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Ca^{2+}-INDUCED HYDROPHOBIC SITE ON CALMODULIN: APPLICATION FOR PURIFICATION OF CALMODULIN BY PHENYL-SEPHAROSE AFFINITY CHROMATOGRAPHY

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Calmodulin binds quantitatively to phenyl-Sepharose and octyl-Sepharose affinity columns in the presence of micromolar concentrations of Ca^{2+}. In addition to EGTA, calmodulin also can be eluted from these affinity columns with low ionic strength buffer, non-ionic detergent (i.e., 1% Triton X-100), or ethylene glycol (50%), suggesting hydrophobic interaction. Using hydrophobic interaction chromatography calmodulin can be purified to homogeneity from bovine brain homogenate in a single step. For large-scale purification the protein fraction containing calmodulin was concentrated by isoelectric precipitation prior to application to the affinity column. The yield obtained by this procedure (160-180 mg calmodulin per kg brain) is significantly greater, and the time required ($\sim$ 5 hr) is substantially less, than that of previously described procedures for calmodulin purification. It is apparent that phenyl-Sepharose offers several advantages over phenothiazine-Sepharose for affinity purification of calmodulin.

INTRODUCTION

Calmodulin is a low molecular weight Ca^{2+}-binding protein which serves to mediate numerous Ca^{2+}-regulated enzyme systems and cellular processes (see refs. 1, 2). The site(s) through which calmodulin interacts with target enzymes, and the nature of this interaction, are not clearly understood. Norman (3) noted a correlation between octanol:water partition coefficients of antipsychotic phenothiazine drugs and their ability to inhibit calmodulin activation of cyclic nucleotide phosphodiesterase, and suggested these drugs probably bind to calmodulin through hydrophobic interaction. This was supported by the demonstration of a Ca^{2+}-induced hydrophobic region on calmodulin in experiments using a fluorescent probe for hydrophobic sites (4).

By employing hydrophobic interaction chromatography (agarose conjugated with hydrophobic ligands) it is possible to demonstrate hydrophobic sites on

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proteins and to determine the relative strength of hydrophobicity. This information can be extended to purification of the protein of interest (5, 6). In this communication results are presented of studies designed to characterize the Ca^{2+}-sensitive hydrophobic binding site of calmodulin using phenyl- and octyl-Sepharose. The rapid purification of calmodulin to homogeneity, and in high yield, can be achieved in a single step using this hydrophobic interaction chromatography.

