

The Preparation of Calmodulins from Barley (*Hordeum* sp.) and Basidiomycete Fungi

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(Received 30 July 1979)

1. Calmodulin-like proteins were purified from the fruiting bodies of higher (basidiomycete) fungi and barley (*Hordeum* sp.) shoots. 2. These calmodulins have electrophoretic mobilities on 10% (w/v) polyacrylamide gels at pH 8.3 in the presence of 6 M-urea and at pH 8.3 in the presence of 0.1% sodium dodecyl sulphate similar to that of bovine brain calmodulin. They interacted with rabbit skeletal-muscle troponin I in the presence of Ca^{2+} . 3. Barley and fungal calmodulins activated myosin light-chain kinase and phosphodiesterase in the presence of Ca^{2+} , although the amounts needed were at least an order of magnitude greater than is required to produce the same effect with mammalian calmodulin. 4. Amino acid analyses indicated a number of differences from the mammalian protein, most notably the absence of trimethyl-lysine. 5. By using ^{125}I -labelled calmodulin, a small amount of calmodulin-binding protein was detected in homogenates of barley and fungi. 6. No protein corresponding to calmodulin could be found in *Escherichia coli* or yeast, although a relatively high concentration of a protein that bound calmodulin was detected in *E. coli* by this technique.

Calmodulin was originally identified by Cheung (1970) and Kakiuchi & Yamazaki (1970) as an activator of bovine brain phosphodiesterase. This protein has since been shown to be necessary for full activity of a number of other Ca^{2+} -requiring enzymes, namely adenylate cyclase (Brostrom *et al.*, 1975), the membrane-bound ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase (Gopinath & Vincenzi, 1977), myosin light-chain kinase (Yagi *et al.* 1978), phosphorylase kinase (Cohen *et al.*, 1978) and NAD^+ kinase (Anderson & Cormier, 1978).

Calmodulin is present in high concentrations (150–700 mg/kg) in all mammalian tissues (Cheung *et al.* 1975; Grand & Perry, 1979) with the exception of striated muscle, where the amounts are almost an order of magnitude lower (Yagi *et al.* 1978; Nairn & Perry, 1979). Vanaman *et al.* (1976) have also shown calmodulin to be present in the brains of birds and reptiles. The amino acid sequence is strongly conserved, for the calmodulins isolated from bovine brain (Vanaman *et al.* 1977), bovine uterus (Grand & Perry, 1978) and rat testis (Dedman *et al.*, 1978) differ only in three or four residues.

A protein similar to calmodulin that is capable of increasing the activity of phosphodiesterase is present in a number of invertebrate species such as

Abbreviations used: Bicine, *NN*-bis-(2-hydroxyethyl)glycine; SDS, sodium dodecyl sulphate.

the starfish and sea anemone (Waisman *et al.*, 1975), and the protein isolated from the earthworm (*Lumbricus terrestris*) is very similar to mammalian cardiac-muscle calmodulin (Waisman *et al.*, 1978).

Although the role of calmodulin in activating a number of Ca^{2+} -requiring enzymes is clearly established, it appears to be present in most cells in greater amounts than would be required to activate those enzymes currently known to require calmodulin for activity (Grand & Perry, 1979). In mammalian tissue extracts calmodulin is associated with one or more of a number of calmodulin-binding proteins as complexes that are stable in high urea concentrations and that are dissociated when Ca^{2+} is removed by EGTA (Head *et al.*, 1977; Grand & Perry, 1979). This would suggest that, in mammalian systems at least, calmodulin functions by a complex series of interactions with a number of proteins.

With the aim of investigating the wider implications of the presence of calmodulin and calmodulin-binding proteins in living systems we have studied the system in several eukaryotes and a prokaryote. In the present paper we report the purification and characterization of calmodulin-like proteins from the green shoots of barley (*Hordeum* sp.) seedlings and from the fruiting bodies of three species of basidiomycete fungi.

Materials and Methods

Preparation of urea homogenates

Barley (Hordeum sp.). Shoots from barley seedlings grown on vermiculite for about 10 days were harvested when about 10 cm high, homogenized in 10 vol. of buffer A (9 M-urea/1 mM-CaCl₂/15 mM-β-mercaptoethanol/75 mM-Tris, adjusted to pH 8.0 with 1 M-HCl), and centrifuged at 11000g for 30 min. The supernatant was filtered through cheese-cloth.

