Purification and Characterization of a Novel Ca\textsuperscript{2+}-binding Protein (CBP-18) from Bovine Brain\textsuperscript{*}

Allan S. Manalan and Claude B. Klee
From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

A novel Ca\textsuperscript{2+}-binding protein (CBP-18) has been identified and purified from bovine brain. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified protein consists of a single band of apparent M, 18,000 in the presence of Ca\textsuperscript{2+} or 20,000 in the absence of Ca\textsuperscript{2+}. CBP-18 contains one high affinity Ca\textsuperscript{2+}-binding site, measured at 10\textsuperscript{-5} M Ca\textsuperscript{2+} in the presence of 1 mM Mg\textsuperscript{2+} and 0.1 M K\textsuperscript{+}. The amino acid composition and UV absorption spectrum distinguish CBP-18 from other Ca\textsuperscript{2+}-binding proteins identified in brain. The protein has an extinction coefficient \(\epsilon_{\text{UV}}\) at 280 nm = 4.9 and contains 1 tryptophan/mol, 5 tyrosines/mol, and no trimethyllysine. CBP-18 does not interact with or activate calmodulin-stimulated phosphodiesterase. However, available evidence suggests that CBP-18 binds to other component(s) present in the brain extract in a Ca\textsuperscript{2+}-dependent manner.

Many biochemical effects of Ca\textsuperscript{2+} are mediated by a family of homologous Ca\textsuperscript{2+}-binding proteins (see Refs. 1–3 for reviews). Several such proteins, including calmodulin, calcineurin B, parvalbumin, S-100a, and S-100b proteins, are found in brain (4–9). These proteins exhibit sequence homology, particularly evident in the "E-F hand" Ca\textsuperscript{2+}-binding loops (1, 2). They also undergo conformational changes on binding Ca\textsuperscript{2+} (1–3) and, even under denaturing conditions, exhibit alterations in electrophoretic mobility in the presence or absence of Ca\textsuperscript{2+} (6). The characteristic Ca\textsuperscript{2+}-dependent electrophoretic shift can be used to detect these Ca\textsuperscript{2+}-binding proteins in crude protein mixtures. The present report describes the purification and characterization of a novel Ca\textsuperscript{2+}-binding protein detected in bovine brain extracts using this method.

**EXPERIMENTAL PROCEDURES**

**Materials**—\textsubscript{45}CaCl\textsubscript{2} (specific activity 27.54 mCi/mg) was obtained from New England Nuclear. CAPP\textsuperscript{*} was generously provided by Dr. Albert Manalan, Psychopharmacology Research Branch, National Institute of Mental Health. CAPP Affi-Gel 10 was prepared as described (10). It was then equilibrated in column buffer (2000 cpm/nmol) was dialyzed overnight against 100 volumes of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl, 1 mM MgCl\textsubscript{2}, and 0.1 mM dithiothreitol, with two changes of dialysis fluid. After dialysis, the solution was adjusted to the same total \textsubscript{45}CaCl\textsubscript{2} concentration as that of the column buffer, applied and eluted with identical buffer at a flow rate of 3 ml/h, collecting 0.4-ml fractions.

**RESULTS**

A new Ca\textsuperscript{2+}-binding protein (CBP-18) was initially identified in fractions obtained by DE23 chromatography of a bovine brain extract (Fig. 1). When column fractions were subjected to SDS-polyacrylamide gel electrophoresis, three low molecular weight proteins were found to exhibit Ca\textsuperscript{2+}-induced alterations in mobility. Two of these proteins were calcineurin B and calmodulin. The third protein, which exhibited apparent M, 18,000 in the presence of Ca\textsuperscript{2+} or 20,000 in the presence of EGTA, had not yet been described. This protein (CBP-18) had a larger M, and eluted earlier from DE23 than calcineurin B or calmodulin. When a calmodulin-Sepharose column was deliberately overloaded with pooled material from the DE23 column, CBP-18 was recovered in flow-through fractions eluting in the presence of Ca\textsuperscript{2+}.

