Vol. 127, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1985 Pages 31-36

# HIGH-AFFINITY CALCIUM-BINDING PROTEINS IN ESCHERICHIA COLI*

Alice C. Harmon, Douglas Prasher, and Milton J. Cormier§

Department of Biochemistry, University of Georgia, Athens, Georgia 30602

Received January 3, 1985

Crude extracts of Escherichia coli contain at least three heat stable proteins of $M_r$, 33,000, 47,000, and 60,000, which bind $^{45}Ca^{2+}$ in buffers containing micromolar calcium and physiological salt concentrations. Fractions containing these proteins neither activated the calmodulin-dependent enzyme, NAD kinase, nor inhibited the activity of this enzyme in the presence of brain calmodulin. Radioimmunoassay of crude extracts for calmodulin indicated the presence of a calmodulin-like antigen. Crude extracts also contain proteins that interact with 2-trifluoromethyl-10H-(3'-aminopropyl)phenothiazine-Sepharose in a calcium-dependent manner, but proteins eluted from this resin did not bind calcium with high affinity. 

Calcium-binding proteins play an important role in the physiology of eukaryotic cells. Many cellular responses are ellicited through the second messenger, calcium, and these proteins serve as the receptors and transducers of the calcium message. These proteins, such as calmodulin and troponin C, bind calcium when internal free calcium levels rise from the resting level of less than $10^{-7}$ M to greater than $10^{-6}$ M. Upon binding calcium, they are able to interact with other proteins, and alter their function. Calmodulin is found in every eukaryotic cell type and is able to affect many cellular processes by binding to a variety of enzymes and stimulating their activity (See 1 and 2 for reviews).

Calmodulin has not been found in prokaryotes (3-7). However, a calmodulin-like activity (8) as well as an inhibitor of brain calmodulin-dependent phosphodiesterase (9) have been found in extracts of E. coli. Also, cell-free extracts of the cyanobacterium, Oscillatoria limnetica, contained a

*This work was supported by a grant from the National Science Foundation, PCM 8213177.
§To whom correspondence should be addressed.
Abbreviations used: TAPP, 2-trifluoromethyl 10H-(3'-aminopropyl)phenothiazine; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid.

0006-291X/85 $1.50

factor that was able to compete with calmodulin in a calmodulin RIA (10). None of these proteins has been directly shown to be calcium-binding proteins. We report here that extracts of E. coli do contain high-affinity calcium-binding proteins.

## MATERIALS AND METHODS

Sodium chloride, potassium chloride, potassium hydroxide, and magnesium chloride used for the calcium-binding studies were all Analar grade (BDH). $^{45}CaCl_2$ (17.5 mCi/mg Ca) was obtained from ICN.

E. coli Extracts. Twelve grams of E. coli cells (SK1592) was lysed at 0° C in 10% sucrose, 50mM Tris, pH 8.025 µg/ml phenylmethylsulfonyl fluoride, 12.5 mM EDTA, 2 mg/ml lysozyme, 20 µg/ml RNase. After 45 min the mixture was centrifuged at 120,000 x g for 1 h. The supernatant was made 1 mM in EGTA and heated to 90° C for 5 min. The coagulated protein was removed by centrifugation and the supernatent was divided into three 10 ml aliquots. One of the aliquots was immediatedly frozen and will be referred to as the crude extract. The second aliquot was made ≈2 mM in free calcium by the addition of 140 µmol of $CaCl_2$, and then 2 mg DNase, 0.2 mg RNase A, and 200 U RNase T1 were added. The mixture was incubated 3.5 h at 37° C then frozen on dry ice. This aliquot will be referred to as the DNase-treated extract. To the third aliquot, 2 ml of STEP buffer (0.5% SDS, 50 mM Tris, pH 7.5, 0.1 M EDTA, and 1 mg/ml proteinase K) was added, and this mixture was incubated 17 h at 50° C then frozen on dry ice. This aliquot will be referred to as the proteinase K-treated extract.