MATERIALS AND METHODS

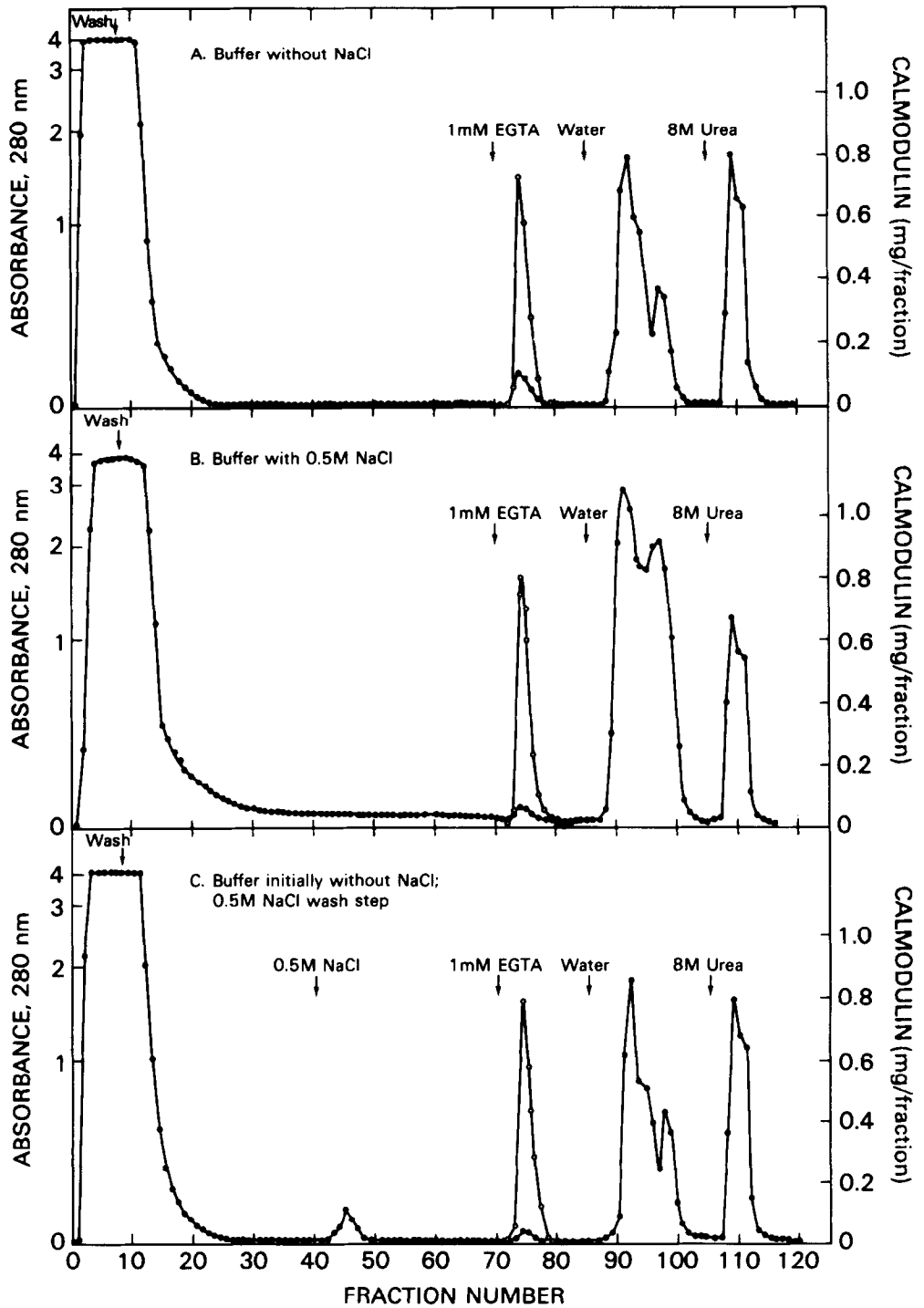
Materials--Phenyl-Sepharose and octyl-Sepharose (each at 40 μmol ligand per ml), and Sepharose 4B were obtained from Pharmacia. 2-Chloro-10(3-aminopropyl) phenothiazine was kindly provided by Dr. Albert Manian, Psychopharmacology Research Branch, National Institute of Mental Health. Calmodulin-dependent bovine brain cyclic nucleotide phosphodiesterase was a generous gift from Dr. Claude Klee, National Cancer Institute.

Preparation of phenothiazine-Sepharose--Phenothiazine was coupled through its amino group to Sepharose 4B using either the cyanogen bromide activation procedure (7) or the bisoxirane method (8). The amount of phenothiazine coupled was estimated after hydrolyzing the gel (8).

Purification of calmodulin from bovine brain--Unless otherwise stated, all procedures were carried out at 4°C and centrifugations were at 15000 g for 30 min. Bovine brain from Pel Freeze (~ 300 gms) was homogenized with a Waring blender in two volumes of buffer A (0.05 M Tris-HCl, pH 7.5-1 mM EDTA-1 mM 2-mercaptoethanol-0.5 mM phenylmethylsulfonyl fluoride). The pellet obtained after centrifugation of this homogenate was re-extracted as above and the combined supernatants were passed through four layers of cheese cloth to remove lipids. This tissue extract was used for phenyl-Sepharose chromatography directly, or was concentrated by isoelectric precipitation or 50% ammonium sulfate precipitation at pH 4.3 (9) to facilitate purification from greater amounts of tissue (up to 1 kg).

