Calcium-dependent Affinity Chromatography of S-100 and Calmodulin on Calmodulin Antagonist-coupled Sepharose*

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Two different calcium-binding proteins, S-100 and calmodulin, have been isolated from bovine brain by calcium-dependent affinity chromatography on calmodulin antagonist-coupled Sepharose. Calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) has been coupled to epoxy-activated Sepharose 4B or cyanogen bromide-activated Sepharose 4B. S-100-like protein bound to W-7 coupled to epoxy-activated Sepharose 6B in the presence of Ca$^{2+}$ was eluted with the buffer containing 4 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) instead of Ca$^{2+}$. This protein did not stimulate cyclic nucleotide phosphodiesterase and was identified as S-100 by demonstrating its cross-reactivity with rabbit antisera to bovine S-100. Amino acid composition together with electrophoretic evidence shows that S-100-like protein is a mixture of S-100a and S-100b. Calmodulin-like protein bound to W-7 coupled to cyanogen bromide-activated Sepharose 4B in the presence of Ca$^{2+}$ was eluted with buffer containing 4 mM EGTA. This protein could stimulate cyclic nucleotide phosphodiesterase and had an amino acid composition and electrophoretic mobility very similar to those of calmodulin, indicating that this protein is calmodulin. When N-4-aminohexyl)-1-naphthalene sulfonamide (W-5), a chloro-deficient analogue which has lower affinity for calmodulin than W-7, was coupled to epoxy-activated Sepharose 6B, calmodulin instead of S-100 did bind to this Sepharose in the presence of Ca$^{2+}$, and was eluted with the buffer containing 4 mM EGTA. These results indicate that different immobilized naphthalenesulfonamide derivatives interact differently with S-100 and calmodulin in a calcium-dependent manner and provide a rapid purification procedure for S-100 and calmodulin.

Since Moewes and Kreisenger demonstrated that the crystal structure of ovalbumin contained three homologous domains, called EF-hands (1), many other calcium-binding proteins, e.g. calmodulin, troponin-C, myosin light chain, intestinal calcium-binding protein, and S-100 have been shown to have amino acid sequences that could form an EF-hand (2).

In the nervous tissue, two such different but homologous calcium-binding proteins exist, namely, S-190 and calmodulin. The S-100 group of proteins is brain specific and localized primarily to glial elements. To the predominant components of this group are called S-100a and S-100b (3). Although the exact biological function of S-100 remains unknown, it may be suggested that S-100 plays an important role in the control of membrane permeability (4) or in the maturation of glial cells (5). On the other hand, calmodulin, which was originally discovered as an activator of cyclic nucleotide phosphodiesterase (6, 7), has been revealed to activate several other different enzymes and to bind to several enzyme proteins such as microtubule-associated proteins (8). Calmodulin activity has been found in many species from both animal and plant kingdoms (9-12), suggesting that this protein is ubiquitous among the eukaryotes.

We previously reported that N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) interacted with calmodulin in a calcium-dependent fashion and inhibited Ca$^{2+}$-dependent 3'5'-cyclic nucleotide phosphodiesterase (13), (Ca$^{2+}$ + Mg$^{2+}$)-ATPase from human erythrocyte (14), myosin light chain kinase from chicken gizzard (15), and myelin basic protein phosphorylation (16).

In the present paper, we utilized immobilized W-7 and its derivatives to develop a calcium-dependent affinity chromatographic isolation procedure of S-100 protein and calmodulin.

EXPERIMENTAL PROCEDURES

Materials—Cyanogen bromide-activated Sepharose 4B and epoxy-activated Sepharose 6B were obtained from Pharmacia Fine Chemicals, Sweden. W-7 and N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) were synthesized according to method of Hidaka et al. (17). S-100a and S-100b were prepared from bovine brain by the method of Isobe et al. (3). Calmodulin was prepared from bovine brain by ammonium sulfate and isoelectric precipitation, heat treatment, DEAE-cellulose, and Sephadex G-100 column chromatography according to Teo et al. (18) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the isolated calmodulin indicated that the final purified calmodulin was free from S-100. Calmodulin-deficient Ca$^{2+}$-dependent 3'5'-cyclic nucleotide phosphodiesterase from bovine brain was prepared by method of Kies (19). S-100 antisera was obtained as described previously from rabbits after immunization with bovine S-100 (20). The antisera reacted with S-100a and S-100b but did not react with calmodulin. All other reagents were laboratory grade.

