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Isolation and Properties of Calmodulin from *Dictyostelium discoideum*

MARGARET CLARKE,* WENDY L. BAZARI, AND SAMUEL C. KAYMAN

Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

A calcium-dependent regulatory protein (calmodulin) was purified from vegetative amoebae of *Dictyostelium discoideum*. The properties of *Dictyostelium* calmodulin are similar but not identical to those of bovine brain calmodulin. Calmodulin activity was not detected in extracts of *Saccharomyces cerevisiae* or *Escherichia coli*.

A Ca^{2+}-dependent regulatory protein, calmodulin (5), occurs in a wide variety of eucaryotic organisms, although the protein from mammalian sources has been most extensively studied. First described as a heat-stable activator of cyclic 3',5'-nucleotide phosphodiesterase (4, 19), calmodulin has since been shown to modulate the activity of a variety of enzymes. These include brain adenylate cyclase (3, 5), erythrocyte (Ca^{2+}-Mg^{2+}) ATPase (13, 17), skeletal-muscle phosphorylase kinase (8), myosin light-chain kinase from vertebrate skeletal- (27) and smooth-muscle (9) and nonmuscle (14, 28) cells, and NAD kinase in plants (2). The structure of the protein has been so highly conserved that even calmodulin isolated from a eucaryotic microorganism is an effective modulator of mammalian enzymes (20).

The distribution and biological roles of calmodulin in microorganisms are not known. It may participate in the regulation of actin-myosin interaction, as it appears to do in higher organisms. In vertebrate smooth muscle (9), platelets (14), and BHK-21 cells (28), calmodulin in the presence of Ca^{2+} has been shown to activate a kinase that catalyzes the phosphorylation of a myosin light chain. This phosphorylation stimulates the actin-activated ATPase activity of the myosin. A similar mechanism could provide Ca^{2+} regulation of actomyosin activity in eucaryotic microorganisms, potentially affecting several types of cell motility.

The cellular slime mold *Dictyostelium discoideum* is a particularly favorable system for examining this possible role of calmodulin. Myosin and actin have been purified from *Dictyostelium* amoebae and found to be similar to the corresponding muscle proteins (7, 26). The enzymatic interaction of pure *Dictyostelium* myosin and actin is affected by Ca^{2+} only in the presence of an uncharacterized protein fraction (23). There is evidence that *Dictyostelium* myosin can be phosphorylated (24; E. R. Kuczmarski and J. A. Spudich, Fed. Proc. **38**:629, 1979). Furthermore, the isolation of *Dictyostelium* motility mutants (6) suggests that genetic analysis of motility is possible in this organism.

We show in this report that *Dictyostelium* amoebae contain a substantial amount of a protein closely resembling bovine brain calmodulin. We have purified and partially characterized this protein, referred to here as *Dictyostelium* calmodulin. Calmodulin has previously been isolated from only one other eucaryotic microorganism, the acellular slime mold *Physarum polycephalum* (20, 21).

We tested the ability of *Dictyostelium* cell extracts to activate cyclic nucleotide phosphodiesterase from bovine brain. We found that a 30,000 × *g* supernatant from sonicated amoebae could fully activate the brain enzyme. This activity was expressed only in the presence of Ca^{2+} and was not destroyed by heating the extract to 90°C, behavior characteristic of a calmodulin-like protein (5). In contrast, comparable extracts from yeast (*Saccharomyces cerevisiae*) or from *Escherichia coli* exhibited no activity even when five times as much protein (10 μg) was tested. This lack of activity was not due to the presence of an inhibitor, since the same quantity of these extracts did not inhibit activation of brain phosphodiesterase by the *Dictyostelium* extract (Clarke and Silverman, unpublished data).

We purified *Dictyostelium* calmodulin as described in Table 1. The initial steps of this procedure also lead to purification of *Dictyostelium* actomyosin (7, 26), enabling us to determine the extent to which the calmodulin-like activity cofractionated with actomyosin. The cells were disrupted by sonication in a buffer containing 20% sucrose, which minimizes breakage of the lysozomes and thus reduces proteolysis. It also renders actomyosin soluble under low-salt conditions that would otherwise cause it to aggregate. When the sucrose is removed by dialysis, actomyosin precipitates and can be col-

TABLE 1. *Fractionation of Dictyostelium calmodulin*

Fraction[a]	Total protein (mg)	Total activity[b] (kU)	Sp act (U/μg)
Homogenate	7,100	51	0.0072
100,000 × *g* supernatant	4,300	43	0.0099
Postdialysis 16,000 × *g* supernatant	3,800	38	0.010
Postdialysis 16,000 × *g* pellet (actomyosin)	340	1.7	0.005
Supernatant after heating to 90°C	1,300	27	0.21
Bio-Rad Cellex D (pooled active fractions)	18	20	1.1
DEAE-Sephadex A-25 (pooled peak fractions)	3.6	14	4.0
Bio-Rad P-60 (pooled peak fractions)	0.6	10	16.8

