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Purification and Properties of an Intracellular Calmodulinlike Protein from *Bacillus subtilis* Cells

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Although calcium ions are crucial in a variety of bacterial processes, including spore development, reports of calmodulin in procaryotes have been few. We have purified to homogeneity a calmodulinlike protein (CaLP) from sporulating cells of *Bacillus subtilis* grown in a chemically defined sporulation medium; purification involved heat treatment, fractionation with ammonium sulfate, affinity chromatography, and gel filtration on high-performance columns. The protein was eluted from a phenothiazine affinity column in a calcium ion-dependent manner, stained poorly with Coomassie blue and silver stain dyes, bound poorly to nitrocellulose filters, and was not an inhibitor of the major intracellular serine proteinase. It stimulated bovine brain phosphodiesterase in a dose- and Ca^{2+}-dependent manner and stimulated NAD kinase from peas in a dose-dependent manner. The *B. subtilis* calmodulin reacted with anti-bovine brain calmodulin antibodies in enzyme-linked immunoabsorbance assays. The amino acid composition data showed it to be distinctly different from eucaryotic calmodulins, having particularly high levels of serine and glycine. The pI of the protein was estimated to be 4.9 to 5.0. The molecular weight was estimated to be 23,000 or 25,000, based on amino acid composition and detergent gel electrophoresis, respectively. The protein reacted with rhodamine isothiocyanate, which blocked its enzyme-activating capacity and greatly increased its electrophoretic mobility and Coomassie dye-binding ability.

Calmodulin, a small, acidic, heat-stable, Ca^{2+}-binding protein, has been found in a variety of eucaryotic organisms (7, 17, 24, 25, 48). Calmodulin-Ca^{2+} complex activates several enzymes of physiological importance: cyclic nucleotide phosphodiesterase (7), myosin light-chain kinase (33), adenylate cyclase (4), and Ca^{2+}-Mg^{2+} ATPase (21). The amino acid sequence of calmodulin is very remarkably conserved in diverse eucaryotic organisms (29).

Several authors have reported finding calmodulinlike proteins (CaLPs) in procaryotes (18, 20, 22). The analogy drawn between calmodulin and these procaryotic proteins, however, has relied on physical properties characteristic of calmodulin, such as amino acid sequence motifs (18, 41, 42), Ca^{2+} binding (27), and binding to anticalmodulin antibodies (22).

In our previous report (13), we described the discovery of such a CaLP in the gram-positive organism *Bacillus subtilis* grown in chemically defined sporulation medium (CDSM) (15). The CaLP of *B. subtilis*, unlike those found in other bacteria, was able to activate bovine heart or brain phosphodiesterase. We report here the further ability of the CaLP to activate NAD kinase from pea shoot tissue and report for a highly purified preparation several physical and chemical properties, such as isoelectric point, molecular weight, amino acid composition, and UV spectrum. We also compare these parameters for CaLP with those of brain calmodulin to assess the similarities and differences between these two proteins.

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MATERIALS AND METHODS

Chemicals and reagents. Bacto-agar, gelatin, α-D-glucose, and related growth medium supplies were obtained from Difco Laboratories, Detroit, Mich. The inorganic salts and DL-lactate for the growth medium CDSM were from Mallinckrodt Chemical Co., St. Louis, Mo. Gelcode silver stain kit was from Pierce Chemical Co., Rockford, Ill. Dalton Mark VIIL standard marker proteins were from Sigma Chemical Co., St. Louis, Mo. Prestained standard marker proteins were from Diversified Biotechnology, Inc., Newton Centre, Mass. Bovine brain calmodulin was from Calbiochem, San Diego, Calif.; bovine heart calmodulin and bovine brain and heart activator-deficient cyclic nucleotide phosphodiesterase were from Sigma Chemical Co. Protein determination reagent, Bio-Gels, Affigel-phenothiazine, and Dowex 1X-8 anion-exchange resin were from Bio-Rad Laboratories, Richmond, Calif. Pharmalytes and Polybuffer used in isoelectric focusing and chromatofocusing were from Pharmacia, Piscataway, N.J. Tetramethyl-rhodamine-5 and -6 isothiocyanate (TMRITC) were purchased from Molecular Probes, Inc., Eugene, Oreg. Anti-sheep calmodulin antibodies were from Polysciences, Inc., Warrington, Pa., and rabbit anti-sheep immunoglobulin G (IgG) and rabbit anti-sheep IgG-horseradish peroxidase conjugate antibodies were from Boehringer Mannheim, Indianapolis, Inc. Immulon II Removawells were from Dynatech Laboratories, Inc., Alexandria, Va. ^{3}H-cyclic AMP ([^{3}H]cAMP) was from Amersham, Arlington Heights, Ill., and aqueous complete counting cocktail (3a70b) was from Research Products International, Mt. Prospect, Ill.