$Ca^{2+}$-binding studies. Buffer A (150 mM KCl, 15 mM NaCl, and 10 mM Hepes, pH 7.5) was depleted of $Ca^{2+}$ (<1 µM) by passing it through a column of Chelex 100. Buffer B was made by adding 1 mM $MgCl_2$, 2 µM $CaCl_2$, and 0.1 mCi/L $^{45}CaCl_2$ to buffer A. Free calcium concentrations were determined with a calcium selective electrode (Orion) standardized with $Ca^{2+}$/EGTA or $Ca^{2+}/Mg^{2+}$/EGTA buffers. The free calcium in the standard solutions was calculated by a computer program based on that of Perrin and Sayce (11). Calcium-binding activity was determined by the method of Hummel & Dreyer (12) as modified by MacManus (13). Each of the E. coli extracts was lyophilized, redissolved in 2.5 ml $H_2O$, and dialyzed extensively at 4° C for 48 h against 2 L of Buffer A made 0.1 mM in EGTA. After concentration to approximately 1 ml in an Amicon Centricon 10, the extract was dialyzed against 100 ml of Buffer B. The extracts were applied to a Sephacryl S-200 column (1.5 x 90 cm) equilibrated in buffer B. Two-ml fractions were collected and the radioactivity was determined in 10 µl aliquots.

Calmodulin assays. Calmodulin-like activity was determined by activation of NAD kinase according to the method of Harmon et al. (14). Inhibitor activity was determined by assaying NAD kinase activity in the presence of 4.4 ng/ml brain calmodulin. This concentration of calmodulin gave 50% activation in the normal assay. Calmodulin radioimmunoassays were performed with a kit obtained from CAABCO according to the package directions. Bovine brain calmodulin purified as previously described (15) was used as standard.

TAPP-Sepharose Chromatography. Crude extract was chromatographed on TAPP-Sepharose as described by Charbonneau et al. (15).

## RESULTS

Heat-stable proteins from E. coli extracts that were able to bind calcium with high affinity were observed in experiments illustrated in Fig. 1.

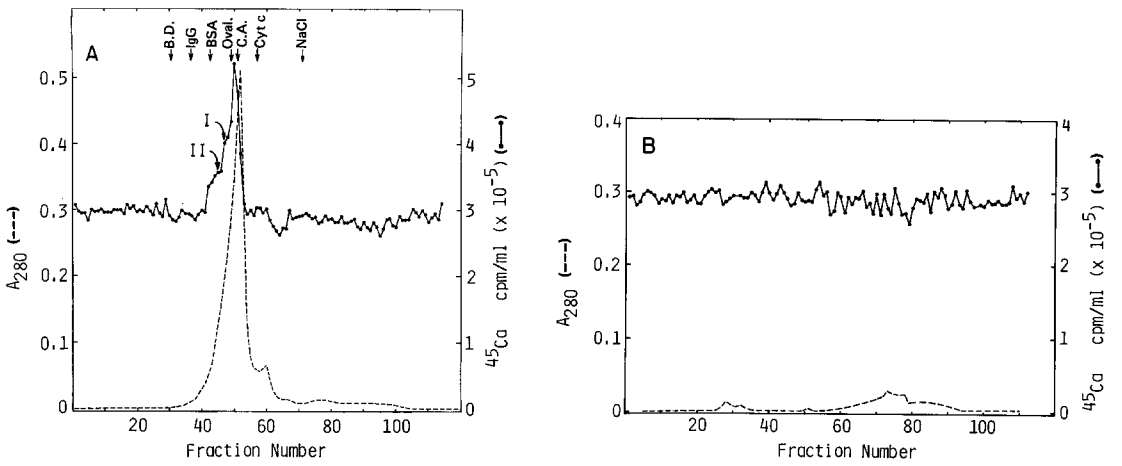


Figure 1. Chromatography of $Ca^{2+}$-binding activity in E. coli extracts. Extracts prepared and dialyzed against $^{45}Ca^{2+}$-containing buffer as described in Materials and Methods were chromatographed on Sephacryl S-200 equilibrated in the same buffer. Elution profiles are shown for the crude extract (A), and the proteinase K-treated extract (B). Elution positions of blue dextran (B.D.), bovine IgG (IgG), bovine serum albumin (BSA), ovalbumin (Oval.), carbonic anhydrase (C.A.), and cytochrome c (Cyt. c) and sodium chloride (NaCl) are indicated.