[a] Amoebae of *D. discoideum* AX3 were grown to a density of 10^7 cells per ml in 6 liters of HL-5 medium (per liter: glucose, 10 g; yeast extract, 5 g; proteose peptone, 10 g; $Na_2HPO_4 \cdot 7H_2O$, 0.35 g; KH_2PO_4, 0.35 g; dihydrostreptomycin sulfate, 50 mg; final pH 6.4 to 6.6). The cells were collected by centrifugation at 2,000 × *g* for 5 min and washed twice with 10 mM Tris-hydrochloride (pH 7.5). The packed cells (117 g) were suspended in 234 ml of homogenization buffer (10 mM Tris-hydrochloride [pH 7.5], 40 mM sodium pyrophosphate, 0.1 mM dithiothreitol [DTT], 0.1 mM EGTA, 30% [wt/vol] sucrose) and lysed by sonication. The homogenate (340 ml) was centrifuged at 100,000 × *g* for 90 min. The resulting supernatant (270 ml) was dialyzed overnight against Buffer E (20 mM Tris-hydrochloride [pH 7.5], 0.1 mM EGTA, 0.1 mM $MgCl_2$, and 0.1 mM DTT) and then centrifuged at 16,000 × *g* for 30 min to collect the precipitated crude actomyosin. The supernatant (510 ml) was heated to 90°C for 1 min, then centrifuged for 10 min at 16,000 × *g* to remove denatured protein. The supernatant (450 ml) was applied to a DEAE-cellulose column (Bio-Rad Cellex D, 3 by 18 cm) equilibrated with Buffer E. The calmodulin activity was eluted with Buffer E containing 0.4 M KCl. The active fractions (80 ml) were pooled and dialyzed against Buffer S (20 mM sodium acetate, pH 5.0, 0.1 mM DTT, 0.1 mM $CaCl_2$) and then applied to a DEAE-Sephadex A-25 column (2 by 4 cm) equilibrated with the same buffer. The column was developed with a linear gradient of 0 to 0.6 M KCl in Buffer S and a total volume of 150 ml. The calmodulin eluted at about 0.4 M KCl. The active fractions (20 ml) were concentrated to 2.6 ml by pressure filtration (Amicon PM10), and 1.2 ml was applied to a Bio-Rad P-60 column (1 by 100 cm) equilibrated with 20 mM Tris-hydrochloride (pH 7.5), 0.1 M NaCl, 0.1 mM $CaCl_2$, and 0.1 mM DTT. Calmodulin eluted slightly behind the main peak of protein. The yield reported for the last step was adjusted to reflect the amount of material that would have been recovered if the entire DEAE-Sephadex sample had been chromatographed on the P-60 column.

TABLE 1. *Continued*

[b] Calmodulin was assayed by its ability to stimulate the hydrolysis of cyclic AMP to 5'-AMP catalyzed by bovine brain cyclic nucleotide phosphodiesterase. One unit (U) of calmodulin activity is defined as the amount required to increase the rate of cyclic AMP hydrolysis by 1 nmol/min under the conditions of the assay. Each 50-μl reaction mix contained 20 mM Tris-hydrochloride (pH 7.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 2 mM DTT, 50 μg of bovine serum albumin, 0.5 mM cyclic [^{3}H]AMP (50 μCi/μmol), and sufficient phosphodiesterase to hydrolyze 10% of the cyclic AMP during the assay in the absence of calmodulin. The addition of calmodulin resulted in a maximal increase in cyclic AMP hydrolysis of four- to fivefold. Calmodulin activity was calculated from assays containing samples of calmodulin that gave approximately half-maximal stimulation of the phosphodiesterase. The samples were incubated at 30°C for 15 min, and the reaction was terminated by heating at 90°C for 2 min. Reaction products were separated by thin-layer chromatography (25) and quantitated by scintillation spectrometry in Hydromix (Yorktown Research).

lected by low-speed centrifugation. Most of the calmodulin activity separated from actomyosin at this step: more than 95% of the activity remained soluble, whether the dialysis step was carried out in the presence of Ca^{2+} or of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA). At present we do not know whether this distribution is physiologically significant or a result of the fractionation conditions. It certainly does not rule out the possibility that calmodulin is involved in regulating actin-myosin interaction in *Dictyostelium*; even the small fraction of activity that precipitated with the crude actomyosin corresponds to about 1 calmodulin molecule for each 10 myosin molecules. Furthermore, the protein fraction reported to render *Dictyostelium* actomyosin ATPase activity sensitive to Ca^{2+} also remained soluble under these conditions (23).