Bacterial strains and culturing methods. *B. subtilis* 168 *trpC2* was used exclusively in this work. Cells were cultured in CDSM (15) unless the use of Hanson's supplemented nutrient broth (HSNB) (16) was indicated. Bacterial cells were grown in 12 2-liter baffled shaker flasks (Bellco, Vineland, N.J.) at 220 rpm and 37°C until 3 h after the onset of

sporulation, unless otherwise noted. Culturing flasks were then placed on ice to arrest growth, and 90 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol was added to a final concentration of 1 mM. The cells were centrifuged from the growth medium at 2,000 × *g* and 4°C. The culture medium was discarded, and to minimize protease contamination, the cells were washed once with iced 20 mM Tris-Cl–2 mM $CaCl_2$–1 M KCl, pH 7.3, and again with the same buffer without KCl. The cell pellet was rapidly frozen in a dry ice-ethanol bath and stored at −20°C.

Purification of CaLP. Thawed *B. subtilis* cell paste was resuspended in breakage buffer (40 mM Tris-Cl, 1 mM $CaCl_2$, 1 mM PMSF [pH 7.5]) at a ratio of 1:4 (vol/vol). Cells were ruptured with two passes through an ice-cold French pressure cell at 20,000 lb/in^2. The lysate was cleared by centrifugation at 17,000 × *g* and 4°C for 1 h. The supernatant fraction (crude intracellular extract) was heated to 80°C for 10 min and then cooled in an ice-water bath. After cooling, the heated crude extract was digested at 4°C for 4 h after the addition of a 12% (vol/vol) solution of 50 mM Tris-Cl (pH 7.0)–1 mg of pancratic DNase I per ml–0.5 mg of RNase A per ml. The nucleases were then inactivated by another heating step, as indicated above. The heat-denatured material was centrifuged from the heated, digested crude extract at 17,000 × *g* for 20 min at 4°C. Solid, finely ground ammonium sulfate was added gradually to the extract (at 0°C, under constant stirring) to a final saturation of 80% (wt/vol). The ammonium sulfate-precipitable material was removed by centrifugation at 30,000 × *g* and 4°C for 1 h, and the ammonium sulfate was dialyzed from the supernatant fraction by dialysis in autoclaved 40 mM Tris-Cl–1 mM $CaCl_2$, pH 7.5, until the conductivity of this fraction equaled that of the dialysis buffer. CaLP was removed from the dialyzed supernatant fraction by Affigel-phenothiazine affinity chromatography as described by Gopolkrishna and Anderson (14). Fractions were dialyzed against the dialysis buffer above and assayed for phosphodiesterase activation and anti-calmodulin IgG cross-reactivity.

CaLP was purified to apparent homogeneity with a fast protein liquid chromatography (FPLC) Superose 12 gel filtration column (30 by 1 cm; Pharmacia). The elution buffer was 40 mM Tris-Cl–1 mM $CaCl_2$, pH 7.5. Samples were prepared by dialysis in the above buffer, lyophilized to dryness, and then resuspended in 600 μl of elution buffer. Fractions (1 ml) were eluted at a flow rate of 1 ml/min. Eluted protein was detected with an in-line A_{280} monitor. The presence of CaLP was monitored throughout the purification by the ability of a fraction both to activate cyclic nucleotide phosphodiesterase and to cross-react with anti-brain calmodulin antibodies.

Protein determination. For the purification steps up to and including the ammonium sulfate treatment, total protein in the samples was estimated by the method of Bradford (3); however, we found that CaLP itself reacted poorly or not at all with any of the usual protein reagents tested. Thus, the protein content of the most highly purified CaLP was determined by amino acid analysis, and the amounts of CaLP protein in various fractions obtained during purification were estimated by enzyme-linked immunosorbent assay (ELISA) with authentic beef brain calmodulin as a standard.

Enzymological assays. The procedure for assaying 3′-5′-cyclic nucleotide phosphodiesterase was modified from that of Thompson et al. (45). Final concentrations of the reaction components, in a total volume of 240 μl, were 42 mM Tris-Cl, 5.2 mM $MgCl_2$, 1.3 mM cAMP (21,000 cpm of [^{3}H] cAMP), 1 mM $CaCl_2$, and 0.0026 U of bovine heart or brain activator-deficient phosphodiesterase. Samples containing calmodulin (50 μl) were incubated with the reaction mixture minus cAMP for 30 min at 0°C. Reactions were initiated with cAMP and incubated for 30 min at 30°C. Reactions were stopped by boiling reaction tubes for 45 s and immediately placing them on ice for 1.75 min. Any 5′-AMP formed in the assay was converted to adenosine by the addition of 70 μl of 1-mg/ml king cobra snake venom and incubation at 30°C for 10 min. The reaction was stopped by the addition of 1 ml of absolute methanol, and the contents of the reaction tube were applied to a column of Dowex 1 × 8 anion-exchange resin (0.38-ml bed volume) equilibrated with methanol (45) and washed with 1 ml of methanol. The eluate was collected in a 20-ml polypropylene scintillation vial, and the residual methanol was allowed to evaporate overnight. Samples were counted for tritium in 10 ml of 3a70b complete counting cocktail for 2 min.

Calcium ion dependence of CaLP. Samples to be assayed were treated with EGTA (ethylene glycol tetraacetic acid, 10 mM) and subsequently dialyzed overnight at 4°C with two changes of buffer (autoclaved 40 mM Tris-Cl [pH 7.5]) and one change of autoclaved, distilled, deionized water (ddH_2O). Calcium-free samples were incubated with the desired concentration of calcium ion in the form of aqueous $CaCl_2$ for 3 h at 4°C prior to performing the standard assay for phosphodiesterase stimulation.