Escherichia coli. E. coli K12 was grown anaerobically in continuous culture at pH 7.2. Glycerol was the carbon source and nitrite the nitrogen source. The dilution rate was 0.04. Bacteria were harvested from the growing medium by centrifugation at 5000g for 10 min. The packed cells (40 g) were resuspended in 75 mM-Tris adjusted to pH 8.0 with 1 M-HCl (300 ml) and re-centrifuged at 5000g for 10 min. The washed pellet was homogenized in 70 ml of buffer A and the cells were broken by using a Hughes press at a pressure of 60 MPa (3.8 tons/in²). The suspension was diluted to 300 ml with buffer A and then centrifuged at 31000g for 45 min.

Yeast (Saccharomyces cerevisiae). Yeast cells (10 g wet wt.) were suspended in 25 ml of buffer A and mixed with 40 g of glass beads. Cells were broken by subjecting this mixture to four 30 s bursts in a Braun (3508 Melsungen, Germany) shaker at full speed. The suspension was centrifuged at 31000g for 30 min.

Fungus. The fruiting bodies (100 g) from two wild species of Basidiomycetes tentatively identified as members of the *Russula* and *Cortinarius* genera and from the cultivated mushroom (*Agaricus bisporus*) were each washed in cold water to remove particulate material, homogenized in 500 ml of buffer A and centrifuged at 11000g for 30 min.

Preparation of calmodulin

Each of the homogenates prepared as described above was used for the preparation of calmodulin by the organic-solvent method described by Grand *et al.* (1979).

Bovine brain calmodulin was prepared by the same method. Bovine brain calmodulin was labelled with Bolton & Hunter (1973) reagent [*N*-succinimidyl-3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propionate] as described by Grand & Perry (1979) and was detected on polyacrylamide gels using Kodak Blue Brand X-ray films (Grand & Perry, 1979).

Phosphodiesterase assays

Bovine brain 3':5'-cyclic AMP phosphodiesterase, deficient in calmodulin, was prepared by the method of Wang & Desai (1977). The enzymic assay used was the linked 5'-nucleotidase assay system described by Watterson *et al.* (1976).

Incubations were carried out for 5 min at 30°C and the reaction was stopped by boiling; 5'-nucleotidase from the venom of the snake *Atrax crotalus* (grade V; Sigma Chemical Co., Poole, Dorset, U.K.; 10 μl of solution containing 0.25 mg/ml) was added and the whole incubated for 10 min at 30°C. P_i release was determined by the method of Sanui (1974).

Myosin light-chain kinase assays

Myosin light-chain kinase was prepared from rabbit fast-skeletal muscle by the method of Pires & Perry (1977) and enzymic assays were carried out as described by Nairn & Perry (1979).

Polyacrylamide-gel electrophoresis

Samples were run on 10% (w/v) polyacrylamide gels in 14 mM-Tris/90 mM-glycine, pH 8.3, either in the presence or absence of 6 M-urea. Samples were also run on 10% polyacrylamide gels in 0.1 M-Tris/0.1 M-Bicine (pH 8.3)/0.1% SDS. Gels were stained with Coomassie Brilliant Blue R for 30 min and then destained for 48 h.

Affinity chromatography

Troponin I was prepared from rabbit fast-skeletal muscle by the method of Perry & Cole (1974) and was coupled to Sepharose 4B by the method of Head *et al.* (1977).

Digestion with CNBr

Barley and basidiomycete-fungal calmodulins (0.5 mg) were dissolved in 70% (v/v) formic acid (200 μl). Approx. 1 mg of CNBr was added to each. After 16 h the samples were dried and then re-dissolved in buffer A. Portions were run on 10% (w/v) polyacrylamide gels in 6 M-urea/14 mM-Tris/90 mM-glycine, pH 8.3.

