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PURIFICATION AND CHARACTERIZATION OF 81K, HEAT STABLE CALMODULIN-BINDING PROTEIN FROM BOVINE BRAIN

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Heat stable calmodulin-binding protein has been purified from Triton X-100 soluble particulate fraction of bovine brain. Considerable purification was achieved with calmodulin coupled Sepharose 4B affinity chromatography. SDS-PAGE of the purified protein revealed the apparent homogeneity being 92 % at Mr 81,000. Isoelectric focusing of purified 81K protein gave isoelectric point of 4.3. The amino acid composition was notable for high contents of acidic amino acids (15.0 mol% of glutamic acid and 8.1 mol% of aspartic acid) and 17.4 mol% of alanine. On alkaline 1 M urea gel electrophoresis, mobility of the purified 81K protein in the presence of Ca^{2+} and calmodulin became lower than 81K protein alone toward the anode; however, Ca^{2+} solely did not affect the mobility of this protein. Similarly, S-100 protein and troponin C showed the interaction with 81K protein and a decrease of mobility in the presence of Ca^{2+} in alkaline urea PAGE. Binding assay of ^{125}I-labeled calmodulin revealed that 81K protein could bind to an equimolar of ^{125}I-calmodulin as apparent dissociation constant (Kd) of $0.65 \times 10^{-6}M$.

Calmodulin has been recognized to play an important role in physiological responses in many tissues or cells and to mediate a number of actions of Ca^{2+} as an intracellular messenger. We reported the possibility that Ca^{2+} binding produced conformational

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[2] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CaM, calmodulin; MAP2, microtuble associated protein 2.

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changes of calmodulin that exposed hydrophobic regions on the surface of the calmodulin molecule and that acted as active sites of interaction with its binding proteins using calmodulin antagonists and hydrophobic fluorescent probes (1,2). It is known that Ca^{2+}-calmodulin complex regulates the activity of various enzymes including phosphodiesterase (3), adenylate cyclase (4), phosphorylase b kinase (5), myosin light chain kinase (6), Ca^{2+}/CaM dependent protein kinase II (7) and calcineurin (8). One of other functions of calmodulin is thought to modulate interactions between cytoskeletal proteins. It was reported that calmodulin exhibited a potent inhibitory effect on microtuble assembly in the presence of Ca^{2+} (9) and inhibited both MAP2-actin and Tau-actin interactions (10). However, existence of other calmodulin-binding proteins and their roles are still obscure. We report here that a new calmodulin binding protein was purified from the membrane fraction of bovine brain and was characterized about its calmodulin binding ability.

Materials & Methods

Materials : Catalytic subunit of cAMP-dependent protein kinase from bovine heart was purified by the method of Beavo et al (11). Calmodulin and S-100 protein were purified from bovine brain (12). Rabbit skeletal muscle troponin C was prepared as described (13). CaM coupled Sepharose 4B was prepared as described (14). Calmodulin was iodinated with Bolton-Hunter reagent according to manifactual instructions (15). Specific activity of the ^{125}I-CaM solution was 7.6 x 10^4 cpm/μl. Anti-caldesmon$_{77}$ monoclonal antibody was kindly provided from Dr. Ohwada, M. K. (Kyoto Pharmaceutical Univ.).

Purification of 81K protein from bovine brain : All procedures were carried out at 4 °C unless otherwise indicated. Bovine brain (500 g) stored at -80 °C was thawed and homogenized with 2 volumes of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 10 mM EGTA, 50 mg/*l* trypsin inhibitor, 0.1 mM PMSF, 10 mg/*l* leupeptin, 0.5 mM DTT (Buffer A) in a Mixer. The homogenate was centrifuged at 10,000 x g for 30 min. The pellet fraction was rehomogenized with 2 volumes of Buffer A containing 1.5 % Triton X-100, and then centrifuged at 30,000 x g for 30 min. Supernatant was heated in a boiling water bath at 100 °C for 15 min and immediately cooled down in ice for at least 15 min. The heat treated solution was centrifuged at 30,000 x g for 30 min. Solid ammonium sulfate (30% saturation) was slowly added to the supernatant. The precipitate that formed was removed by centrifugation at 10,000 x g for 30 min. To the supernatant fluid, additional ammonium sulfate was added to give a final concentration of 70 % (w/v). The precipitate was recovered by centrifugation at 10,000 x g for 30 min and was resuspended with 75 ml of 20 mM