NAD kinase assays. Partially purified NAD kinase was obtained from extracts of pea shoots by an adaptation (32) of the method of Marshak et al. (28). Reaction components were 3 mM ATP, 10 mM $MgCl_2$, and 2 mM NAD. This reaction was stopped by heating reaction tubes at 100°C for 5 min, followed by immersion in an ice-water bath. NADP was reduced by NADP-dependent glucose 6-phosphate as described by Marshak et al. (28), and NADPH production was monitored by the increase in A_{340}.

ELISA. The sample to be assayed was bound to the wells of a 96-well microtiter plate and assayed by the procedure of Engvall (11). Primary sheep anti-bovine brain calmodulin IgG was diluted 1:200 with physiologically buffered saline (PBS), and 100 μl of this solution was applied to the sample wells. The secondary antibody (goat anti-sheep IgG-horseradish peroxidase) was diluted 1:200 in PBS, and 100 μl of this solution was added to the sample well after the washing step. Primary and secondary antibody incubation periods were 1 h. The color development solution placed in each well was composed of 200 μl of 10-mg/ml *o*-phenylenediamine, 0.013% H_2O_2, and 0.1 M sodium citrate, pH 4.5. After 1 h at room temperature, the solution was removed from the wells, diluted 1:5 in ddH_2O, and the A_{540} was measured. The sample results were quantitated with brain calmodulin (0 to 1 μg) as a standard, and the A_{450} was plotted against the logarithm of the concentration of calmodulin. The affinity of the *B. subtilis* CaLP was judged to be only 10% of that of bovine calmodulin towards the antibodies based on standard dilutions of each and with the amino acid analysis data used to estimate the protein concentrations of the *B. subtilis* protein. Standard dilutions of bovine brain calmodulin were run with each ELISA experiment.

Continuous, denaturing polyacrylamide electrophoresis. Rod gels were run by the method of Laemmli (26), but without the stacking gel. The acrylamide concentration of the gels was 15%, with an acrylamide-to-bisacrylamide ratio of 1:30. Samples were diluted 1:4 in 0.05% bromphenol blue–50% glycerol–10% β-mercaptoethanol–1% sodium dodecyl sulfate (SDS), boiled for 5 min, and then put on ice. The sample volume loaded onto the tube gels was between

100 and 200 μl. Gels were fixed overnight in 50% methanol–5% acetic acid, stained in 0.05% Coomassie brilliant blue R-250–10% acetic acid–50% methanol, and destained with 7% (vol/vol) acetic acid.

Isoelectric focusing. Isoelectric focusing gels were prepared as 15% polyacrylamide native rod gels by the method of Davis (8) with the addition of Pharmalyte (Pharmacia), pH 3 to 10, to a final concentration of 1% (vol/vol). Samples were prepared by diluting them 1:3 in 5% sucrose–1% Pharmalyte. Gels were prefocused for 30 min at 100 V constant power at 4°C, with 0.4% (vol/vol) ethanolamine as the cathode buffer and 0.1% H_2SO_4 as the anode buffer. Samples were focused at 200 V for 23 h at 4°C at constant power. A blank gel was run concurrently and was sliced into 1-mm slices, which were placed in separate tubes with 1 ml of ddH_2O. The pH was measured from the water used to soak the slices after 1 day. Rod gels were fixed in 50% ethanol–5% acetic acid for 1.5 days. Pharmalyte was removed by several changes of 10% ethanol–5% acetic acid in water. Gels were stained for 3 days in 50% methanol–10% acetic acid–0.05% Coomassie brilliant blue and destained for 1 to 3 months in 7% acetic acid.

Chromatofocusing. Chromatofocusing was performed with a Mono-P anion-exchange column fitted to a Pharmacia FPLC system. The Mono-P column was equilibrated with 0.025 M histidine chloride (pH 6.3). The sample volume injected was 500 μl of the activity peak fractions from the phenothiazine affinity column. Limit buffer was 1:8 Polybuffer 74 (Pharmacia). The equilibration buffer was applied isocratically for 6 min to the Mono-P column at 0.5 ml/min. A linear gradient of the Polybuffer 74 was applied to the column at the same rate for 72 min and run isocratically for the next 42 min. The pH of every fraction was checked with a pH meter with a glass electrode. Proteins eluted from the column were monitored with an in-line A_{280} flow cell. All fractions were dialyzed against 40 mM Tris-Cl–1 mM $CaCl_2$, pH 7.5, overnight at 4°C. Polybuffer was removed from the samples by binding the CaLP in the fractions by batch phenothiazine affinity chromatography.

UV absorbance spectrum of CaLP. The UV absorption of CaLP was measured on a sample which was purified through the gel filtration step on a Hitachi-Perkin Elmer 320 spectrophotometer. Samples were scanned against a dialysis buffer blank from 300 to 200 nm with a bandpass of 1 nm in matched quartz cells with a 1-cm path length.