Amino acid analysis

Amino acid analysis was carried out after hydrolysis for 24 and 72 h in 6 M-HCl as described by Wilkinson *et al.* (1972). Analyses for trimethyllysine were by the method of Kuehl & Adelstein (1969), with 'system C', in which the analyser column (55 cm) is eluted with sodium citrate buffer, pH 5.28, at 27°C.

Results

Electrophoresis of whole extracts

We have shown elsewhere that the presence of calmodulin (Grand & Perry, 1979) and similar proteins such as troponin C (Head *et al.*, 1977) in tissues can be demonstrated by the appearance of a band migrating rapidly to the anode when urea extracts of whole tissues are subjected to electrophoresis on polyacrylamide gels at pH 8.6 in the presence of EGTA. If the EGTA is replaced by CaCl₂, the fast band corresponding to calmodulin or troponin C disappears and the Ca²⁺-binding pro-

teins migrate very much more slowly in the form of complexes either with calmodulin-binding proteins in non-muscle tissues or troponin I in the case of striated muscle.

Application of this test to urea extracts of whole barley, basidiomycete fruiting bodies, *E. coli* and yeast gave little evidence of a band of similar mobility to calmodulin if 2–5 mg wet wt. of tissue of cells was applied to 10% gels at pH 8.3 in the presence of 6 M-urea. If the precipitate obtained on addition of 80% (v/v) ethanol to the extract of barley and basidiomycete fungi [see the preparative procedure in Grand *et al.* (1979)] was tested in this way, however, the presence of calmodulin-like proteins was suggested by the appearance of a faint fast-migrating band of the appropriate mobility on electrophoresis in the presence of EGTA. These bands disappeared when Ca^{2+} was added to the buffer. No such band was visible in similar fractions from *E. coli* and yeast. If ^{125}I -labelled bovine brain calmodulin was added to the urea extract of barley and the basidiomycete fungi, all the radioactivity was present in the fast-migrating band obtained on electrophoresis in the presence of EGTA. In the presence of Ca^{2+} , a proportion of the radioactivity was present at the origin, but most of the remainder migrated with the mobility of free calmodulin.

Similar studies were carried out with ^{125}I -labelled calmodulin added to urea extracts of yeast and *E. coli*. For yeast, no difference in protein band pattern or distribution of radioactivity was apparent whether CaCl_2 or EGTA was present in the urea buffer. With *E. coli* all the radioactivity was present at the origin when Ca^{2+} was present. When the Ca^{2+} was replaced with EGTA the radioactive calmodulin could be seen as a single fast-moving band on the gel.

Properties of calmodulins from barley and basidiomycete fungi

Application of the procedure used for the preparation of vertebrate calmodulin (see the Materials

and Methods section) to barley and basidiomycete fungi gave proteins very similar in properties to bovine brain calmodulin. Yields were approx. 20–50 mg/kg for barley and basidiomycete fungi. A mixture of proteins was obtained from yeast or *E. coli* when the same procedure was used. None of these proteins clearly corresponded to calmodulin and all were present in low concentrations.

When subjected to electrophoresis in 0.1 M Tris/0.1 M-Bicine (pH 8.3)/0.1% SDS or 14 mM-Tris/90 mM-glycine (pH 8.3)/6 M-urea, the barley and basidiomycete calmodulins were shown to be reasonably pure when up to 20 μg was applied. The calmodulins migrated with the same mobility as bovine brain calmodulin on electrophoresis in urea (Fig. 1) and SDS. As is the case with calmodulins isolated from mammalian tissues, their mobility was slightly decreased when the CaCl_2 was replaced by EGTA (Fig. 1) in the sample run in 6 M-urea, pH 8.3.

The amino acid analyses of the two calmodulins showed some differences from that of bovine brain calmodulin, particularly in the absence of trimethyllysine (Table 1). The differences in composition were reflected in the band patterns obtained on electrophoresis of digests with CNBr. The electrophoretic band pattern of the CNBr digest of basidiomycete extracts was very different from that of bovine brain calmodulin, whereas that of barley bore obvious similarities to brain protein. It is considered that the relatively low values obtained for the number of methionine residues may be due to oxidation during hydrolysis of the barley and fungal calmodulins.