When crude and DNase-treated extracts were chromatographed on a gel filtration column that had been equilibrated in buffer which contained 2 μM $CaCl_2$, identical elution profiles were obtained (Fig. 1A). The major peak of $Ca^{2+}$-binding activity corresponded to $M_r$ 33,000, and the two shoulders (I and II in Fig. 1) corresponded to $M_r$s of 47,000, and 60,000, respectively. When the proteinase K-treated extract was similarly chromatographed, no $Ca^{2+}$-binding activity was observed. Therefore it was concluded that the $Ca^{2+}$-binding factors are proteins.

To examine whether these calcium-binding proteins possessed calmodulin-like activity, the column fractions from the experiment in Fig. 1A were tested for ability to activate the calmodulin-dependent enzyme, NAD kinase. None of these fractions stimulated the enzyme's activity. Nor was any fraction able to inhibit NAD kinase activity in the presence of brain calmodulin. However, the crude extract contained a heat stable factor that did compete with calmodulin in the calmodulin radioimmunoassay (Fig. 2). This calmodulin-like antigen was present at a concentration of 43 ng per mg of protein..

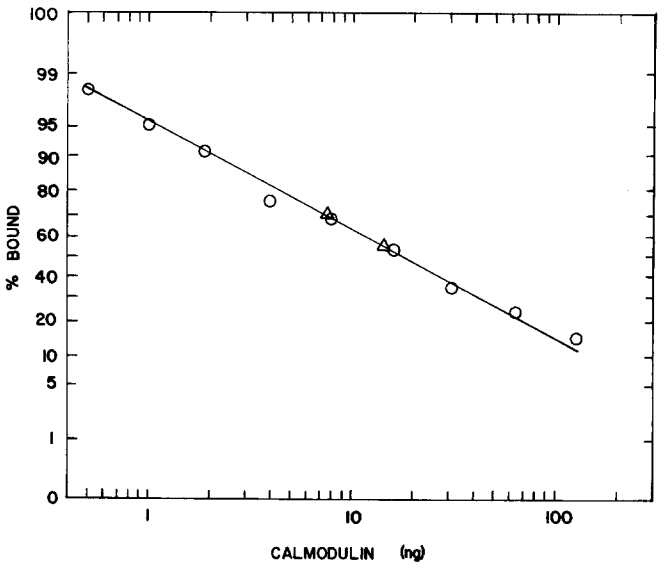


Fig. 2. Calmodulin radioimmunoassay. Bovine brain calmodulin standards are indicated by circles and the dilutions of the E. coli extract are indicated by triangles.

Calmodulin binds to TAPP-Sepharose in the presence of calcium and is eluted by EGTA-containing buffers (15). When an E. coli extract was passed through two successive TAPP-Sepharose columns several proteins were recovered in the EGTA eluate. These proteins bound to the resin in the presence of 0.5 mM $Ca^{2+}$; however, these proteins did not bind $^{45}Ca^{2+}$, present at a concentration of 2 μM, when they were extensively dialyzed against buffer B.

## DISCUSSION

We have shown that heat-treated extracts of E. coli contain factors that bind $Ca^{2+}$ at micromolar levels. The binding of calcium by these factors is highly selective since it was observed in the presence of 150 mM KCl, 15 mM NaCl, and 1 mM $MgCl_2$. This observation suggests that the $K_d$ for calcium is $\approx 10^{-6}$ M. Since the factors are resistant to treatment with nucleases, but are destroyed by treatment with proteinase K, the factors must be protein.

No factor was found that was able to activate the calmodulin-dependent enzyme, NAD kinase. This enzyme is more sensitive to calmodulin (14) than the enzymes previously used for calmodulin detection in prokaryotes (4-8), but we were unsuccessful in finding an activator. Iwasa et al. (9) reported that a

factor is present in E. coli that inhibits calmodulin-activated phoshphodiesterase. Such a factor could mask the activity of a calmodulin-like protein, so we also looked for inhibitory activity in our extracts. No factor that inhibited calmodulin-activated NAD kinase was found.

None of the proteins that bound to TAPP-Sepharose in the presence of milimolar $Ca^{2+}$ bound calcium with high affinity. Perhaps this observation and the lack of biological activity in our extracts are related, in that the exposure of a hydrophobic site upon binding calcium by calmodulin is thought to be required for both processes. If no such site is exposed on the high-affinity $Ca^{2+}$-binding proteins, then they would neither bind to TAPP-Sepharose nor to the hydrophobic binding sites of enzymes.