Amino acid composition of purified CaLP. The amino acid composition was determined on Superose 12-purified calmodulin (Fig. 1, peak B). Samples were prepared for analysis by extensive dialysis in sterile ddH_2O. After dialysis, samples were lyophilized to dryness in a sterile, 1.5-ml polypropylene microfuge tube. The Protein Sequencing Laboratory of the University of Michigan Medical School, Ann Arbor, Mich., hydrolyzed the protein samples and analyzed them for amino acids with a Waters Pico-Tag amino acid analyzer. Tryptophan was destroyed during the hydrolysis process, and glutamine and asparagine content were reported as combined glutamate (Glx) and aspartate (Asx), respectively.

Fluorescent labeling of CaLP with TMRITC. FPLC-purified CaLP was fluorescently labeled in the dark with TMRITC by the procedure of Dedman and Kaetzel (10). A 1-mg sample of CaLP was dialyzed overnight in 100 mM borate–75 mM NaCl, pH 8.4. After incubation of CaLP with TMRITC and quenching of the TMRITC with excess ethanolamine, the unincorporated label was removed by spin column gel filtration chromatography. This was done in a 1-ml disposable spin column with a 0.9-ml bed volume of Biogel P-25 equilibrated with 10 mM imidazole–200 mM NaCl, pH 6.1. Samples were added to each spin column in 100-μl aliquots and spun at top speed in a Sorvall GLC-2 clinical centrifuge for 1 min. The column was then washed seven times with successive additions of 100 μl of the equilibration buffer. Fractions were collected in a 1.5-ml microfuge tube for each wash. The fractions were checked for the presence of the labeled protein by illumination with a long-wavelength UV lamp. The fluorescent fractions were pooled and dialyzed in 40 mM Tris-Cl–1 mM $CaCl_2$, pH 7.5, before application to polyacrylamide gel electrophoresis (PAGE) columns.

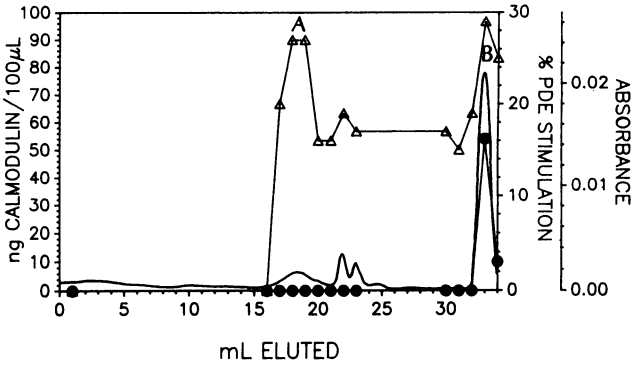

FIG. 1. Gel filtration on Superose 12 of phenothiazine affinity-purified CaLP from *B. subtilis* cells. Phenothiazine affinity-purified fractions (Table 1) were pooled, lyophilized, and resuspended in 600 μl of water. This sample was injected (3.5 μg of antigen) at 200 μl per injection onto a Superose 12 column and chromatographed on a Pharmacia FPLC system. Elution buffer was 40 mM Tris-Cl–1 mM $CaCl_2$, pH 7.5, with a flow rate of 0.5 ml/min and a back pressure of 1.2 mPa. The path length of the in-line UV monitor was 3 mm. A_{280} (solid line), percent stimulation of bovine heart phosphodiesterase (two peaks of activity, A and B) caused by 50 μl of eluant (△), and antigenic material determined by anticalmodulin ELISA (●) are shown. Methods for ELISA and phosphodiesterase-stimulating activities are described in the text. Maximum standard deviation for the phosphodiesterase data points was ±2.5% of the stimulated value.

RESULTS

Isolation of CaLP from *B. subtilis*. An intracellular protein with the ability to activate activator-deficient bovine heart and brain cyclic nucleotide phosphodiesterase and pea NAD kinase was purified by the protocol outlined in Table 1. This protocol was modified from one used for purification of calmodulin from the slime mold *Physarum polycephalum* (16a).

The initial heat step served to precipitate much of the soluble protein and inactivate proteinases while not appreciably affecting the CaLP activity. The second heat step was included to inactivate the nucleases which were added to digest DNA during the previous purification step and which can interfere with the phosphodiesterase assays. Up to 80% saturation with ammonium sulfate left most of the calmodulinlike activity in the supernatant fraction and rather dramatically increased the apparent antigenicity (358% recovery of antigen) and the phosphodiesterase-stimulating ability. We assume that the ammonium sulfate treatment removes a component from CaLP, perhaps another protein, which blocks the antigenic as well as the phosphodiesterase-binding process. This apparent activation by ammonium

TABLE 1. Purification of CaLP from *B. subtilis* cells[a]

Step	Sample	Protein concn (mg/ml)	Total vol (ml)	Total antigen (ng)	Recovery of antigen (%)	Stimulated phosphodiesterase sp act[b] (μmol)	
						Per ng of antigen	Per ng of protein[c]
1	Crude extract	10.50[c]	480	1.2×10^5	100	2.0×10^{-5}	6.2×10^{-11}
2	Heated crude extract	1.85[c]	380	3.8×10^4	30	6.5×10^{-5}	1.8×10^{-11}
3	Nuclease-treated reheated extract	0.88[c]	480	6.2×10^4	49	4.3×10^{-5}	6.2×10^{-11}
4	80% ammonium sulfate pellet fraction	2.30[c]	87	2.3×10^4	18	3.9×10^{-5}	4.4×10^{-10}
5	80% ammonium sulfate supernatant fraction	0.08[c]	1,120	4.5×10^5	358	2.5×10^{-5}	1.3×10^{-8}
6	Phenothiazine affinity chromatography	ND[d]	12	3.5×10^4	27	4.3×10^{-5}	ND
7	Superose 12 chromatography (peaks)	0.007[e]	2	1.4×10^3	1	1.4×10^{-6}	1.7×10^{-5}

