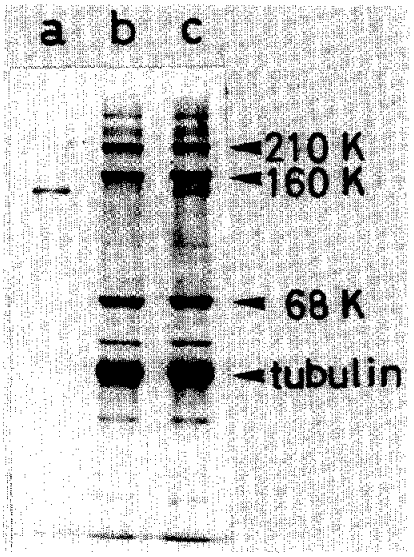

Fig.3. SDS–polyacrylamide gel electrophoresis of the 155 000 M_r protein and neurofilaments: (a) 155 000 M_r calmodulin-binding protein; (b) purified neurofilaments; (c) a + b.

migrated on the gel slightly slower than a 150 000 M_r protein caldesmon [11,12] but faster than the 160 000 M_r component of the neurofilaments (fig.3). The binding of the 155 000 M_r protein to calmodulin, either upon the calmodulin–Sepharose chromatography or upon the polyacrylamide gel electrophoresis, was Ca^{2+}-dependent in the presence or absence of 6 M urea (section 2).

The neurofilaments from mammalian brain consist primarily of 3 polypeptide components [13,14]: M_r-values of 210 000, 160 000 and 68 000 were estimated on SDS–polyacrylamide gel electrophoresis for this triplet from bovine brain [7]. Therefore, a possible identity between the 160 000 M_r neurofilament component and the 155 000 M_r calmodulin-binding protein deserved investigation. Upon SDS–polyacrylamide gel electrophoresis, the 160 000 M_r protein band of the purified bovine brain neurofilaments did not migrate with the 155 000 M_r calmodulin-binding protein (fig.3). In addition, essentially no protein component of the bovine brain neurofilaments was bound to calmodulin–Sepharose in the presence of Ca^{2+} (fig.4). Small amounts of protein