For isoelectric precipitation, 6 M acetic acid was added dropwise to the supernatant with constant stirring to adjust to pH 4.3. After 1 hr at pH 4.3 precipitated protein was recovered by centrifugation and the supernatant discarded. The pelleted protein was resuspended in a minimal volume of buffer A (300 to 400 ml per kg brain) and the pH adjusted to 7.5 with 1 M Tris base. This suspension was centrifuged to remove insoluble proteins. The supernatant was subjected to hydrophobic interaction chromatography directly, or after heat denaturation at 100°C for 5 min; heat denatured protein was removed by centrifugation after cooling the preparation to 4°C in ice.

Hydrophobic interaction chromatography--This was carried out at room temperature using either a phenyl- or octyl-Sepharose column (15 ml bed vol.; 1.5 x 8.8 cm) equilibrated with buffer I (0.05 M Tris-HCl, pH 7.5-1 mM 2-mercaptoethanol-0.1 mM $CaCl_2$). Prior to application sufficient 0.1 M $CaCl_2$ was added to the sample to adjust to 5 mM $CaCl_2$ final concentration. This sample was applied to the column and the column then washed with 10 bed volumes of buffer I followed by another wash with 10 bed volumes of buffer I containing 0.5 M NaCl. Calmodulin then was eluted from the column with modified buffer I (containing 1 mM EGTA in place of 0.1 mM $CaCl_2$). For renewal the column was washed with water, then with 8 M urea, to remove other pro-


Wash
A. Buffer without NaCl
1mM EGTA
Water
8M Urea
ABSORBANCE, 280 nm
CALMODULIN (mg/fraction)
Wash
B. Buffer with 0.5M NaCl
1mM EGTA
Water
8M Urea
ABSORBANCE, 280 nm
CALMODULIN (mg/fraction)
Wash
C. Buffer initially without NaCl;
0.5M NaCl wash step
0.5M NaCl
1mM EGTA
Water
8M Urea
ABSORBANCE, 280 nm
CALMODULIN (mg/fraction)
0 10 20 30 40 50 60 70 80 90 100 110 120
FRACTION NUMBER

teins bound hydrophobically to the column (see Fig. 1), and finally reequilibrated with buffer I. To decrease even further the time required for column elution, or to scale up the procedure for large-scale purification, the above steps can be carried out batch-wise using a coarse scintered glass funnel to retain the phenyl-Sepharose.

RESULTS AND DISCUSSION

Initial results indicated that calmodulin would bind to phenyl-Sepharose in the presence of Ca^{2+} ($\geq$ 10 μM) but would not bind in the presence of EGTA or in the presence of other divalent metal ions, including Mg^{2+}. Calmodulin also was found to bind in a Ca^{2+}-dependent manner to octyl-Sepharose, indicating that this interaction is not restricted to aromatic compounds. To study the nature of calmodulin binding to phenyl-Sepharose we determined the ability of various agents to elute bound calmodulin from the column within 3 column volumes (unpublished results). As presented in Fig. 1C, calmodulin was not eluted with 2 M NaCl, indicating that binding is not due to ionic interactions. When Triton X-100 solutions of 0.2, 0.5, and 1% were used, only the highest (1%) concentration eluted calmodulin. Ethylene glycol (50%) also was found to elute calmodulin; 10% and 20% ethylene glycol were ineffective. Temperature also alters binding; when applied to the column at room temperature calmodulin is completely adsorbed to the matrix, while at 4°C binding was incomplete. The temperature dependency for binding, and the elution of calmodulin by detergents and low polarity solvents, but not by high ionic strength buffers, clearly indicate that this binding must be due to hydrophobic forces.