Coupling of the Ligands to the Sepharose—The coupling of W-7 to cyanogen bromide-activated Sepharose 4B was carried out as follows. W-7 (265 μmol) was dissolved in 20 ml of 30% dimethylformamide, pH 9.8, and added to 7 ml of settled cyanogen bromide-activated Sepharose 4B. After overnight incubation at 40 °C, the resin was then washed with 70 ml of distilled water, alternating 20 mM Tris-HCl (pH 7.5) containing 5 mM MgCl$_2$, 100 μM CaCl$_2$, and 150 mM NaCl (Buffer A). The coupling of W-7 or W-5 to epoxy-
activated Sepharose 6B was essentially the same as above. The amount of W-7 coupled to cyanogen bromide-activated Sepharose 6B was determined to be 12.3 μmol/ml by the decrease of the amounts of W-7 measured by absorbance at 295 nm in the supernatant of the reaction mixture after the coupling reaction. The amounts of W-7 or W-5 coupled to epoxy-activated Sepharose 6B were 19.9 μmol/ml, respectively.

Preparation of Acidic Protein Solution of Bovine Brain—All subsequent operations were performed at 0–4 °C. Bovine brain obtained from a local slaughterhouse was homogenized for 2 min with a mixer in 2.5 volumes of 50 mM Tris-HCl (pH 8.0) containing 3 mM MgCl₂, 1 mM EGTA, and 4 mM 2-mercaptoethanol. The homogenate was centrifuged at 12,000 × g for 10 min, and the supernatant fluid was filtered through glass wool. The filtrate was subsequently centrifuged at 100,000 × g for 60 min. The supernatant was brought to 55% (NH₄)₂SO₄ saturation and stirred for 30 min, followed by centrifugation. The pellet obtained after centrifugation was discarded, and the supernatant was brought to pH 4.0 by the slow addition of 1.0 N HCl. The material precipitated by this procedure was collected by centrifugation and dissolved in a small volume of Buffer A. The solution was dialyzed overnight against Buffer A and loaded on the ligand-coupled Sepharose.

Analytical Procedures—Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried in 15% gels as described by Laemmli (21) containing 0.1% sodium dodecyl sulfate. Samples to be applied were treated with 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol for 2 min in a boiling water bath. Molecular weights of proteins were estimated on the sodium dodecyl sulfate gels by using chicken gizzard actin (42,000) (15), chicken gizzard myosin light chain (20,000) (15), bovine brain calmodulin (16,700), cytochrome c (Boehringer Mannheim) (12,500), and β-chain of bovine brain S-100 (10,500) as standards. Electrophoresis of S-100 was carried out in 20% polyacrylamide slab gels in the buffer system of Ornstein (22) and Davis (23) containing either CaCl₂ or EGTA at a final concentration of 0.2 mM. Analytical analysis of protein hydrolysate was carried out with an Hitachi amino acid analyzer model KLA 5, according to Spackmann et al. (24). Proteins were hydrolyzed at 100 °C for 24 h in sealed tubes. Immunochemical analysis was performed by Ouchterlony's double diffusion (25), and staining with Coomassie brilliant blue.

Assay for Activity of Phosphodiesterase of Bovine Brain—Activity of bovine brain cyclic nucleotide phosphodiesterase using cyclic GMP as substrate was measured as described by Hidaka and Asano (26, 27).

Protein Determinations—Protein amounts were determined according to Lowry et al. (28) with bovine serum albumin as a standard.

RESULTS

Isolation Procedure—Five ml of acidic protein solution (19.0 mg/ml) obtained from 16.6 g wet weight of bovine brain were applied to a W-7-coupled epoxy-activated Sepharose 6B (W-7 epoxy Sepharose) column (1 × 6.4 cm) or a W-7 coupled to cyanogen bromide-activated Sepharose 4B (W-7 CNBr Sepharose) column (1 × 7.7 cm) which was pre-equilibrated with Buffer A. The column was washed with Buffer A until UV absorbancy at 280 nm returned to the base-line level. Then the buffer was changed to Buffer B (same composition as Buffer A but 4 mM EGTA instead of 100 μM CaCl₂). Flow rate was 6 ml/h in each chromatography. Figure 1, a and b, shows the typical elution profiles for chromatography on W-7 epoxy Sepharose and W-7 CNBr Sepharose. In both elution profiles an EGTA-elutable protein peak was observed which could not be observed when the acidic protein solution containing 1 mM EGTA was applied to the columns equilibrated with Buffer B. EGTA-elutable proteins from the W-7 epoxy Sepharose column (abbreviated W-7 epoxy protein) and from the W-7 CNBr Sepharose column (abbreviated W-7 CNBr protein) were pooled individually and dialyzed against distilled water. The protein amounts of W-7 epoxy protein and W-7 CNBr protein were 8.5 mg and 3.6 mg, respectively.