Effect of Ca\textsuperscript{2+} on Ammonium Sulfate Precipitation of CBP-18—When aliquots of fraction 1 were subjected to ammonium sulfate fractionation in the presence of Ca\textsuperscript{2+}, approximately two-thirds of CBP-18 was precipitated at 35% saturation (Table I). In contrast, in the presence of EGTA, most of the CBP-18 remained in the supernatant. At 45% saturation with ammonium sulfate, CBP-18 still remained soluble in EGTA,
A Novel Ca**+-binding Protein from Bovine Brain

Fig. 1. SDS-gel electrophoretic pattern of fractions after each step of the purification of CBP-18. Aliquots of the pooled fractions were made 2 mM Ca**+ or 2 mM EGTA as indicated prior to SDS-gel electrophoresis. DE23-cellulose peak (DE-23), 24 μg; Sephadex G-200 pool (G200), 11 μg; CAPP-Affi-Gel: flow-through fractions (F/T), 9 μg; EGTA eluate (EGTA), 2.5 μg; urea eluate (urea), 6.4 μg; HPLC purified protein (HPLC), 1.3 μg. Three proteins exhibited a Ca**+-dependent change in mobility: calcineurin B (small arrows), calmodulin (arrowhead), and CBP-18 (large arrows). Curve of the CBP-18, calcineurin B, and calmodulin bands resulted from electrophoresis of samples containing EGTA in adjacent lanes. Electrophoretic mobilities of molecular weight standards are indicated at the left of the figure.

Ammonium sulfate precipitation of CBP-18 in the presence of Ca**+ or EGTA

Aliquots of calmodulin-Sepharose flow-through were made 2 mM CaCl2 or 2 mM EGTA and brought to the indicated per cent saturation with ammonium sulfate at 2 °C. Precipitate (Ppt) and supernatant fluid (Sup) were separated by centrifugation. The amount of Ca**+-binding protein, quantified by densitometric analysis, is expressed as a per cent of the total Ca**+-binding protein prior to fractionation.

| Ammonium sulfate (% saturation) | 2 mM Ca**+ | 2 mM EGTA |
|---------------------------------|------------|-----------|
|                                 | Ppt | Sup | Ppt | Sup |
| 35                              | 66  | 33  | 22  | 78  |
| 45                              | 93  | —   | —   | 88  |

* None detected.

but was quantitatively precipitated in the presence of Ca**+ (Table I). The differential effect of Ca**+ on the ammonium sulfate fractionation was exploited in the purification of CBP-18. Fraction I (10.6 liters derived from 9.5 kg of bovine brain) in 0.04 M Tris-HCl buffer, pH 7.5, containing 0.2 mM CaCl2, 3 mM MgCl2, 0.05 M NaCl, 0.1 mM dithiothreitol, 1 μg/ml of leupeptin, and 10 μg/ml of soybean trypsin inhibitor, was brought to 45% saturation with ammonium sulfate. The precipitate, which contained CBP-18, was isolated by centrifugation, resuspended in the original volume of buffer containing 2 mM EGTA instead of Ca**+, and again brought to 45% saturation with ammonium sulfate. CBP-18 was recovered in the supernatant fraction. This material was dialyzed overnight against 10 volumes of 0.05 M (NH4)2HCO3 with one change of dialysis fluid. The protein was lyophilized, resuspended in a minimal volume of 0.04 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.5 mM dithiothreitol, and centrifuged at 8000 × g for 30 min to remove insoluble material.

The partially purified CBP-18 was then applied to a column (2.5 × 79 cm) of Sephadex G-200 (superfine) which had been equilibrated with 0.04 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl, 1 mM MgCl2, 0.1 mM EGTA, and 0.5 mM dithiothreitol. The column was eluted with the same buffer at a flow rate of 4 ml/h, collecting 4.8-ml fractions.

Interaction of CBP-18 with CAPP-Affi-Gel—Fractions from the Sephadex G-200 column which contained CBP-18 were pooled, made 2 mM CaCl2, and applied to a column (1.5 × 3.5-cm) of CAPP Affi-Gel equilibrated with 0.04 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl, 1 mM MgCl2, 2 mM CaCl2, and 0.05 mM dithiothreitol (buffer 1). The column was washed with 70 ml of buffer 1 followed by 70 ml of buffer 1 made 0.5 M NaCl. A small amount of calmodulin was removed from the column on substitution of 2 mM EGTA for CaCl2 in the eluting buffer (Fig. 1). CBP-18 was also selectively bound to the column but, unlike calmodulin, CBP-18 was retained on the column in the absence of Ca**+ and was recovered only when 6 M urea was added to the eluting buffer (Fig. 1).

