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CALMODULIN-LIKE PROTEIN FROM BACILLUS SUBTILIS†

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The first example of a calmodulin-like activity in a Gram-positive bacterium, Bacillus subtilis, is reported. A calcium ion-dependent, 3',5' cyclic-AMP phosphodiesterase-stimulating activity was found in the soluble fraction of cell-free extracts of cells sporulating in a chemically-defined medium; activation was reversed by trifluoperazine. The activity was heat stable, bound to phenothiazine-agarose in a calcium ion-dependent manner and was eluted therefrom with buffer containing EGTA, and displaced authentic beef brain calmodulin from its antibody in a radioimmunoassay.

Calmodulin was first reported as a protein activator of bovine brain phosphodiesterase by Cheung (1). It has since been recognized to be an ubiquitous intracellular calcium ion receptor in virtually all eukaryotic cells. This receptor is an acidic, heat stable, low-molecular-weight (15,000-22,000) protein that has no known enzymatic activities (2). It binds calcium ions and is thereby activated to regulate the activity of a number of enzymes vital to cellular process (3,4). Calmodulin has been found in lower eukaryotes such as yeasts and fungi (5,6); seldom has it been reported in prokaryotes. Calmodulin-like proteins have been reported in only two procaryotic species, the Gram-negative bacteria, Escherichia coli (7) and Myxococcus xanthus (8,9). We present evidence here that cell of the Gram-positive bacterium, Bacillus subtilis, grown in a chemically-defined medium produce a calmodulin-like protein.

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MATERIALS AND METHODS

Chemicals. Affigel-phenothiazine and Dowex 1x8 anion exchange resin were obtained from Bio-Rad Corp. (Richmond, CA). [^{125}I]Calmodulin RIA kit and [^{3}H]cAMP were from New England Nuclear (Boston, MA). All other enzymes and chemicals were from Sigma Chemical Co. (St. Louis, MO). Bacterial maintenance and growth supplies were from Difco (Detroit, MI).

Bacterial Strains and Media. Bacillus subtilis 168(trp$^-$) was used in all experiments (10). Bacteria were grown in chemically-defined sporulation medium (11).

Assay of Activation of cAMP Phosphodiesterase. The cAMP phosphodiesterase activation assay was adapted from that of Thompson et al. (12). The standard assay volume was 250 µL. The calcium ion concentration was 0.035 mM in all assays unless otherwise stated.

Purification of Calmodulin-like Protein from B. subtilis. A scheme for purification of calmodulin was modified from that developed for the protein from Physarum polycephalum (Kuehn and Heerdt, unpublished observations). B. subtilis cells were harvested at 3 h after the end of log-phase growth by centrifugation at 8,000 x g for 10 min at 4 °C. The cell pellet was washed twice, first with a buffer containing 20 mM Tris-HCl, 2 mM CaCl, 1 M KCl, pH 7.3, then with the same buffer lacking KCl. Washed cells were broken in a buffer containing 40 mM Tris-HCl, 75 mM NaCl, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2% isopropanol, using a French pressure cell at 20,000 psi. The slurry of broken cells was centrifuged at 17,000 x g, 4 °C, for 1 h. Nucleic acids in the clarified crude extract were digested by treatment with 12% (v/v) of a solution containing 1 mg/mL pancreatic DNAse, 0.5 mg/mL RNAse A, and 50 mM $MgCl_2$, in 0.5 M Tris-HCl, pH 7.0, for 4 h at 4 °C. Nuclease-treated fractions were then heated in a water-bath at 85 °C for 10 min, cooled on ice, and the denatured proteins were removed by centrifugation as before. The supernatant solution were treated with 80% or 95% saturation (13) of solid ammonium sulfate at 0°C. Calmodulin activity in the supernatant fractions was detected by the capacity to stimulate beef heart cAMP phosphodiesterase. The calmodulin activity remaining in the 95% ammonium sulfate-treated supernatant solution was bound to the phenothiazine column and eluted with EGTA by the procedure of Wells et. al. (14) as described in the Bio-Rad manual which accompanies Affigel-phenothiazine.

Radioimmunoassay of Calmodulin. The quantitative capacity of various protein fractions obtained from B. subtilis cells to compete with [^{125}I]-labeled beef brain calmodulin for binding to a monospecific antibody preparation was determined using a radioimmunoassay kit (Catalog No. NEK-018) and procedures obtained from New England Nuclear (Boston, MA).