[a] This CaLP purification started with 20 liters of *B. subtilis* cell culture grown to 3 h after the onset of sporulation in CDSM. Calmodulin-specific antigenic material was determined by anti-bovine brain calmodulin ELISA as described in Materials and Methods.
[b] Specific activity defined as micromoles of 5'-AMP per minute.
[c] Protein concentration determined by the method of Bradford (3).
[d] ND, Not determined.
[e] Protein concentration estimated from amino acid analysis.

sulfate and the subsequently increased antigenicity occurred proportionally; the phosphodiesterase specific activity per nanogram of antigen reported for step 5 in Table 1 did not change substantially from the value shown in the previous purification step.

As we reported previously, CaLP bound to a phenothiazine column and could be eluted by chelation of calcium ion, but large amounts apparently stick to this column. A similar observation was made when phenyl-Sepharose was used as the affinity matrix. Greater recoveries were obtained when 2 M urea was included in the elution buffer (45a).

When the major peak from the Affigel-phenothiazine affinity column (Table 1, step 6) was applied to a Superose-12 gel filtration column, two phosphodiesterase-stimulating proteins were resolved (Fig. 1, peak A and peak B), only one of which (peak B) cross-reacted with anti-calmodulin IgG. Peak B, which appeared to be a single protein, was designated CaLP and has been further characterized chemically and physically.

Production of CaLP during sporulation. To determine the time of production of CaLP in *B. subtilis* cells during sporulation, it proved necessary to purify the protein through step 6 (Table 1). Cell extracts obtained from cells grown in supplemented nutrient broth (16) were harvested at t_0, t_1, t_3, and t_5 (the subscript denotes hours after the end of exponential growth). Extracts were purified through step 5 (Table 1), quantitatively applied to phenothiazine columns, and eluted with EGTA. Since cells harvested at t_3 appeared to have the greatest quantity of phosphodiesterase-stimulating activity, this time of harvesting was used in subsequent preparations. As the phosphodiesterase-stimulating activity at t_3 was only slightly more than at t_5 and about two times that at t_0 and t_1, and as not all of the phosphodiesterase-stimulating activity cross-cuts with antibody (Fig. 1), these results have been interpreted with caution.

Enzymatic activation of calmodulin-dependent enzymes by CaLP. The ability of CaLP preparations to activate bovine heart cyclic nucleotide phosphodiesterase and pea NAD kinase was tested. The semilogarithmic dose-response plot of the activation of bovine heart phosphodiesterase with partially purified CaLP (through step 5 of Table 1) is shown in Fig. 2 and is comparable to other calmodulin activation plots reported for this enzyme (43).

When the fraction described in Fig. 2 was dialyzed against EGTA to remove Ca^{2+} and tested in the standard assay, a requirement for Ca^{2+} for stimulation by the CaLP preparation was established with an apparent K_{ca} of 6 μM, in the same range (2 to 10 μM) reported for bovine brain calmodulin (31, 43). In view of an irreversible loss of appreciable amounts of phosphodiesterase-stimulating activity upon dialysis with EGTA, presumably as a result of removing Ca^{2+}, and the presence of more than one phosphodiesterase-stimulating activity (Fig. 1), we take this result as very tentative. Removal of calcium ion from homogeneous CaLP (peak 3, Fig. 1) by chelation and dialysis led to completely irreversible loss of phosphodiesterase-stimulating activity, which is not typical of eucaryotic calmodulins.

Preparations of the *B. subtilis* CaLP were strongly stimulatory of pea NAD kinase (Fig. 3). The half-maximal stimu-

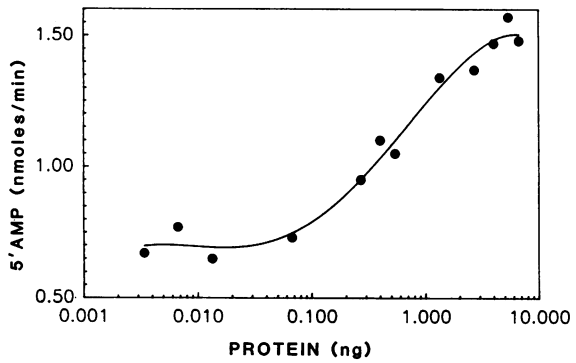

FIG. 2. Activation of bovine heart phosphodiesterase with partially purified CaLP from *B. subtilis* cells. The intracellular extract from 6 liters of CDSM cell culture harvested at t_3 was treated with nucleases, heated, treated with 80% saturating $(NH_4)_2SO_4$, and centrifuged, and the soluble layer was dialyzed against 40 mM Tris-Cl–1 mM $CaCl_2$, pH 7.5. The dialyzed $(NH_4)_2SO_4$ supernatant fraction was lyophilized, resuspended in 1/43 the original volume, and dialyzed in the above buffer. The protein concentration of the concentrated 80% $(NH_4)_2SO_4$ supernatant fraction was 134 μg/ml. This sample was diluted with the dialysis buffer to give the amount per assay (3) indicated with a sample volume of 50 μl. The samples were assayed for standard phosphodiesterase stimulation as indicated in the text in the presence of 1 mM $CaCl_2$. Phosphodiesterase assays were performed in duplicate, and the average values were plotted with a SigmaPlot computer program. Unstimulated activity was 0.65 nmol of 5'-AMP per min. Maximum standard deviation for the phosphodiesterase data points was ±0.10 nmol of 5'-AMP per min.