| Table II
| Purification of CBP-18 from bovine brain |
|----------------------------------------|
| Volume | Protein | CBP-18 Recover- | Purifica- |
|        |        | % fold |
| __________ | __________ | __________ |
| Calmodulin-Sepharose flow-through (fraction I) | 10,600 | 11,500 | 55 | 100 |
| Ammonium sulfate (45% supernatant) | 15.5 | 138 | 35 | 64 | 53 |
| Sephadex G-200 | 96 | 54 | 15 | 27 | 58 |
| CAPP Affi-Gel | 25 | 8.3 | 9.6 | 17 | 240 |
| HPLC | 5 | 1.9 | 3.9 | 7 | 430 |

* CBP-18 was quantified by densitometric analysis of Coomassie blue stained gels, as described under "Experimental Procedures."  
| CBP-18 was also selectively bound to the column but, unlike calmodulin, CBP-18 was retained on the column in the absence of Ca**+ and was recovered only when 6 M urea was added to the eluting buffer (Fig. 1).
A Novel Ca\(^{2+}\)-binding Protein from Bovine Brain

TABLE III

| Amino acid       | CBP-18* | Calmodulin* | Calcinurin B* | Parvalbumin* | S-100 α | S-100 β |
|------------------|--------|-------------|---------------|--------------|---------|---------|
|                  | residues/mol | residues/mol | residues/mol | residues/mol | residues/mol | residues/mol |
| Lysine           | 12.4   | 7           | 15            | 15.5         | 9       | 8       |
| Histidine        | 2.0    | 1           | 2             | 2.2          | 2       | 5       |
| Arginine         | 7.2    | 6           | 6             | 1.0          | 0       | 1       |
| Aspartic acid    | 21.5   | 23          | 23            | 14.6         | 13      | 0       |
| Threonine        | 12.7   | 23          | 11            | 10.6         | 5       | 3       |
| Serine           | 12.7   | 14          | 22            | 9.5          | 15      | 19      |
| Glutamic acid    | 21.4   | 27          | 3             | 0            | 0       | 0       |
| Proline          | 4.1    | 4           | 2             | 0            | 0       | 0       |
| Glycine          | 12.0   | 11          | 14            | 9.4          | 6       | 4       |
| Alanine          | 8.0    | 11          | 5             | 11.4         | 6       | 5       |
| Valine           | 6.5    | 7           | 14            | 5.2          | 8       | 7       |
| Methionine       | 5.6    | 9           | 6             | 2.4          | 3       | 3       |
| Isoleucine       | 7.6    | 8           | 11            | 5.0          | 1       | 4       |
| Leucine          | 14.3   | 9           | 14            | 9.6          | 11      | 8       |
| Tyrosine         | 4.6    | 2           | 3             | 0            | 2       | 1       |
| Phenylalanine    | 10.2   | 8           | 12            | 8.5          | 5       | 7       |
| Trimethyllysine  | 0      | 0           | 0             | 0            | 0       | 0       |
| Cysteine         | ND*    | ND*         | ND*           | ND*          | ND*     | ND*     |
| Tryptophan       | +**    | +**         | +**           | +**          | +**     | +**     |

\( M, \) 18,000** 16,700 19,000 12,000 10,400 10,587

** Values correspond to a molecular weight of 18,000.
* Based on the amino acid sequence of calmodulin (16), calcineurin B (Altken A., Klee, C. B., and Cohen, P. (1984) Eur. J. Biochem, in press), S-100 α (17), and S-100 β (18).
* Amino acid composition of rat brain parvalbumin (19).
* Not determined.
* Tryptophan was detected spectrophotometrically (see "Results").

Fig. 3. Calcium binding to CBP-18. Gel filtration of CBP-18 was performed in the presence of 13 \( \mu \)m 4\(^{45}\)CaCl\(_2\) (specific activity 29,000 cpm/nmol). Protein concentrations were determined by analysis of UV absorption spectra of peak fractions. Ca\(^{2+}\) concentration was measured directly in tubes 7, 16, 35, 45, and 47 by atomic absorption spectrophotometry.