RESULTS

Crude cell-free extracts of washed cells of B. subtilis were found to contain a non-dialyzable material which stimulated beef-brain cAMP phosphodiesterase 5 to 10-fold and beef heart cAMP phosphodiesterase 2 to 4-fold. When cell-free extracts were heated for 10 min at 85 °C and centrifuged, the protein concentration in them fell from 30 mg/mL to 1 mg/mL, but they lost no phosphodiesterase-stimulating activity. Such heat stability is typical of calmodulin (3). Partially purified fractions of these extracts caused a typical stimulation of cAMP phosphodiesterase (Fig. 1). Addition of calmodulin antagonists to the phosphodiesterase assays (MATERIALS AND METHODS)

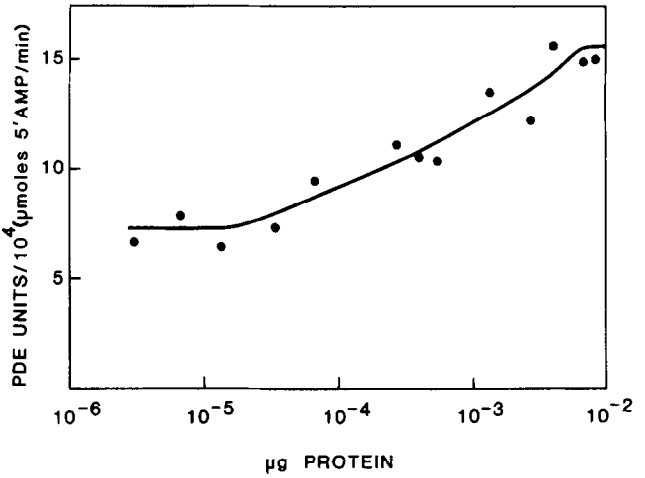

Figure 1. Bovine Heart Phosphodiesterase Activation by Partially Purified Cell-Free Extracts of Bacillus subtilis. Activity was measured as described in MATERIALS AND METHODS; the stimulatory activity present in varying added amounts of the 80% ammonium sulfate supernatant fraction was assayed in a standard volume of 240μL. Rates are average values with standard deviations no greater than 1.03×10^{-4} μmol 5-AMP/min. Ca^{2+} concentration was constant at 32 μM. Non-stimulated phosphodiesterase activity was 6.5×10^{-4} μmol 5-AMP/min.

reduced the stimulation of this enzyme caused by the B. subtilis extracts; trifluoperazine (28 μM) reduced activation by 45%, while W-7 (74 μM) reduced activation by 51%. These results are similar to those described for calmodulins from fungi (6). Calcium ion was essential for the stimulation of phosphodiesterase by the B. subtilis preparations, about 8 μM causing 50% activation.

Another typical behavior of calmodulin is its calcium-dependent absorption to phenothiazine-derivatized insoluble supports. Phosphodiesterase-stimulating activity in the ammonium sulfate fractions (MATERIALS AND METHODS) bound to Affigel-phenothiazine columns in a calcium-dependent manner and could be eluted with solutions of EGTA. Reproducible elution of phosphodiesterasestimulating activity from Affigel-phenothiazine was seen in 10 separate experiments.

Finally, the capacity of fractions of B. subtilis extracts, at various stages of purification, were tested for competion with authentic bovine calmodulin in a standard radioimmunoassay. In all cases competition was found; two examples are shown in Table 1. In addition, a positive correlation

Table I: Calmodulin Levels in Partially Purified Fractions of Bacillus subtilis Extracts Detected by Radioimmunoassay

Additions to Complete Assay Mixture[a]	Volume (µl)	Radioactivity in Antibody-Antigen Precipitate (cpm)	$\frac{B}{B_o}$ %	Calmodulin in Sample (ngm)[b]
Experiment 1				
None	0	5148	100	---
Crude extract from cells grown in CDSM (nuclease and heat treated)[c]	100	2515	49	1.60
"	15	4982	97	0.28
Experiment 2				
None	0	6795	100	---
Peak fraction from Affigel-phenothiazine column:	100	1621	23	6.40
"	25	2798	42	2.70

[a] Assay mixture contained 10 nCi of [^{125}I]-labeled bovine brain calmodulin, 100 µL of sheep anti-bovine brain calmodulin IgG, in presence of the extract volumes indicated,; 500 µL of secondary antibody was added 24 h later and the amount of radioactivity remaining in the immunoprecipitate was measured. Primary and secondary antibody solutions were prepared as described in the RIA kit. B_o values represent the counts in the immunoprecipitate in the same assay mixture as above incubated without the competing antigen.

[b] Cross-reacting antigen in sample listed was quantitated by means of a standard curve using bovine brain calmodulin as the competing antigen for [^{125}I]-calmodulin from bovine brain.

[c] CDSM denotes chemically-defined sporulation medium (11).

between levels of phosphodiesterase-stimulating activity detected in various fractions and levels of competition for calmodulin in the radioimmunoassays (data not shown).