Tris-HCl (pH 7.5), 1.0 % Triton X-100 (Buffer B) and dialyzed against 4 *l* of buffer B overnight. Dialyzed sample was applied to DEAE-cellulose column (Whatman DE52, 3 x 19 cm), previously equilibrated with Buffer B and then the column was washed with 1 *l* of Buffer B. Elution was carried out with a salt linear gradient of NaCl (0-1.0 M; total volume, 1*l*). The eluted fractions were phosphorylated by catalytic subunit of cAMP-dependent protein kinase. A major phosphoprotein judged by autoradiography was eluted at 0.24 M to 0.34 M of NaCl concentration. The corresponding fractions were pooled and concentrated with ultrafiltration (Amicon UK-10) to 12 ml. Concentrated fraction was dialyzed overnight against two changes (1 *l* each) of 0.6 M NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA (Buffer C). Dialyzed fraction was loaded onto a column of Superose 6 (1.6 x 50 cm) previously equilibrated with Buffer C. A single peak of phosphorylatable 81K protein by cAMP-dependent protein kinase was pooled and dialyzed overnight against 3 *l* of 0.2 M NaCl, 40 mM Tris-HCl (pH 7.5), 0.2 mM $CaCl_2$ (Buffer D). A half of the dialyzed sample was applied to a column of CaM coupled Sepharose 4B (1.5 x 5 cm) previously equilibrated with Buffer D. The column was washed with 120 ml of Buffer D containing 1 M NaCl to remove nonspecifically bound proteins and washed again with 36 ml of Buffer D. Elution of calmodulin binding protein was carried out with 50 ml of solution containing 0.2 M NaCl, 40 mM Tris-HCl (pH 7.5), 2 mM EGTA.

Other Methods : The phosphorylation of the preparation by cAMP-dependent protein kinase was carried out as described previously (16). 10% SDS-PAGE was carried out according to the method of Laemmli (17). Calmodulin binding property of 81K protein was analyzed on 7.5 % polyacrylamide gel with 1 M urea in the presence of 2 mM $CaCl_2$ or 2 mM EGTA using a Tris-glycine buffer (pH 8.3) system as described by Head and Perry (18). Isoelectric point of purified 81K protein was determined by isoelectric focusing (19) on a LKB FBE-3000 apparatus. Amino acid analysis was performed on a Biotronic LC-5000 amino acid analyzer. Protein sample was hydrolyzed in 6N HCl at 100 °C for 24 hrs (20). Protein was determined by the method of Bradford (21) using bovine serum albumin Fr. V as a standard.

Results

Purification of 81K protein by calmodulin coupled Sepharose 4B chromatography : 81K, calmodulin binding protein was purified to apparent homogeneity from bovine brain membrane fraction. The purification procedure included Triton X-100 extraction, heat treatment, ammonium sulfate precipitation and chromatography on DEAE-cellulose, Superose 6 and CaM coupled Sepharose 4B. The final step of purification was affinity chromatography on a column of CaM coupled Sepharose 4B (Fig. 1A). The major peak of 81K protein