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FIG. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the isolated proteins. A, electrophoresis was performed on a 0.1% sodium dodecyl sulfate/15% polyacrylamide slab gel. a, bovine brain crude acidic protein fraction (50 µg); b, W-7 epoxy protein (2.0 µg); c, W-7 CNBr protein (2.0 µg); d, bovine brain calmodulin (2.5 µg). B, molecular weight calibration curve for 0.1% sodium dodecyl sulfate/15% polyacrylamide gel. The standards are chicken gizzard actin (42,000), chicken gizzard light chain (20,000), bovine brain calmodulin (16,700), cytochrome c (12,500), and β-chain of bovine brain S-100b (10,500).

Isolated Proteins—Fig. 2 illustrates the ability of the isolated proteins to activate preparations of calmodulin-deficient phosphodiesterase. The degree of activation of phosphodiesterase by W-7 CNBr protein was the same as that obtained with bovine brain calmodulin (0.4 pg/ml), but W-7 epoxy protein could not stimulate phosphodiesterase activity, suggesting that W-7 epoxy protein was another calcium-binding protein different from calmodulin. To test the ability of calmodulin to bind to the W-7 epoxy Sepharose column, 10 mg of purified calmodulin dissolved in 2 ml of Buffer A was applied to the column equilibrated with Buffer A and eluted with Buffer A (Fig. 1c). Calmodulin did not bind to the column, and calmodulin activity was not detected in EGTA-eluted fractions but was detectable in the flow through.

Electrophoretic Analyses of Isolated Proteins—Fig. 3A shows a sodium dodecyl sulfate polyacrylamide slab gel electrophoretic pattern of the isolated proteins, stained with Coo massie brilliant blue. W-7 epoxy and W-7 CNBr proteins were essentially homogenous. The M, of W-7 epoxy protein migrating the most rapidly was apparently smaller than that of purified bovine brain calmodulin. Molecular weights estimated from the mobility of proteins on this electrophoresis are 10,500 and 16,700, respectively (Fig. 3B). Isobe et al. previously showed that S-100 protein is a mixture of two closely related components, S-l00a (M, = 20,900) and S-100b (M, = 21,000) proteins; each of the components has the subunit composition (alpha (M, = 10,400), beta (M, = 10,500)), or (beta, beta) (29). W-7 epoxy protein (M, = 10,500 in sodium dodecyl sulfate polyacrylamide gel (Fig. 3, a and b)) has an M, similar to that of the subunits of S-100 protein. Electrophoresis of S-100a, S-100b, and W-7 epoxy protein was carried out in 20% polyacrylamide gel in the buffer system of Ornstein (22) and Davis (23) (Fig. 4). W-7 epoxy protein consisted of two predominant bands which co-migrated with S-100a and S-100b.

Amino Acid Analysis of the Isolated Proteins—The amino acid compositions of W-7 epoxy protein, W-7 CNBr protein, S-100, and calmodulin are listed in Table I. The amino acid compositions were determined using peptide mapping. The results were consistent with the expected compositions of S-100 and calmodulin. The amino acid compositions of W-7 epoxy protein and W-7 CNBr protein were similar, with the exception of the glutamic acid/lysine ratio, which was higher in W-7 epoxy protein. The amino acid compositions of these proteins are consistent with their known functions as calcium-binding proteins.

TABLE I

<table>
<thead>
<tr>
<th>Amino acid contents of isolated proteins</th>
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<tbody>
<tr>
<td>W-7 Epoxy protein</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Lys 9.7</td>
</tr>
<tr>
<td>His 3.5</td>
</tr>
<tr>
<td>Arg 1.0</td>
</tr>
<tr>
<td>Asp 12.3</td>
</tr>
<tr>
<td>Thr 4.3</td>
</tr>
<tr>
<td>Ser 5.9</td>
</tr>
<tr>
<td>Glu 18.5</td>
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<tr>
<td>Pro 0.0</td>
</tr>
<tr>
<td>Gly 6.3</td>
</tr>
<tr>
<td>Ala 7.0</td>
</tr>
<tr>
<td>Cys/2 1.0</td>
</tr>
<tr>
<td>Val 7.0</td>
</tr>
<tr>
<td>Met 0.8</td>
</tr>
<tr>
<td>Ile 3.3</td>
</tr>
<tr>
<td>Leu 11.1</td>
</tr>
<tr>
<td>Thr 1.8</td>
</tr>
<tr>
<td>Phe 6.6</td>
</tr>
<tr>
<td>Trp* 0.8</td>
</tr>
</tbody>
</table>

* Taken from Isobe et al. (3).
† Taken from Dannies and Levine (30).
‡ Taken from Kasai et al. (32).
§ Determined spectrophotometrically (33).
composition of W-7 epoxy protein coincides well with that of S-100 reported by Dannies and Levine (30). On the other hand, W-7 CNBr protein lacks tryptophan and cysteine and is quite similar in overall amino acid content to bovine brain calmodulin (31, 32).