DISCUSSION

We report here the first example of a calmodulin-like protein in a Gram-positive bacterium, B. subtilis. Although calmodulin is widely distributed in eukaryotic cells, including protists such as Dictyostelium discoideum (15), its occurrence in bacteria remains controversial (16). Iwasa et al. have reported a phosphodiesterase-stimulating activity in E. coli cells (7), but they grew these cells in nutrient broth, which we have found upon

concentration contained a calmodulin-like activity even after autoclaving. Other workers have reported being unable to find a stimulator of myosin light chain kinase in crude extracts of E. coli (17) or in crude extracts of E. coli or Pseudomonas putida cells which had been partially purified by chromatography (6). Harmon et al. (16) have reported finding several Ca^{2+}-binding proteins in E. coli extracts, all of which were larger than calmodulins, but they did find calmodulin-like antigens in crude extracts. These workers also found hybridization of a cDNA probe for eel calmodulin genes to the DNA of several bacterial species.

A calmodulin-like protein has been reported in another Gram-negative bacterium; the developmentally associated S-protein of M. xanthus has some striking similarities in its presumed Ca^{2+}-binding sites to calmodulins (8). The S-protein, however, has a higher molecular weight (23,000) than typical calmodulins and several major differences in amino composition compared to the calmodulins (8,18).

What function a calmodulin-like protein may play in B. subtilis, a Gram-positive spore former, is unknown. Greene and Slepecky (19) have shown that Bacillus megaterium cells have an absolute minimal requirement for Ca^{2+} in order to develop normal heat-resistant spores, and we have found a similar requirement for B. subtilis cells growing in the chemically-defined medium used here (O'Hara and Hageman, unpublished observations). Furthermore, Ordal has demonstrated chemotactic responses in B. subtilis cells linked to changes in their intracellular Ca^{2+} concentrations (20). B. subtilis cells also appear to have a requirement for Ca^{2+} to carry out intracellular proteolysis (21, O'Hara and Hageman, unpublished observation). Whether the calmodulin-like activity reported here is structurally related to the known calmodulins or whether it plays any role in these Ca^{2+}-dependent steps in B. subtilis cells will require further work.

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REFERENCES

1. Cheung, W.Y. (1971) J. Biol. Chem. 246, 2859-2869.
2. Cheung, W.Y. (1980) Science 207, 19-27.
3. Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) Annu. Rev. Biochem. 49, 489-515.
4. Cheung, W.Y. (1984) Fed. Proc. 43, 2995-2999.
5. Hubbard, M., Bradley, M., Sullivan, P., Shepherrd, M. and Forrester, I. (1982) FEBS Lett. 137, 85-88.
6. Nakamura, T., Fujita, K., Eguchi, Y. and Yazawa, M. (1984) J. Biochem. 95, 1551-1557.
7. Iwasa, Y., Yonemitsu, K., Matsui, K., Fukunaga, K. and Miyamoto, E. (1981) Biochem. Biophys. Res. Comm. 98, 656-660.
8. Inouye, S., Harada, W., Zusman, D. and Inouye, M. (1981) J. Bacteriol. 148, 678-683.
9. Inouye, S., Franceschini, T. and Inouye, M. (1983) Proc. Natl. Acad. Sci. USA 80, 6829-6833.
10. Hageman, J.H., Carlton, B.C. (1970) Arch. Biochem. and Biophys. 139, 67-79.
11. Hageman, J.H., Shankweiler, G.W., Wall, P.R., Franich, K., McCowan, G.W., Cauble, S.M., Grajeda, J. and Quinones, C. (1984) J. Bacteriol. 160, 438-441.
12. Thompson, W.J., Terasaki, W.L., Epstein, P.M. and Strada, S.J. (1979) Adv. in Cyclic Nucl. Res. 8, 69-92.
13. DiJeso, F. (1968) J. Biol. Chem. 243, 2022-2033.
14. Wells, J.N., Baird, C.E., Wyl Y.J. and Hardman, J.G. (1975) Biochem. Biophys. Acta 384, 430-438.
15. Marshak, D.R., Clarke, M., Roberts, D.M. and Watterson, D.M. (1984) Biochemistry 23, 2891-2899.
16. Harmon, A.C., Prasher, D. and Cormier, M.J. (1985). Biochem. Biophys. Res. Comm. 127, 31-36.
17. Grand, R.J.A., Nairn, A.C. and Perry, S.V. (1980) Biochem. J. 185, 755-760.
18. Burgess, W.H., Schleicher, M., Van Eldik, L.J. and Watterson, D.M. (1983) In Cheung, W.Y. (Ed). "Calcium and Cell Function", Vol. 6, Academic Press, N.Y., N.Y.
19. Greene, R.A., Slepecky, R.A. (1972) J. Bacteriol. 111, 557-565.
20. Ordal, G.W. (1977) Nature 270, 66-67.
21. Nakamura, M., Okamura, S., Izaki, K. and Takahashi, H. (1980) J. Gen. Appl. Microbiol. 26, 119-132.