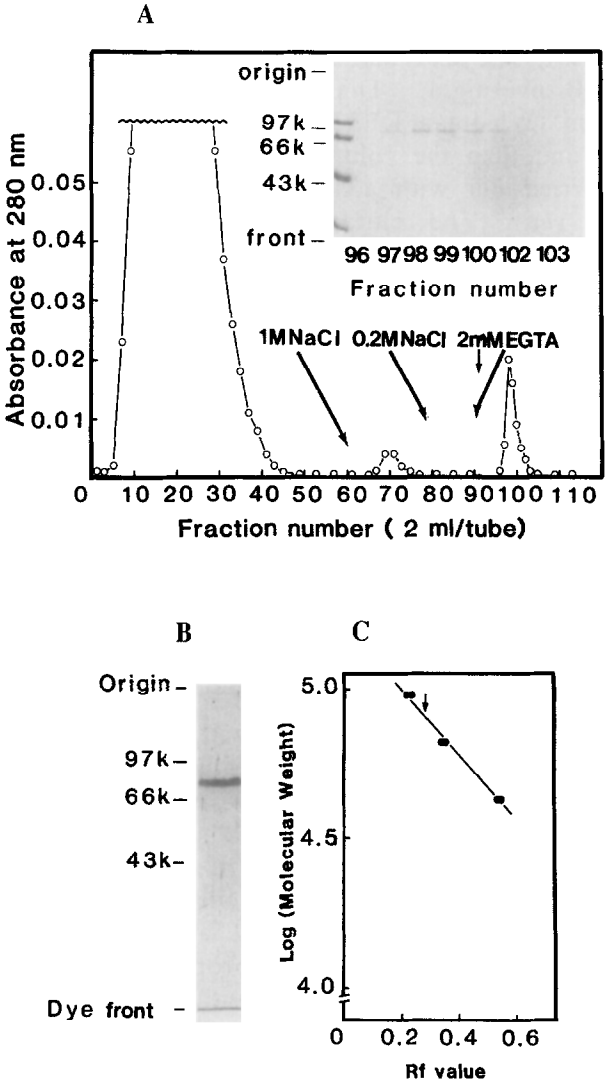

Fig. 1. Purification of bovine brain 81K protein by calmodulin-affinity chromatogaraphy.
A: Representative elution profile of calmodulin affinity chromatography of bovine brain 81K protein, ○ , absorbance of 280 nm. inset; 10 % SDS-PAGE of eluted fractions.
B: Commassie Blue staining of 10 % SDS-PAGE of purified 81K protein.
C: Determination of molecular weight of 81K protein. Molecular weight marker: upper, phosphorylase b: Mr. 97,400; middle, albumin: Mr. 66,200; lower, ovalbumin; Mr. 42,699.
81 K protein was indicated by arrow.

(fraction No.96 to 102) was eluted when 2mM EGTA was introduced into the eluting buffer (see Materials & Methods). The peak of 81K protein was pooled, divided into 0.1ml aliquots and kept frozen at -80 °C until ready to use. Approximately 160μg of purified 81K protein could be prepared from 500g of bovine brain. SDS-PAGE

revealed that the presence of a single polypeptide with a Mr of 81,000 estimated by comparison with proteins known Mr and that more than 92% of purity was achieved (Fig. 1B, 1C). Isoelectric focusing of purified 81K protein gave isoelectric point of 4.3. Thus 81K protein was very acidic. The amino acid composition of 81K protein was notable for high contents of acidic amino acids (8.1 mol % of aspartic acid and 15.0 mol % of glutamic acid) and 17.4 mol % of alanine, accounting for the pI value (4.3).

Calmodulin binding property of 81K protein : To test the Ca^{2+}-dependent interaction between purified 81K protein and calmodulin, alkaline 1M urea PAGE was carried out in the presence and absence of Ca^{2+} (Fig. 2A). The mobility of 81K protein on the gel was not altered in the absence or presence of Ca^{2+} (Fig. 2A lanes c and d). In the presence of Ca^{2+} and calmodulin, 81K protein formed a complex with calmodulin and migrated toward the anode with lower mobility than 81K protein alone (Fig. 2A lanes e and f). Similarly to calmodulin, S-100 protein and troponin C showed in alkaline 1M urea PAGE a decrease of mobility in the presence of Ca^{2+} and an interaction with 81K protein (Fig. 2B, lanes g-j). These findings may suggest that the conservative regions of each Ca^{2+}-binding protein