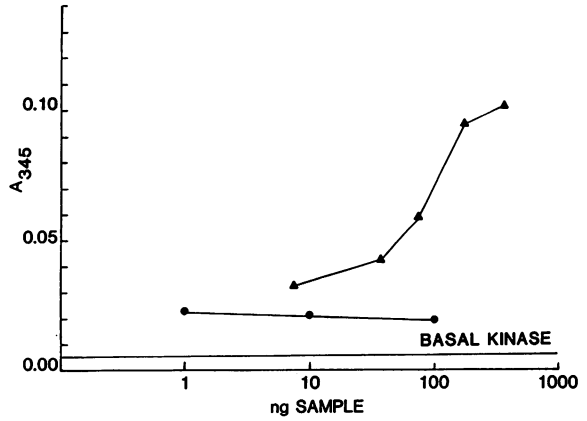

FIG. 3. Activation of pea NAD kinase by partially purified CaLP from *B. subtilis* cells. Partially purified NAD kinase was obtained from the extracts of pea shoots according to the protocol adapted from Muto and Miyachi (32) by Marshak et al. (28). Increasing concentrations of partially purified CaLP from *B. subtilis* cells (see Fig. 2) were incubated in the presence of ATP (3 mM), $MgCl_2$ (10 mM), and NAD^+ (2 mM). The reaction was stopped by heating in a 100°C water bath for 5 min, followed by chilling the tubes in an ice bath. The $NADP^+$ formed was assayed by converting to NADPH with the $NADP^+$-dependent glucose 6-phosphate dehydrogenase, and the change in A_{340} was monitored spectrophotometrically. Symbols: ▲, *B. subtilis* CaLP fraction; ●, bovine brain calmodulin.

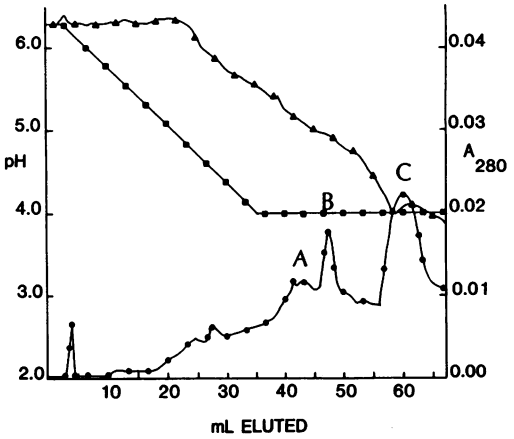

FIG. 4. Chromatofocusing of CaLP from *B. subtilis* cells on an FPLC Mono-P column. Six liters of cells were grown in CDSM, harvested at t_3, washed, and broken with a French pressure cell; the intracellular extract was heated, digested with nucleases, heated again, treated with 80% $(NH_4)_2SO_4$, and centrifuged, and the soluble fraction was dialyzed in 40 mM Tris-Cl–1 mM $CaCl_2$, pH 7.5. The dialyzed 80% $(NH_4)_2SO_4$ supernatant fraction was further purified by Affigel-phenothazine affinity chromatography. Phosphodiesterase-activating and anticalmodulin ELISA antigenic, affinity-purified material was applied to the Mono-P column (5 μg of antigen per 500 μl of sample), and the column was eluted isocratically for 6 min at 0.5 ml/min with buffer A (0.025 M histidine chloride [pH 6.3]). A linear gradient was established with 100 to 0% buffer A and 0 to 100% buffer B (1:8 Polybuffer-Cl 74 [pH 4.0]) at 0.5 ml/min for 36 min. After the gradient was complete, the column was eluted isocratically with buffer B for 1 h at 0.5 ml/min, which eluted three peaks of protein, A, B, and C. Symbols: ■, pH gradient applied; ▲, pH of eluted fractions; ●, A_{280} (in-line UV monitor).

lation of NAD kinase in the standard assay occurred at a CaLP level of about 100 ng, a value lower than those reported for pea (0.4 μg) and bovine brain (1.0 μg) calmodulins (1). Roberts et al. (39) have suggested that calmodulins isolated from non-mammalian sources have a higher capacity to stimulate NAD kinase because they lack trimethyllysine. The absence of trimethyllysines in procaryotes may account for the higher capacity to stimulate pea NAD kinase than was seen for the authentic bovine brain calmodulin.

Because of several similarities between CaLP and the small, acidic, heat-stable inhibitor (30) of the major intracellular serine proteinase (ISP-1) of *B. subtilis*, which undergoes an apparent activation during sporulation (6), we tested CaLP as a proteinase inhibitor. The ISP-1 proteinase was not inhibited by any of the amounts of CaLP preincubated with the proteinase before assaying against azocasein.