Fractons of this urea eluate containing CBP-18 were pooled (30 ml) and dialyzed overnight against 2 liters of 0.05 M \( \text{NH}_4\text{HCO}_3\), and desalted by chromatography on a 9-ml column of Sephadex G-25 (PD-10 column, Pharmacia) equilibrated and eluted with 0.05 M \( \text{NH}_4\text{HCO}_3\). Fractions containing CBP-18 were pooled and stored at -70 °C. SDS-gel electrophoresis shows a shoulder at 290 am, suggesting the presence of tryptophan. The amino acid composition (Table III) indicates that CBP-18 is rich in acidic residues and contains 12 lysines, 2 histidines, and 5 tyrosines/mol. No trimethyllysine was detected. A molar extinction coefficient (\( \epsilon_{279\text{nm}} = 8900\)) based on a \( M, \) of 18,000, is consistent with the presence of one tryptophan and 4 to 5 tyrosines/mol.

Direct evidence of Ca\(^{2+}\)-binding was obtained by gel filtration of the purified protein in the presence of Ca\(^{2+}\) by the method of Hummel and Dreyer (14), as shown in Fig. 3. The protein eluted in the void volume associated with a peak of 4\(^{45}\)Ca\(^{2+}\). Binding of 4\(^{45}\)Ca\(^{2+}\) to the protein resulted in depletion of Ca\(^{2+}\) from the eluting buffer, manifested by a Ca\(^{2+}\) trough in the included volume. Based on radioactivity and absorbance at 279 nm, CBP-18 bound 0.9 ± 0.3 mol of Ca\(^{2+}\)/mol at a Ca\(^{2+}\) concentration of 13 \( \mu \)M.

**DISCUSSION**

A new Ca\(^{2+}\)-binding protein (CBP-18) has been identified in bovine brain. Comparison of the amounts of CBP-18, calcineurin B, and calmodulin identified in DE23 fractions suggests that CBP-18 is present at a substantial level in brain. Uncertainty in recovery at early steps of extraction and ion-
A Novel Ca\(^{2+}\)-binding Protein from Bovine Brain

exchange chromatography precludes a reliable estimate of the total amount of the protein present.

CBP-18 exhibits properties typical of other intracellular Ca\(^{2+}\)-binding proteins, including Ca\(^{2+}\)-induced changes in electrophoretic mobility (6), and binding to CAPP Affi-Gel (15). However, based on present results, CBP-18 has several characteristics which distinguish it from the other Ca\(^{2+}\)-binding proteins identified in brain (4-9, 16-20). In addition to its unique electrophoretic mobility and amino acid composition, the protein fails to elute from CAPP Affi-Gel in the presence of EGTA. CBP-18 contains one high affinity Ca\(^{2+}\)-binding site, measured at 10\(^{-9}\) M Ca\(^{2+}\) in the presence of physiological concentrations of Mg\(^{2+}\) and K\(^{+}\). In crude extracts, CBP-18 is quantitatively precipitated on heating to 100 °C in the presence of EGTA under conditions in which added calmodulin remains soluble (data not shown). CBP-18 does not activate calmodulin-regulated cyclic nucleotide phosphodiesterase, nor does it inhibit activation of the enzyme by calmodulin (data not shown). Thus, CBP-18 does not interact with the calmodulin-binding site of this enzyme.

In crude extracts, the Ca\(^{2+}\)-dependent precipitation of CBP-18 with low concentrations of ammonium sulfate suggests a Ca\(^{2+}\)-dependent interaction with another constituent in the brain extract. Chromatographic behavior of CBP-18 provides further evidence of such an interaction. First, on gel filtration of a brain extract, CBP-18 elutes in the void volume in the presence of Ca\(^{2+}\), and in the included volume in the presence of EGTA. In contrast, free CBP-18 elutes in the included volume even in the presence of Ca\(^{2+}\), thus excluding Ca\(^{2+}\)-dependent self-association as an explanation for this behavior (data not shown). Second, purified CBP-18 does not interact with calmodulin-Sepharose. However, if a crude extract containing CBP-18 is applied to a calmodulin-Sepharose column in the presence of Ca\(^{2+}\), and in the included volume in the presence of EGTA, CBP-18 elutes in the void volume in the presence of Ca\(^{2+}\), and in the included volume in the presence of EGTA. CBP-18 contains one high affinity Ca\(^{2+}\)-binding site, measured at 10\(^{-9}\) M Ca\(^{2+}\) in the presence of physiological concentrations of Mg\(^{2+}\) and K\(^{+}\). In crude extracts, CBP-18 is quantitatively precipitated on heating to 100 °C in the presence of EGTA. CBP-18 contains one high affinity Ca\(^{2+}\)-binding site, which is capable of simultaneous interaction with two different Ca\(^{2+}\)-binding proteins. Such an interaction is reminiscent of phosphorylase kinase (22) and calcineurin (6), which are capable of simultaneous interaction with two different Ca\(^{2+}\)-binding proteins. Efforts are in progress to identify constituents which bind to and are potentially regulated by this novel Ca\(^{2+}\)-binding protein.