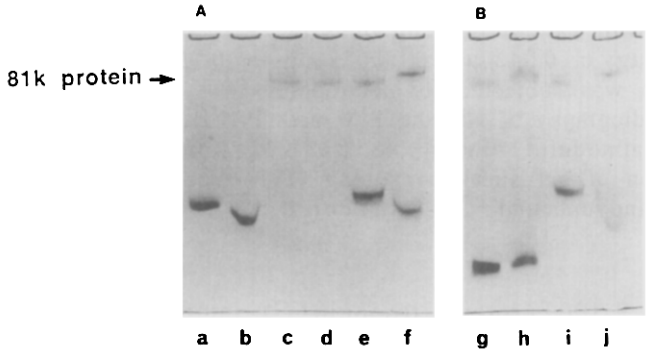

Fig. 2. Interaction of bovine brain 81K protein with Ca^{2+}-binding proteins.

Electrophoresis was carried out using 7.5% polyacrylamide gel containing 1 M urea as described "Materials and Methods". Samples were incubated at 30°C for 30 min in a solution containing 1 M urea, 20 mM Tris-glycine (pH 8.3) in the presence of 2 mM $CaCl_2$ (lanes b, d, f, h and j) or in the presence of 2 mM EGTA (lanes a, c, e, g and i) and subjected to electrophoresis. Gels were stained with Commassie Blue.

A: lanes a and b, calmodulin (0.8 μg); c and d, purified 81K protein (0.5 μg); lanes e and f purified 81K protein (0.5 μg) + calmodulin (0.8 μg).

B: lanes g and h, purified 81K protein (0.5 μg) + S-100 protein (1.25 μg); lanes i and j, purified 81K protein (0.5 μg) + troponin C (1 μg).