Determination of isoelectric point of CaLP. The isoelectric point of CaLP was estimated by chromatofocusing the protein on a Pharmacia FPLC apparatus and by isoelectric focusing. Phosphodiesterase-activating material eluted from an affinity column (step 6, Table 1) was chromatofocused on a Mono-P column (Fig. 4). Of the three protein peaks which eluted from the Mono-P column, only peak B (pI 4.93) reacted with anti-calmodulin IgG. To confirm this finding, isoelectric focusing was carried out on the antigenic and phosphodiesterase-stimulating CaLP purified by gel filtration (Fig. 1, peak B); an isoelectric point of 5.0 was estimated by this method. It may be noted that the pI of 4.93 to 5.0 for the *B. subtilis* calmodulin is considerably less acidic than that (pI 3.9) determined for bovine brain calmodulin (29).

Molecular weight determination. The molecular weight of CaLP was estimated by continuous SDS-PAGE and by amino acid analysis. Other methods of molecular weight determination (discontinuous SDS-PAGE, SDS-urea-PAGE, and gel filtration) gave apparently anomalous results. The molecular mass of the purified CaLP (Fig. 1, peak B) determined by continuous SDS-PAGE was 25 kDa (Fig. 5A); only one very weakly staining band was visible. A similar value (23 kDa) was calculated from the data in Table 2 by assuming that there was one methionine per molecule of CaLP. This protein is larger than mammalian calmodulin, which has a molecular mass of 16.7 kDa (46) and than the value of 17 kDa reported for a CaLP from a cyanobacterium (38).

Amino acid analysis. The amino acid compositions of CaLP and bovine brain calmodulin are shown in Table 2. The two proteins differ sharply in amino acid composition; differences in aspartate, serine, glycine, proline, methionine, tyrosine, and histidine are notable. The lower relative content of acidic residues in CaLP than in bovine calmodulin was consistent with the higher relative pI of the former protein. In contrast, the compositions of the calmodulins from the lower eucaryotes *Saccharomyces cerevisiae* (37) and *Dictyostelium discoideum* (28) are much more like that of bovine calmodulin than that of the *B. subtilis* CaLP. As is true of other calmodulins, CaLP apparently lacks cysteine.

Rhodamine labeling of CaLP and estimation of molecular weight of CaLP. A sample of the same protein shown electrophoresed in Fig. 5A was treated with TMRITC, which has been reported to specifically label calmodulin (10). When the treated sample was subjected to the same electrophoretic analysis as before, an intensely fluorescent band appeared (Fig. 5B, right-hand lane) with an apparent molecular weight of only 1,000. Remarkably, this protein now stained intensely with Coomassie brilliant blue R-250 dye (Fig. 5B, left-hand lane). In addition, we found that the rhodamine-labeled CaLP (RCaLP) lost both its antigenicity towards

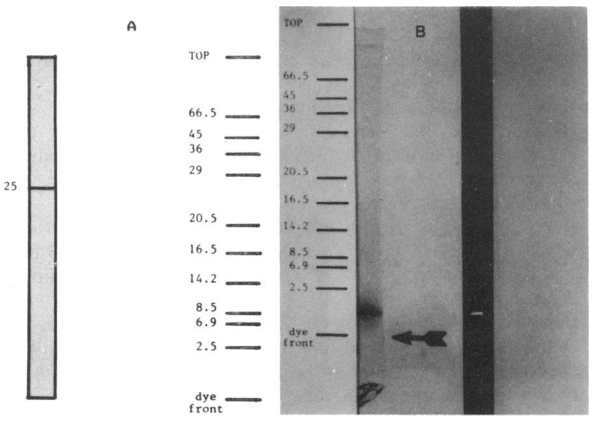

FIG. 5. Electrophoresis of CaLP and RCaLP from *B. subtilis* cells by continuous SDS-PAGE. Panel B represents the sample protein in panel A (Fig. 1, peak B) but labeled with rhodamine isothiocyanate. Samples were prepared by lyophilization of 2.2 μg of CaLP and 0.5 μg of RCaLP and resuspension of each sample in 200 μl of SDS-PAGE sample buffer. Electrophoresis was run in the dark to avoid possible photobleaching of the label. Polyacrylamide concentration was 15%, with 1:30 cross-linking. Molecular mass standards were run concurrently as indicated in Materials and Methods and are represented in kilodaltons in the figure. RCaLP was photographed after electrophoresis with Polaroid 667 film and a Wrattan 66A filter over a long-wave UV transilluminator. CaLP and RCaLP were stained with Coomassie brilliant blue R-250 and destained in 7% acetic acid. The artifact at the top of the gel in panel B (Coomassie blue-stained gel) was caused by tearing the gel upon removal from the glass tubes used for casting.