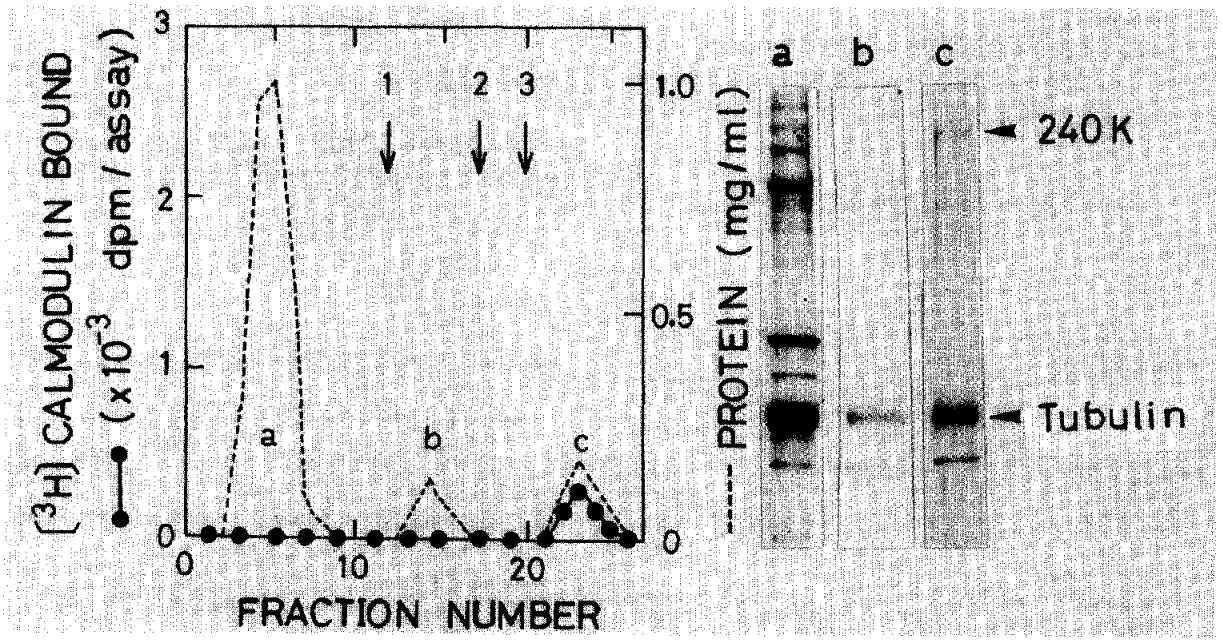

Fig.4. Affinity chromatography of urea-solubilized neurofilaments on calmodulin–Sepharose. Neurofilaments purified by the procedures of [7] were solubilized with medium III consisting of 40 mM Tris–HCl (pH 7.5), 15 mM 2-mercaptoethanol, 2 mM $CaCl_2$ and 6 M urea. The solution was clarified by centrifugation for 60 min at 105 000 × g and the supernatant fluid was applied to a column (0.7 × 1 cm) of calmodulin–Sepharose pre-equilibrated with medium III. The column was eluted with medium III and then with medium III plus 300 mM NaCl (1→) and then with medium III alone (2→). At (3→) $CaCl_2$ in the medium III was replaced by 1 mM EGTA. Samples from protein peaks (a–c) were electrophoresed on SDS–polyacrylamide gels.

were retained by the column in the presence of Ca^{2+} and released from it whit EGTA (fig.4). However, they were identified on SDS–polyacrylamide gel electrophoresis as the 240 000 M_r calmodulin-binding protein and tubulin which contaminated the neurofilament preparation (fig.4c). In [15], we showed that tubulin does not associate with calmodulin–Sepharose. However, in the presence of 6 M urea, tubulin does bind to calmodulin–Sepharose to some extent. This result was confirmed with purified tubulin. The reason for this is unclear at present. At any rate, the 155 000 M_r calmodulin-binding protein was distinct from any neurofilament component.

Densitometric scanning of an SDS–polyacrylamide gel on which a sample of the crude microsomal fraction was electrophoresed showed that the 155 000 M_r protein represented 1.8% of the total protein of this fraction (not shown). This corresponds to ~220 mg/kg brain since the total protein of this fraction was determined to be 12.3 mg/g brain. This is ~60% (on a protein weight basis) or 90% (on a molar basis) of the amount of 240 000 M_r protein (table 1). This ratio was reproducibly obtained in different preparations and is consistent with that calculated from the elution profile of fig.1.

We had found that the particulate fraction derived from 1 kg brain can bind 4.7 μmol calmodulin in the presence of Ca^{2+} [3], which would account for ~20% of the total soluble calmodulin present in brain (table 1). This value is much greater than those reported for calmodulin-binding proteins obtained from the supernatant fraction since 0.61 and 0.03 μmol/kg brain were reported for heat-labile (calcineurin) and heat-stable, respectively, calmodulin-binding proteins [16] (table 1). The interaction between the particulate calmodulin-binding protein and calmodulin is reversible, depends upon μM levels of Ca^{2+}, and is quite specific to calmodulin since the binding was not displaced by a 400–1000-fold excess of other proteins such as troponin C [17]. From these results, ~2/3rds of this particulate calmodulin-binding activity can be attributed to the 240 000 and 155 000 M_r proteins (table 1). Both proteins were devoid of phosphodiesterase, ATPase and GTPase activities, but possessed the ability to bind F-actin. We have purified from a variety of mammalian tissues several cytoskeleton-related calmodulin-binding proteins [8,11,12,15,18]. The possible implication of the brain 240 000 and 155 000 M_r proteins in the Ca^{2+}- and calmodulin-dependent regulation of the cytoskeletal system with a relevance to the synaptic function is under investigation.

Table 1
Calmodulin-binding proteins in bovine brain

Protein	Amount		Ref.
	mg/kg	μmol/kg	
Calmodulin (EGTA-extractable form)	400	24	[2]
Particulate calmodulin-binding proteins	–	4.7 (100)[a]	[3]
240 000 M_r	370	1.6 (34)	[4]
155 000 M_r	220	1.4 (30)	This study
Soluble calmodulin-binding proteins			
85 000 M_r	52	0.61	[16]
70 000 M_r	0.2	0.03	[16]
Phosphodiesterase	10	0.07	[16]

[a] Numbers in parentheses are percentage values taking the total amount of the particulate form calmodulin-binding proteins as 100

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