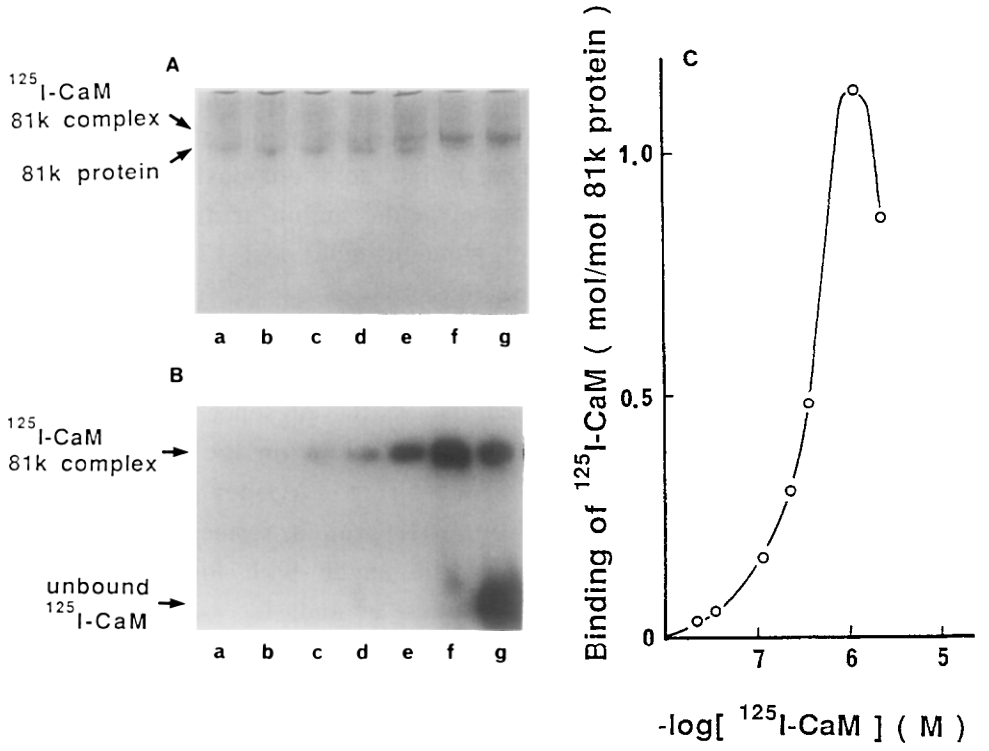

Fig. 3. Interaction of bovine brain 81K protein with various concentrations of ^{125}I-calmodulin.
A: Commassie staining of alkaline 1 M urea PAGE in the presence of 2 mM $CaCl_2$ carried out as described under Fig. 2 legend. 81K protein (1.2 μg) with various concentrations of ^{125}I-calmodulin (a, 0.008 μg; b, 0.012 μg; c, 0.042 μg; d, 0.082 μg; e, 0.124 μg; g, 0.416 μg; g, 0.824 μg) was subjected to electrophoresis.
B: Autoradiography of alkaline 1 M urea PAGE.
C: ^{125}I-calmodulin binding to 81 K protein was determined by quantitation of gel slices containing of ^{125}I-calmodulin-81K protein complex and unbound ^{125}I-calmodulin.

(calmodulin, S-100 protein, troponin C) interacted with 81K protein in a Ca^{2+}-dependent manner. The stoichiometry of 81K protein-calmodulin interaction was determined by alkaline 1M urea PAGE using ^{125}I-calmodulin. Purified 81K protein (1.2μg) was incubated with ^{125}I-calmodulin in the presence of 2mM $CaCl_2$ at 30 °C for 30min and then subjected to electrophoresis. Commassie Blue staining of the gel showed that 81K protein formed a complex with ^{125}I-calmodulin in a dose-dependent manner. Figure 3B shows the autoradiography of the gel above described. For quantitation of ^{125}I-calmodulin binding, both ^{125}I-calmodulin-81K protein complex and free ^{125}I-calmodulin localized by Commassie Blue staining and autoradiography were excised from the gel and ^{125}I radioactivity

was measured by liquid scintillation counting. Figure 3C is a titration curve showing the effect of increasing concentration of ^{125}I-calmodulin on the ^{125}I-calmodulin binding of 81K protein. The figure showed that 81K protein could bind to an equimolar of ^{125}I-calmodulin. When the data plotted according to the method of Scatchard, the apparent dissociation constant (Kd) of 81K protein for ^{125}I-calmodulin was calculated to be 0.65 x 10^{-6}M.

Discussion

We described here the purification procedure and its calmodulin binding ability of 81K protein from bovine brain. In our purification steps, the buffer used for Superose 6 gel filtration step contained 2mM EGTA is necessary to isolate the calmodulin binding 81K protein. When the buffer omitted EGTA was used, most of proteins eluted from Superose 6 column were not adsorbed to CaM affinity column. It may suggest that contaminated Ca^{2+}/calmodulin already bound to 81K protein. The properties of bovine brain 81K protein such as heat stability and high contents in the non-ionic detergent soluble fraction suggested that this protein was one of the membrane bound cytoskeletal proteins. In neuronal cytoskeletal proteins which molecular weight range were between 80 K-90 K, Synapsin I (22) and caldesmon$_{77}$ (23) were reported about their calmodulin binding ability. However, Synapsin I was a basic protein (pI=10.3, 10.2) and was composed of two related subunits (Synapsin Ia, 86 kDa; Synapsin Ib, 80 kDa). By western blotting, anti-caldesmon$_{77}$ antibody did not cross-react with purified 81K protein. These data strongly suggested that 81K protein was a new calmodulin binding protein in bovine brain. Furthermore, we have found that 81K protein in each purification step was phosphorylated not only with cAMP-dependent protein kinase but also with protein kinase C. Further study will reveal the effect of phosphorylation on calmodulin binding ability of this protein.

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