When brain extract was applied to a phenyl-Sepharose column in buffer I the majority of protein was not adsorbed (Fig. 1). Washing the column with

Fig. 1. Phenyl-Sepharose chromatography of calmodulin from brain extract. A, brain supernatant (50 ml, 280 mg protein) was applied to a phenyl-Sepharose column (1.5 x 8.8 cm) equilibrated with buffer I. The column was washed with this same buffer as indicated. Calmodulin was eluted with buffer I containing 1 mM EGTA in place of 0.1 mM Ca^{2+}. Remaining bound proteins were eluted with water, followed by 8 M urea as shown. B, conditions were as in panel A except that the column was equilibrated and washed with buffer I containing 0.5 M NaCl. C, conditions were as in panel A with column equilibration and initial wash with buffer I as noted. The column then was washed with buffer I containing 0.5 M NaCl prior to elution of calmodulin. Fractions of 5 ml were collected through fraction number 70; thereafter 2.5 ml fractions were collected. Protein (●—●) was estimated by 280 nm absorbance. Calmodulin (○—○) was determined by the cyclic nucleotide phosphodiesterase assay (10).

buffer I does lower the 280 nm absorbance of eluted fractions to base level (Fig. 1A). However, following this wash procedure, the calmodulin eluted with 1 mM EGTA contained several minor contaminants as demonstrated by SDS-polyacrylamide gel electrophoresis (Fig. 2, line 2). To prevent basic proteins present in the extract from binding to acidic (pI 4.3) calmodulin adsorbed to the column, the supernatant was applied to a phenyl-Sepharose column equilibrated at higher ionic strength (buffer I containing 0.5 M NaCl), and then washed with this high ionic strength buffer (Fig. 1B). This wash did not lower the 280 nm absorbance of eluted fractions to baseline even after washing with 20 column volumes. Again, calmodulin eluted from the washed column with 1 mM EGTA contained several minor impurities (Fig. 2, lane 3). Since these same contaminating proteins were detected in the 0.5 M NaCl-buffer I wash fractions, these proteins do not appear to bind to the column in a Ca^{2+}-dependent manner.

To obtain homogeneous calmodulin the brain extract was applied to a phenyl-Sepharose column equilibrated with buffer I, and then washed with buffer I (10 column volumes) to elute proteins bound through low affinity hydrophobic interaction. This was followed by a wash with 10 column volumes of high ionic strength buffer I (containing 0.5 M NaCl) to elute proteins bound through ionic interactions to the adsorbed calmodulin (Fig. 1C). Following these two wash steps the calmodulin eluted with 1 mM EGTA contained a single minor contaminant of low molecular weight (Fig. 2, lane 5). This contaminant was removed by dialysis versus a solution containing 1 mM EGTA and 0.2 M NaCl (Fig. 2, lane 6).

To purify calmodulin from greater amounts of tissue it is advisable to concentrate, and partially purify, the calmodulin-containing preparation prior to introduction to the hydrophobic column. This saves time in sample application, and also reduces the size of the column required. The amount of homogeneous calmodulin obtainable from 1 kg brain by application of crude supernatant directly to the phenyl-Sepharose column, or by application after concentrating the preparation by isoelectric precipitation or ammonium sul-

