IDENTITY OF THE PARTICULATE FORM OF CALMODULIN WITH SOLUBLE CALMODULIN

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1. Introduction

Discovery of Ca^{2+}-activatable phosphodiesterase [1] and subsequent demonstration of protein modulator which confers Ca^{2+}-sensitivity upon this enzyme [2,3] coincided with the discovery of a protein activator of brain phosphodiesterase [4]. However, it took several years before these two independent lines of the research merged when the identity of the two proteins as a Ca^{2+}-receptive protein was finally established [5–7]. This protein, now called calmodulin, is considered an intracellular mediator of the stimulus-linked actions of Ca^{2+} in the cell. Although calmodulin is generally regarded as a soluble protein, it was observed [8] that, when calmodulin was prepared from the brain homogenate, inclusion of EDTA in the homogenizing buffer greatly increased the yield of this protein in the soluble fraction. Subsequently, work in our laboratory clarified the existence of calmodulin-binding protein(s) of particulate nature that bind calmodulin in the presence of Ca^{2+} in the brain and other tissues [9,10].

However, an extensive extraction of the particulate fraction with EGTA could not solubilize all the calmodulin activity, leaving a certain amount of its activity in the insoluble fraction [9]. We shall henceforth refer to this residual activity as the particulate form of calmodulin. The concentration of this particulate form calmodulin decreased in hepatoma tissues when compared with the normal liver [11]. Since this change was accompanied by an increased level of the soluble form of calmodulin, the concentration ratio between these two forms in hepatoma tissues decreased to 0.1 from ~1.2 of the normal liver tissue [11]. Thus, the question was raised whether these two forms of calmodulin are the same entity. In the present investigation we obtained evidence indicating that they are the same.

Abbreviations: EDTA, ethylenediamine tetraacetic acid; EGTA ethyleneglycol bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonylfluoride

2. Materials and methods

2.1. *Materials*

DFP and PMSF were obtained from Sigma Chemical Co. Pepstatin A was obtained from Peptide Institute, Osaka. Fluphenazine maleate was a generous gift of the Shionogi Pharmaceuticals, Osaka. Fluphenazine–Sepharose 6B was prepared as in [12]. Calmodulin was prepared from the bovine brain as in [12].

2.2. *Preparation of the particulate form calmodulin*

The following procedure was carried out at 4°C. Fresh bovine brain (200 g) was homogenized in a Waring Blender with 9 vol. (1.8 litre) of 5 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM EGTA, 0.25 mM PMSF, 0.1 mM DFP and pepstatin A (0.05 μg/ml) (medium A). The homogenate was centrifuged at 900 × *g* for 10 min. Then the supernatant was centrifuged at 5000 × *g* for 30 min. The resulting supernatant was centrifuged at 105 000 × *g* for 60 min. The pellet was homogenized with 500 ml medium A and the homogenate was recentrifuged as above. This washing procedure was repeated once more and the final pellet (microsomal fraction) was homogenized with 1 litre medium B consisting of 20 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM EGTA, 0.25 mM PMSF, 0.1 mM DFP, 0.05 μg/

pepstatin A ml and Triton X-100 at 2% final conc. (v/v). The homogenate was stirred gently for 2 h at 4°C and then centrifuged at 105 000 × *g* for 60 min. The resulting pellet was dispersed in 500 ml medium B and then centrifuged as above. The supernatants derived from the two centrifugations (Triton sups 1 and 2; see table 1) were combined and applied to a DEAE-cellulose column (2.5 × 15 cm) equilibrated with medium C consisting of 20 mM Tris–HCl (pH 7.5), 0.1 mM EGTA, 5 mM 2-mercaptoethanol and 50 mM KCl. The column was eluted first, at 'a→' with medium C plus 0.145 M ammonium sulfate, then, at 'b→' with medium C plus 0.3 M ammonium sulfate (fig.1A). Fractions containing calmodulin activity (▬) were combined and then diluted 2-fold by the addition of an equal volume of medium C containing no KCl. $CaCl_2$ was added to the solution to make unchelated Ca^{2+} 0.2 mM. The solution was then applied to a column containing 2 ml of fluphenazine–Sepharose 6B equilibrated with 20 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 0.5 M NaCl and 0.2 mM $CaCl_2$ (medium D). The column was eluted with medium D. At 'c→' 0.2 mM $CaCl_2$ in the medium D was replaced by 1 mM EGTA (fig.1B). The calmodulin activity peak was collected and concentrated.

2.3. *Other methods*

Assay procedures for protein and calmodulin activity in terms of the activation of the bovine brain calmodulin-deficient phosphodiesterase activity were as in [13]. Electrophoresis of proteins was done in 15% polyacrylamide gels in the presence of either 0.2 mM $CaCl_2$ or EGTA at pH 8.3 [13]. Lactate dehydrogenase activity was determined as in [14].