TABLE 2. Amino acid composition of CaLP from *B. subtilis* and bovine brain calmodulin[a]

Amino acid	CaLP		Bovine brain[b]	
	Residues/mol	Mol%	Residues/mol	Mol%
Asp + Asn	11	4.7	23	15.5
Thr	11	4.5	12	8.1
Ser	63	26.4	4	2.7
Glu + Gln	33	13.9	27	18.2
Pro	8	3.2	2	1.4
Gly	48	20.1	11	7.4
Ala	19	7.8	11	7.4
Val	6	2.7	7	4.7
Met	1	0.4	9	6.1
Ile	5	2.3	8	5.4
Leu	5	2.2	9	6.1
Tyr	8	3.2	2	1.4
Phe	4	1.9	8	5.4
His	11	4.6	1	0.7
Lys	3	1.3	7	4.7
Arg	2	0.8	6	4.1
Trimethyllysine	NR[c]		1	0.7
Trp	NR		0	0.0
Cys	0	0.0	0	0.0
Total	238		148	

[a] Purified CaLP (Fig. 1, peak B) was prepared for analysis as in Materials and Methods and sent to the University of Michigan Protein Sequencing Facility for hydrolysis and analysis on a Waters Pico-Tag amino acid analyzer. Tryptophan was destroyed by this method. Glu + Gln and Asp + Asn represent composite analysis of Gln and Glu and Asn and Asp, respectively. A molecular weight of 23,000 was assumed for CaLP.
[b] Data from Watterson et al. (46).
[c] NR, Not reported.

anticalmodulin antibodies and its ability to stimulate phosphodiesterase. Isoelectric focusing of the RCaLP showed that the pI was unchanged from that of the native protein. CaLP has three lysines (Table 2), which might react with the isothiocyanate group of the dye. These results contrast sharply with those of Dedman and Kaetzel (10), who found little alteration in the physical or biological properties of mammalian calmodulin after rhodamine modification.

UV spectrum of CaLP. The UV absorbance spectrum of the most highly purified preparations of CaLP has an absorption maximum of 290 nm, which is quite different from that of bovine brain calmodulin (277 nm). The CaLP spectrum also was missing the fine structure in the 250- to 270-nm region ascribed to the absorbance of phenylalanine in the absence of tryptophan (23). The UV absorbance seen for CaLP suggests that one or more tryptophan residues are present in this protein.

Attempts to identify calmodulin gene in *B. subtilis* chromosome. We isolated DNA from *B. subtilis*, carried out restriction digests, and electrophoresed the digested fragments by standard methods. These fragments were subjected to standard Southern blot analysis with either nick-translated probes or a riboprobe generated by T7 RNA polymerase action on the plasmid pGM-CaMC. The riboprobe plasmid pGM-CaMC, containing two-thirds of the 3′ end (0.28 kb) of the structural gene coding for the calmodulin from chicken muscle, was constructed and kindly provided to us by Colin Rasmussen and Anthony Means.

Neither of the probes was found to anneal appreciably to DNA fragments from *B. subtilis* under a variety of conditions. This negative result is consistent with the differences noted in the proteins of the *B. subtilis* CaLP and mammalian calmodulins.

DISCUSSION

The presence of calmodulin in procaryotes has been disputed (34, 47), and indeed, the evidence that an authentic calmodulin protein occurs in procaryotes has been rather limited. However, in 1986 we presented evidence (12) that sporulating cells of *B. subtilis* produce a protein which has physical, enzyme-stimulatory, and antigenic properties characteristic of calmodulins. We have confirmed the existence of such a protein by purifying it to homogeneity and determining a number of its characteristic properties, some of which are rather different from those of the eucaryotic calmodulins. Recently, Shyu and Foegeding (40) have presented physical and immunological evidence for a CaLP in spores of *Bacillus cereus* and *B. subtilis*. Furthermore, Petterson and Bergman (38) have clearly shown that several species of the cyanobacterial genus *Anabaena* contain a heat-stable, NAD kinase-stimulating activity which cross-reacts with anti-spinach calmodulin antibodies. Thus, it seems fairly certain that CaLPs are found in both gram-positive and gram-negative bacteria.

It might be stressed that it is important to use chemically defined medium and more than one assay procedure in establishing the presence of CaLPs in bacteria. We reported earlier (13) that autoclaved, concentrated nutrient broth contains a phosphodiesterase-stimulating substance; thus, the use of complex medium might introduce artifacts. Furthermore, our present work shows that not all the phosphodiesterase-stimulating activity present in cell extracts (Fig. 1) grown in CDSM can be recognized by antibodies raised against authentic calmodulin. Although we have restricted our work here to a protein displaying both enzyme-stimulating and antibody cross-reacting properties, the first peak of

phosphodiesterase-stimulating activity (Fig. 1) may be a related CaLP protein which has been altered in some way.

In yeast cells, an essential function for calmodulin has been established (9), and chicken calmodulin has been shown to function in place of endogenous calmodulin in yeast cells (36). Although no function for calmodulin in the growth or sporulation of *B. subtilis* is known, we have found that calcium ions are essential for efficient protein degradation during sporulation (34a, 35), and of course for forming heat-stable spores. In addition, the calmodulin antagonist promethazine has been reported to selectively block sporulation of *B. subtilis* cells (5), and we have observed a similar effect with trifluoperazine (38a) at levels similar to those at which it inhibits the *B. subtilis* CaLP (12). We are currently trying to clone the gene for CaLP so that larger quantities of the protein may be prepared to simplify further study of the protein and to prepare specific mutations of the gene to determine the in vivo function of the protein.

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