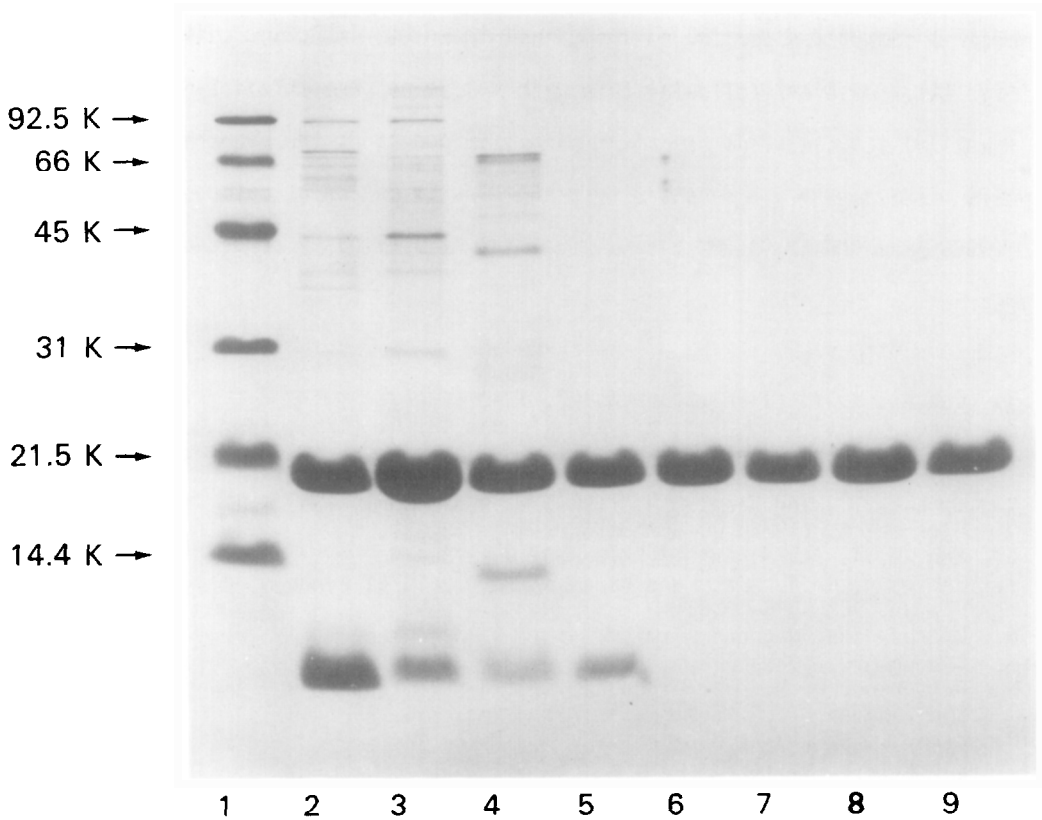


Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of purified bovine brain calmodulin preparations. Preparations of calmodulin (25 to 35 µg protein) were electrophoresed on 15% slab gels in the presence of 1 mM EGTA by the method of Laemmli (12), and stained with Coomassie brilliant blue. Lane 1, molecular weight standards. Lanes 2 to 6, calmodulin preparations obtained by direct application of supernatant to the phenyl-Sepharose column and [2] washing with buffer I; [3] washing with buffer I containing 0.5 M NaCl; [4] washing with buffer I containing 2 M NaCl; [5] washing first with buffer I, followed by a wash with buffer I containing 0.5 M NaCl, and [6] same as 5, with the eluted sample dialyzed *versus* 0.2 M NaCl and 1 mM EGTA. Lanes 7 and 8, supernatants were concentrated by isoelectric precipitation or NH_4SO_4 precipitation, respectively, prior to column purification as in lane 5; lane 9, supernatant was concentrated by isoelectric precipitation and purified by batch phenyl-Sepharose affinity chromatography.

fate precipitation, was 180, 130, and 170 mg, respectively. Among the various concentration procedures attempted, isoelectric precipitation was found to be preferable; this method allowed for 70 to 80% recovery of calmodulin while removing 70% of contaminating proteins. Calmodulin recoveries of at least 95% were attained with the phenyl-Sepharose chromatography step.

Phenothiazine-Sepharose previously has been used to purify calmodulin (7, 8). However, several difficulties are encountered when using phenothiazine including insolubility of the drug during coupling, photosensitivity,

and problems with covalent interaction of the drug with calmodulin. Phenyl-Sepharose thus offers an attractive alternative to phenothiazine-Sepharose for affinity purification of calmodulin. In addition, the present procedure using phenyl-Sepharose allows a more rapid purification of calmodulin to homogeneity, and in higher yield, than that of previously reported methods.

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