Although we were unable to demonstrate the presence of a protein with the activity of calmodulin, we did show that there are high affinity calcium binding-proteins and also calmodulin-like antigens in E. coli extracts. This observation is consistent with the hypothesis that calcium plays a role in the regulation of the physiology of prokaryotes as well as eukaryotes. This hypothesis is also supported by demonstration of the presence of two calcium transport systems in everted vesicles of E. coli (16), which suggests that bacteria are able to regulate their internal calcium levels. Also, Ordal (17) showed that the chemotactic behavior of Bacillus subtilis was related to the internal free calcium concentration. Using a calcium/EGTA buffer system and the ionophore A23187 to control the $Ca^{2+}$ levels, he showed that swimming behavior occurred at $\leq 10^{-8}$ M free $Ca^{2+}$ whereas tumbling behavior occurred at $\geq 10^{-7}$ M. Swimming behavior was observed with external $Ca^{2+}$ concentrations as high as $10^{-4}$ M in the absence of ionophore. These data point to the existance of a high-affinity calcium binding protein that responds to internal calcium concentrations and regulates the chemotactic response.

We have observed DNA fragments that hybridize to eel calmodulin cDNA (18) in Southern blots of genominc DNA from Escherichia coli, Bacillus cereus, Myxococcus xanthus, Pseudomonas putida, Anabaena sp., Methanosarcina barkeri, Desulfovibrio vulgaris, and Acinetobacter calcoaceticus (data not shown).

Whether these DNA sequences code for the high affinity calcium-binding proteins we observed remains to be shown. It will be interesting to see if the high affinity $Ca^{2+}$-binding proteins have a role in either the regulation of internal $Ca^{2+}$ levels or in the $Ca^{2+}$-dependent regulation of other processes.

ACKNOWLEDGMENT

We thank Richard O. McCann for performing the TAPP-Sepharose experiments.

REFERENCES

1. Cormier, M.J. (1983) in Calcium in Biology (Spiro, T.G., ed) pp. 54-106, John Wiley & Sons, Inc.
2. Klee, C.B., and Vanaman, T.C. (1982) in Adv. Prot. Chem (Anfinsen, C.B., Edsall. J.T., and Richards, F.M., eds.) Vol. 35, pp. 213-321, Academic Press, New York.
3. Burgess, W.H. Jemiolo, D.K., and Kretsinger, R.H. (1980) Biochem. Biophys. Acta 623, 257-270.
4. Clarke M.., Bazari, W.L., and Kayman, S.C. (1980) J. Bacteriol. 141, 397-400.
5. Grand, R.J.A., Nairn, A.C., and Perry, S.V. (1980) Biochem. J. 185, 755-760.
6. Yates, L., and Vanaman, T.C. (1981) Fed. Proc., Fed. Am. Soc. Exp. Biol. 40, 1738.
7. Nakamura, T., Fujita. K., Eguchi, Y., and Yazawa, M. (1984) J. Biochem 95, 1551-1557.
8. Iwasa, Y., Yonemitsu, K., Matsui, K., (1981) Biochem. Biophys Res. Commun. 98, 656-660.
9. Iwasa, Y., Yonemitsu, K., Matsui, K., Fukunaga, K., Miyamoto, E., (1981) FEBS Lett. 128, 311-314.
10. Kerson, G. W., Miernyk, J.A., and Budd, K. (1984) Plant Physiol. 75, 222-224.
11. Perrin, D.D., and Sayce, I.G. (1967) Talanta 14, 833-842.
12. Hummel, J.P., and Dreyer, W.J. (1962) Biochim. Biophys. Acta 63, 530-533.
13. MacManus, J.P. (1979) Cancer Res. 39, 3000-3005.
14. Harmon, A.C., Jarrett, H.W., and Cormier, M.J. (1984) Anal. Biochem. 141, 168-178.
15. Charbonneau, H., Hice, R., Hart, R.C., and Cormier, M.J. (1983) Meth. Enzymol. 102, 17-39.
16. Ambudkar, S. V., Zlotnick, B.W., and Rosen, B.P. (1984) J. Biol. Chem. 259, 6142-6146.
17. Ordal, G. W. (1977) Nature 270, 66-67.
18. Lagacé, L., Chandra, T., Woo, S.L.C., and Means A.R. (1983) J. Biol. Chem. 258, 1684-1688.