Acknowledgment—The contributions of Marie H. Krinks are greatly appreciated.

Note Added in Proof—After this paper was accepted, the purification of a similar Ca\(^{2+}\)-binding protein from bovine brain was reported by Waisman et al. (FEBS Lett. (1983) 164, 80-84). The protein described by Waisman et al. exhibits characteristics similar to those of CBP-18, including UV absorption spectrum and early elution from DEAE-cellulose but the M, determined by SDS-gel electrophoresis and the amino acid compositions of the two proteins appear to be different. Waisman et al. have suggested that their Ca\(^{2+}\)-binding protein may be present only in brain tissue. Recent experiments in our laboratory indicate that CBP-18 is brain-specific. Using a polyclonal antibody to CBP-18, the protein was detected in a crude homogenerate of bovine brain by Western blot analysis. It was not detected in homogenates of bovine heart, kidney, skeletal muscle, spleen, liver, stomach, lung, or thymus, even with a 10-fold increase in the amount of homogenate applied.

REFERENCES

1. Kretsinger, R. H. (1980) CRC Crit. Rev. Biochem. 8, 119-174
2. VanEldik, L. J., Zendegui, J. G., Marshak, D. R., and Watterson, D. M. (1982) Int. Rev. Cytol. 77, 1-61
3. Seamon, K. B., and Kretsinger, R. H. (1983) in Metal Ions in Biology (Spiro, T. G., ed) Vol. 6, Wiley, New York, in press
4. Cheung, W. Y. (1979) Biochem. Biophys. Res. Commun. 88, 533-538
5. Kakuchi, S., and Yamazaki, R. (1970) Biochem. Biophys. Res. Commun. 41, 1104-1110
6. Klee, C. B., Crouch, T. H., and Krinks, M. H. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 6270-6273
7. Hetzmann, C. W., and Strehler, E. E. (1979) J. Biol. Chem. 254, 4296-4303
8. Moore, B. W. (1965) Biochem. Biophys. Res. Commun. 19, 739-744
9. Isobe, T., Nakajima, T., and Okuyama, T. (1977) Biochim. Biophys. Acta 494, 222-232
10. Newton, D. L., Oldewurtel, M. D., Krinks, M. H., Shiloach, J., and Klee, C. B. (1984) J. Biol. Chem., in press
11. Klee, C. B., Krinks, M. H., Manalan, A. S., Cohen, P., and Stewart, A. A. (1983) Methods Enzymol. 102, 227-244
12. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685
13. Spackman, D. H., Stein, W. H., and Moore, S. (1958) Anal. Chem. 30, 1159-1206
14. Ackers, G. K. (1973) Methods Enzymol. 27, 441-449
15. Jamieson, G. A., Jr., and Vanaman, T. C. (1979) Biochem. Biophys. Res. Commun. 90, 1048-1056
16. Watterson, D. M., Sharpie, E., and Vanaman, T. C. (1980) J. Biol. Chem. 255, 962-975
17. Isobe, T., and Okuyama, T. (1981) Eur. J. Biochem. 116, 79-86
18. Isobe, T., and Okuyama, T. (1978) Eur. J. Biochem. 89, 379-388
19. Berchtold, M. W., Wilson, K. J., and Heimann, C. W. (1982) Biochemistry 21, 6552-6557
20. Nakamura, Y., Nakahama, T., Ushiywata, A., Takeda, M., and Nakuya, K. (1980) FEBS Lett. 112, 155-158
21. Berl, S., and Puszkin, S. (1970) Biochemistry 9, 2058-2067
22. Cohen, P., Picton, C., and Klee, C. B. (1979) FEBS Lett. 104, 25-30

M. H. Krinks, A. S. Manalan and C. B. Klee, unpublished observation.
Purification and characterization of a novel Ca\(^{2+}\)-binding protein (CBP-18) from bovine brain.

A S Manalan and C B Klee

*J. Biol. Chem.* 1984, 259:2047-2050.

Access the most updated version of this article at http://www.jbc.org/content/259/4/2047

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/4/2047.full.html#ref-list-1