**Immunoechemical Analysis**—To identify W-7 epoxy protein as S-100, immunoechemical analysis was performed. As shown in Fig. 5, W-7 epoxy protein cross-reacted with anti-S 100 sera in double diffusion analysis. The anti-S 100 sera used in this analysis reacted with S-100a and S-100b but did not cross-react with bovine brain calmodulin. W-7 CNBr protein did not cross-react with anti-S 100 sera, indicating that W-7 CNBr protein did not contain S-100.

**Ca\(^{2+}\)**-dependent Affinity Chromatography on Chloro-deficient Analogue of W-7 (W-5)-coupled Sepharose—W-5, a chloro-deficient analogue of W-7, produces weaker inhibition of bovine brain phosphodiesterase and myosin light chain kinase from chicken gizzard than does W-7. The concentrations of W-7 and W-5 which produce 50% inhibition of bovine brain phosphodiesterase are 28 \(\mu\)M and 240 \(\mu\)M, respectively. In order to investigate the nature of immobilized ligand, W-5 was coupled to epoxy-activated Sepharose 6B (W-5 epoxy Sepharose). Five ml of acidic protein solution (19.0 mg/ml) were loaded on a W-5 epoxy Sepharose column (1 \(\times\) 10 cm) pre-equilibrated with Buffer A and followed by washing with Buffer A until UV absorbancy at 280 nm returned to baseline level. The material which bound to the W-5 epoxy Sepharose column was eluted with Buffer B. Fig. 6 shows the elution profile obtained from this separation. The EGTA-elutable proteins were pooled (abbreviated W-5 epoxy protein, amounting to 3.2 mg. Electrophoresis of W-5 epoxy protein with the addition of either Ca\(^{2+}\) or EGTA was carried out using 15% polyacrylamide gels (Fig. 5, inset). W-5 epoxy protein migrated with a mobility identical with that of calmodulin in the presence of EGTA. In the presence of Ca\(^{2+}\), the mobility of W-5 epoxy protein decreased, and it could not be distinguished from that of calmodulin (11). W-5 epoxy protein was capable of activating bovine brain phosphodiesterase in the presence of Ca\(^{2+}\). Maximal stimulation was observed at 200 ng of W-5 epoxy protein (this amount was the same as that of purified calmodulin).

**DISCUSSION**

The immunoechemical as well as electrophoretic evidence, together with the coincidence in amino acid composition and stimulatory activity to cyclic nucleotide phosphodiesterase, show that W-7 epoxy protein and W-7 CNBr protein are S-100 (mixture of S-100a and S-100b) and calmodulin, respectively. Although S-100 has been shown to have an amino acid sequence that could form an EF-hand, our results also suggest that micromolar concentrations of calcium play an important role in the binding of S-100 protein to immobilized naphthalenesulfonamide derivatives.

We previously reported that W-7 interacts with calmodulin in a calcium-dependent fashion and inhibits the calmodulin-dependent activation of phosphodiesterase (13), (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase (14), and myosin light chain kinase from chicken gizzard (15). Therefore, it is of interest that when W-7 was coupled to epoxy-activated Sepharose 6B, S-100 rather than calmodulin binds to W-7 epoxy Sepharose 6B, in a calcium-dependent manner. The reason why S-100 rather than calmodulin binds to the W-7 epoxy Sepharose in a calcium-dependent manner remains unclear. However, the possibility exists that the nature of the affinity matrix might play an important role for S-100 binding to W-7 epoxy Sepharose, since W-7 epoxy Sepharose and W-7 CNBr Sepharose had the same ligand. In addition, it is further suggested that chlorination of the naphthalene ring might also play an important role for S-100 binding to W-7 epoxy Sepharose, since W-7 epoxy Sepharose and W-7 epoxy Sepharose had the same matrix. The calcium-dependent affinity chromatographic isolation procedure presented here provides a rapid purification procedure for S-100 and calmodulin and appears to be of greatest value when a small amount of starting material is available. Furthermore, these naphthalenesulfonamide derivatives may provide a useful tool for elucidating the biological functions of S-100 and calmodulin.

**REFERENCES**

Ca$^{2+}$-dependent Affinity Chromatography of S-100 and Calmodulin