The purification of calmodulin was continued by heating the supernatant to 90°C for 1 min and removing denatured proteins by low-speed centrifugation. The heat-stable proteins were further fractionated by ion exchange and gel filtration chromatography. The resulting calmodulin had been purified about 2,300-fold, indicating that it represents about 0.04% of the total protein in vegetative amoebae. Our recovery by this procedure was about 20%, corresponding to a yield of 0.6 mg of calmodulin from 100 g of amoebae, wet weight.

Dictyostelium calmodulin fully activated brain cyclic nucleotide phosphodiesterase in the presence of Ca^{2+}. However, 2.5 times as much purified *Dictyostelium* calmodulin as bovine

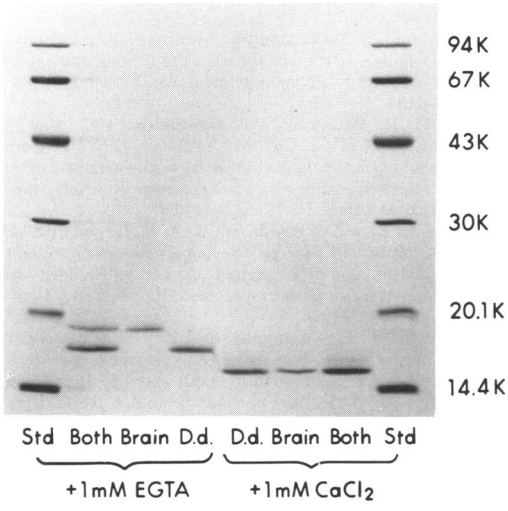

FIG. 1. *SDS-gel electrophoresis of Dictyostelium (D.d.) and brain calmodulin in the presence and absence of Ca^{2+}. The composition of the gel and the chamber buffer were as described by Laemmli (22). The separating gel contained 15% acrylamide. Before application, the protein samples were heated for 2 min at 100°C in a buffer containing 62 mM Tris-hydrochloride (pH 6.8), 2% SDS, 7.5% glycerol (wt/vol), 5% β-mercaptoethanol (vol/vol), and either 1 mM $CaCl_2$ or 1 mM EGTA, as indicated. The outer lanes contained molecular weight standards: phosphorylase b, 94,000; bovine serum albumin, 67,000: ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400 (Pharmacia kit). The inner lanes contained 2 μg of brain or 3 μg of Dictyostelium calmodulin, or these amounts of both, as indicated. Note that incubation in Ca^{2+} or EGTA had no effect on the mobility of the standard proteins.*

brain calmodulin was required to produce the same level of activation. Although this lower activity could be due to inactive molecules present in the *Dictyostelium* preparation, the general stability of calmodulins makes this unlikely. A more probable explanation is that *Dictyostelium* calmodulin has a lower affinity for the brain phosphodiesterase than does brain calmodulin.

A comparison of *Dictyostelium* and brain calmodulins by sodium dodecyl sulfate (SDS)-gel electrophoresis also suggested some divergence between the two proteins (Fig. 1). They did not comigrate on SDS gels under standard (22) conditions. Their mobilities indicated apparent molecular weights of 19,000 (brain calmodulin) and 17,000 (*Dictyostelium* calmodulin). Such a difference has not been found for calmodulins isolated from most other sources (16, 18, 20), although rat testis calmodulin migrates slightly more slowly than the bovine brain protein (10). In the presence of Ca^{2+} the mobilities of both *Dictyostelium* and brain calmodulin increased, and the two proteins did comigrate, with an apparent molecular weight of 15,500. A Ca^{2+}-induced alteration in electrophoretic mobility is characteristic of calmodulin and has been previously reported to occur in alkaline urea gels (11, 15, 16, 20) and in SDS gels (1, 18).

In view of the central role that cyclic AMP plays in development in *Dictyostelium*, calmodulin may also participate in this process. The aggregating amoebae emit and respond chemotactically to pulses of cyclic AMP; this signaling system involves a secreted cyclic AMP phosphodiesterase and its inhibitor, a membrane-bound phosphodiesterase, and an adenylate cyclase (see reference 12 for review). Certain mammalian enzymes with these activities are regulated by calmodulin (5). We therefore tested the ability of *Dictyostelium* calmodulin to stimulate the cyclic AMP phosphodiesterase secreted by aggregation-competent amoebae. This extracellular phosphodiesterase occurs in a low-K_m form in the absence of its inhibitor and in a high-K_m form when complexed with it (19a). Both forms of the enzyme proved to be insensitive to calmodulin.

We conclude that vegetative amoebae of *Dictyostelium discoideum* contain a protein that can be classified as a calmodulin on the basis of its heat stability, its activation of brain cyclic nucleotide phosphodiesterase in a Ca^{2+}-dependent reaction, its co-electrophoresis with brain calmodulin in the presence of Ca^{2+}, and its diminished electrophoretic mobility in the absence of Ca^{2+}. The roles of *Dictyostelium* calmodulin in motility and development of this microorganism can now be studied.

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