3. Results

The particulate form of calmodulin was solubilized from the microsomal fraction of the bovine brain tissue with 2% Triton X-100 and purified by chromatographies using DEAE-cellulose and fluphenazine–Sepharose 6B columns (fig.1A,B). Its elution profiles from these columns were identical to those of the soluble calmodulin. An overall purification of 1700-fold was achieved with a recovery of the protein to be ~80% of the total activity found in the microsomal fraction (table 1). The purified protein was homogenous upon polyacrylamide gel electrophoresis in the presence of either Ca^{2+} or EGTA (fig.2), or sodium dodecyl sulfate (not shown). The particulate form calmodulin showed identical mobilities with the soluble calmodulin upon these electrophoreses (fig.2). Both the particulate form and soluble form of calmodulin activated calmodulin-deficient brain phosphodiesterase with the identical activation curves plotted

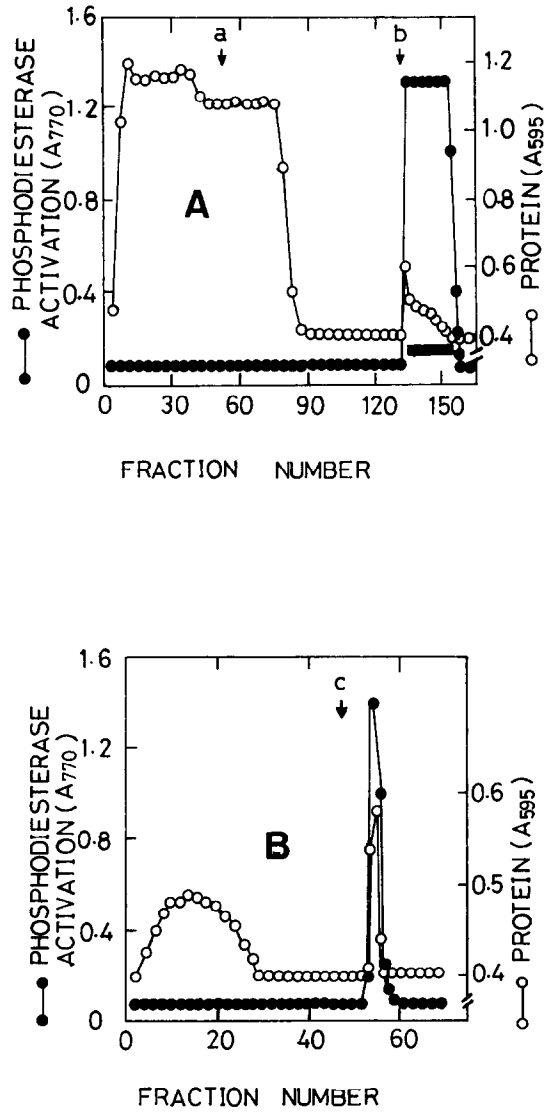

Fig.1. Purification of the particulate form calmodulin by DEAE-cellulose (A) and fluphenazine affinity (B) column chromatographies: (A) fractions of 22 ml each (no. 1–130) or 9 ml each (no. 131–); (B) fractions of 4 ml each (no. 1–50) or 1 ml each (no. 51–) were collected.

Table 1
Summary of purification

Fraction	Protein (mg)	Calmodulin activity[a] (unit/mg protein)	(total unit)	Yield (%)
Microsomal fraction	2594[b]	0.59	1530	100
Triton sup 1	1183	1.21	1431	93.5
Triton sup 2	202	0.25	51	3.3
Triton pellet	1194	n.d.[c]	n.d.	–
DEAE column	34	39.8	1353	88.4
Fluphenazine column	1.2	1011	1213	79.3

[a] One unit of calmodulin activity was defined as the amount equivalent to 1 μg of the purified soluble form calmodulin (bovine brain) in the activation of the calmodulin-deficient brain phosphodiesterase
[b] Derived from 200 g fresh bovine brain; [c] n.d., not detectable

between activation and [protein] (fig.3A) and between activation and [Ca^{2+}] (fig.3B).

The brain microsomal fraction used here and in [9] contained disrupted synaptic membranes and vesicles but was devoid of the intact nerve-ending particles which contain cytoplasm within them. The use of hypo-osmotic medium in combination with a vigorous homogenization at the initial step of the preparation disrupted all such structures. The absence of the intact nerve-ending particles in the microsomal fraction was monitored by the electron microscopic examination and the lactate dehydrogenase activity determination (a marker enzyme for the cytoplasm).

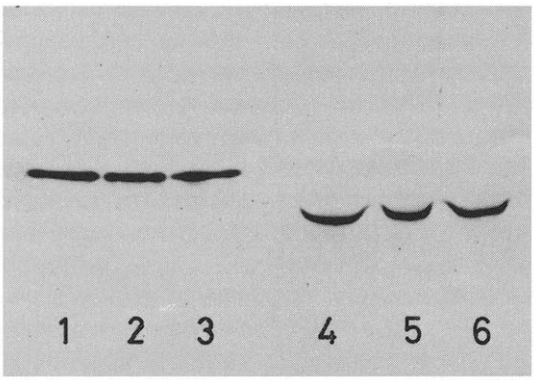

Fig.2. Polyacrylamide gel electrophoresis of proteins in the presence of either $CaCl_2$ (1–3) or EGTA (4–6): (1,4) 5 μg soluble calmodulin; (2,5) 5 μg protein purified from the microsomal fraction; (3,6) 2.5 μg each of both proteins.