Complex-formation

The electrophoretic studies on the whole-tissue extracts indicated that, in the presence of Ca^{2+} , the calmodulins of barley and basidiomycetes existed as complexes with a protein or proteins that are present in the tissue. These proteins are presumably similar to the calmodulin-binding proteins found widely distributed in mammalian tissues (see Grand &

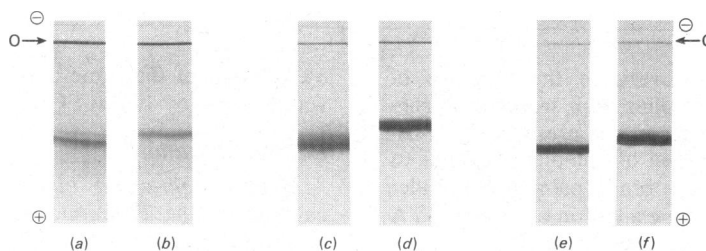


Fig. 1. Changes in mobilities of the calmodulins on electrophoresis in the presence of Ca^{2+} and EGTA. Electrophoresis was carried out with 10% (w/v) polyacrylamide/90 mM-glycine/14 mM-Tris (pH 8.3)/6 M-urea gels. Ca^{2+} and EGTA were present at 5 mM. O, origin. (a) 10 μg of basidiomycete calmodulin + CaCl_2 ; (b) 10 μg of basidiomycete calmodulin + EGTA; (c) 15 μg of barley calmodulin + CaCl_2 ; (d) 15 μg of barley calmodulin + EGTA; (e) 15 μg of bovine brain calmodulin + CaCl_2 ; (f) 15 μg of bovine brain calmodulin + EGTA.

Table 1. *Amino acid analysis of calmodulins*

Analyses were calculated on the basis of a mol.wt. of 16 700. Abbreviations used: Me₃Lys, trimethyl-lysine; n.d. not determined. Proteins were hydrolysed in duplicate for 24 and 72 h. Mean values are given. Serine and threonine values are corrected for breakdown during hydrolysis. The methionine values are low, possibly owing to oxidation during hydrolysis.

	Barley calmodulin (mol/mol) (mean for two preparations)	Basidiomycete (<i>Russula</i> sp.) calmodulin (mol/mol)*	Bovine brain calmodulin (mol/mol)†
Asp	20.7	19.1	24
Thr	8.4	8.8	12
Ser	9.9	11.1	5
Glu	26.5	22.5	29
Pro	5.0	10.1	2
Gly	11.9	16.9	12
Ala	15.2	16.8	12
Val	10.8	9.2	8
Met	3.7	3.6	10
Ile	6.0	6.3	8
Leu	11.5	9.5	10
Tyr	3.2	2.8	2
Phe	4.6	5.3	8
His	2.4	2.5	1
Lys	7.3	8.4	8
Arg	4.0	5.1	7
Me ₃ Lys	—	—	1
Trp	—	—	—
Cys	n.d.	n.d.	—

* Very similar values to those of *Russula* were obtained when a sample of *Agaricus bisporus* calmodulin was analysed.

† From Watterson *et al.* (1976).

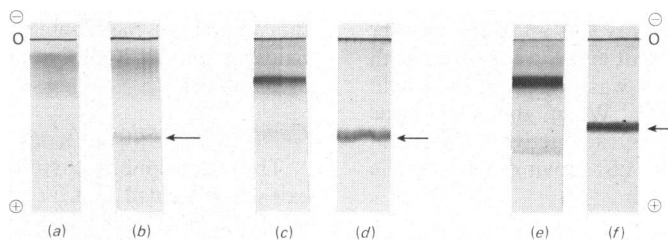


Fig. 2. *Interaction of calmodulins with troponin I from rabbit fast-skeletal muscle*

Electrophoresis conditions were as in Fig. 1. A 10 µg sample of each protein was applied in all cases. O, origin. (a) Basidiomycete calmodulin + troponin I + Ca²⁺; (b) basidiomycete calmodulin + troponin I + EGTA; (c) barley calmodulin + troponin I + Ca²⁺; (d) barley calmodulin + troponin I + EGTA; (e) bovine brain calmodulin + troponin I + Ca²⁺; (f) bovine brain calmodulin + troponin I + EGTA. The arrow indicates the calmodulin band.