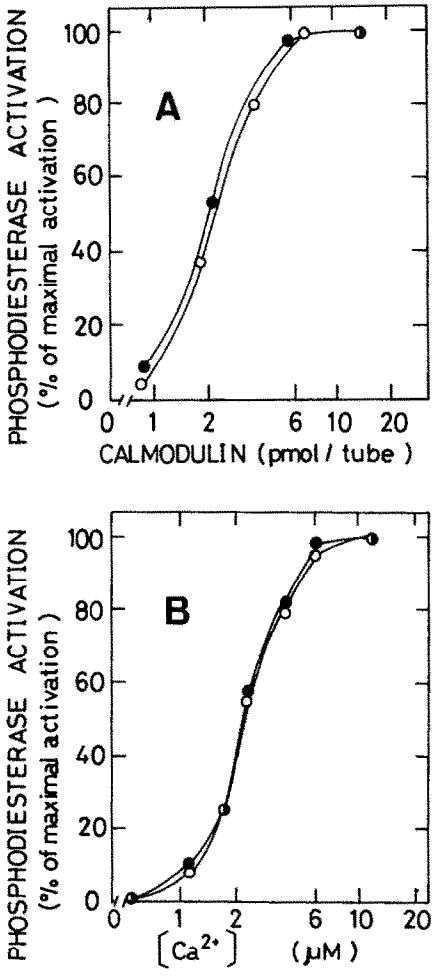

Fig.3. Ca^{2+}-dependent activation of brain phosphodiesterase by calmodulin samples: (A) 0.1 mM $CaCl_2$ or (B) 0.35 μg calmodulin protein was added; (○) soluble calmodulin; (●) protein purified from the microsomal fraction.

4. Discussion

There are two different pools of the particle-associated calmodulin. The 'true' particulate form of calmodulin should be distinguished from the calmodulin which is bound to the calmodulin-binding protein present in the particulate fraction. While the solubilization of the former type required the presence of nonionic detergents, the latter type calmodulin was easily released into the soluble fraction by the exposure of the particulate fraction to a medium containing EGTA [9,10]. The activity of the particulate form of calmodulin (in terms of the ability to activate brain phorphodiesterase), which was latent to a certain extent and was unmasked by the action of nonionic detergents, was found in a variety of tissues that include brain, adrenal gland, liver, kidney and testis (S. K., unpublished). Upon subcellular fractionation of the rat brain tissue, >80% of this particulate form activity was recovered in the microsomal fraction, its specific activity in the microsomal fraction being >10-times that in the mitochondrial fraction [9]. Here, the particulate form calmodulin was purified from the microsomal fraction of bovine brain.

We now provide evidence indicating that the particulate form of calmodulin is identical to the soluble calmodulin. Both were indistinguishable from each other in the two principal criteria for calmodulin [13]; i.e., the mobilities upon electrophoreses in the presence and absence (+EDTA) of Ca^{2+} and the activation of the calmodulin-deficient phosphodiesterase activity by means of its quantitative determination. Thus, the identical specific activity values were obtained for both forms of the protein (table 1). Moreover, the elution profiles of the particulate form of calmodulin from DEAE-cellulose and fluphenazine affinity columns exactly coincided with those of the soluble calmodulin. Here, the yield of the particulate form of calmodulin was ~80% of the original activity found in the microsomal fraction, and no secondary peaks of calmodulin-like activity, other than the major peak, were detected upon the column chromatographies (figs.1A,B). In [15] an acidic Ca^{2+}-binding protein was obtained from rat synaptic plasma membranes which is capable of activating phosphodiesterase but apparently different from calmodulin. According to [15], ~3% of the synaptic membrane protein was accounted for by this protein. However, we were unable to detect such activity from the microsomal fraction rich in the synaptic membranes. The reason for this discrepancy is unclear.

Although the exact mechanism through which the particulate form of calmodulin is linked to the particles remains unclear; this form of calmodulin seems to be embedded in the membrane matrix or tightly bound to an intrinsic membrane protein. The reasons for this are:

(i) Its solubilization requires the presence of nonionic detergents;
(ii) Tryptic digestion of the microsomal fraction failed to solubilize this form of calmodulin while, under the identical condition, the calmodulin bound to the particulate calmodulin-binding protein was solubilized completely [9].

In hepatoma tissues the calmodulin activity decreased in the particulate fraction with a concomitant increase of its activity in the soluble fraction [11]. This strongly suggests the translocation of calmodulin from the particles to the cytoplasm in rapidly growing cells. Using Chinese hamster ovary cells [16] a cell population density-dependent increase in the calmodulin concentration in the supernatant was observed with a concomitant decrease in the particulate fraction. The physiological significance of the translocation of calmodulin between the soluble and particulate phases of the cell remains to be elucidated.

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