Perry, 1979). Electrophoresis in the presence of 6 M-urea of both calmodulins with troponin I from rabbit fast-skeletal muscle in the presence of 1 mM-CaCl₂ led to the formation of complexes similar to that formed by bovine brain calmodulin under identical conditions. On the addition of 5 mM-EGTA the complexes were dissociated (Fig. 2).

When applied to a troponin I-Sepharose affinity column equilibrated with 6 M-urea/50 mM-Tris adjusted to pH 8.0 with 1 M-HCl/1 mM-CaCl₂/10 mM-β-mercaptoethanol, barley and basidiomycete calmodulins were bound to the column, but were eluted

by addition of the same buffer in which the CaCl₂ was replaced by 10 mM-EGTA.

Enzyme activation

Myosin light-chain kinase. In the absence of calmodulin, the 77000-mol.wt. catalytic component of myosin light-chain kinase of skeletal muscle is inactive (Yagi *et al.* 1978; Nairn & Perry, 1979). The enzyme is activated up to 70% of the maximum obtained with large molar excess of calmodulin by equimolar amounts of calmodulin from rabbit fast-skeletal muscle or bovine brain. Calmodulins

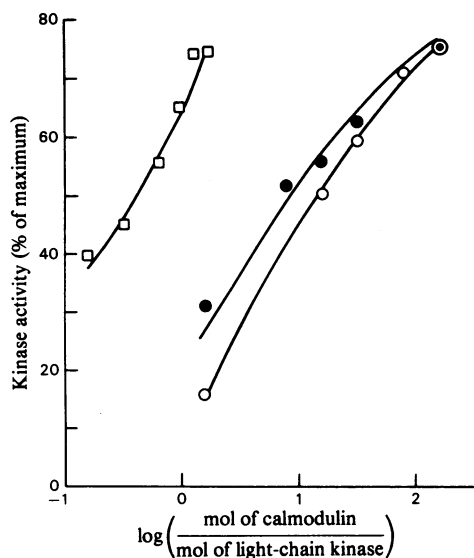


Fig. 3. Effect of calmodulins on the activity of myosin light-chain kinase from rabbit skeletal muscle

The enzymic activity was measured in 50 mM-Tris/HCl (pH 7.6)/12.5 mM-magnesium acetate/0.1 mM-CaCl₂/1 mM-dithiothreitol/5.5 mM-[γ -³²P]ATP as described in the Materials and Methods section, by using myosin light-chain kinase (1.9 nM) and light chain (2 mg/ml) from rabbit fast-skeletal myosin with calmodulin as indicated. Activity is expressed as a percentage of that obtained with a 65-fold molar excess of calmodulin from rabbit fast-skeletal muscle. □, Rabbit skeletal calmodulin; ○, barley calmodulin; ●, basidiomycete calmodulin.

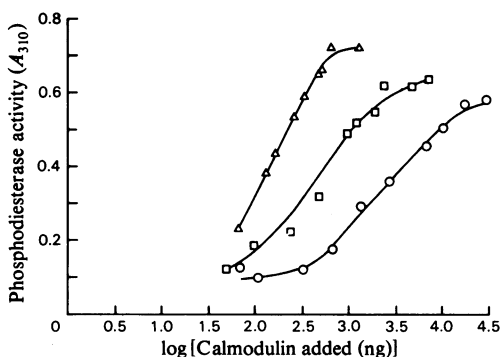


Fig. 4. Effect of calmodulins on the activity of cyclic nucleotide phosphodiesterase from bovine brain

The reaction mixture (0.5 ml) contained 40 mM-Tris adjusted to pH 8.0 with 1 M-HCl/0.4 mM-MnCl₂/1.0 mM-CaCl₂/2 mM-cyclic AMP, bovine brain phosphodiesterase (10 μ l; 1.5 mg/ml) and calmodulins as indicated. Δ , Bovine brain calmodulin; □, barley calmodulin; ○, *Russula* calmodulin.

from barley and the basidiomycete fungi had similar activation effects on the kinase from rabbit fast-skeletal muscle (Fig. 3), but on a molar basis were one to two orders of magnitude less effective than mammalian calmodulin (Fig. 3). No activation was obtained if the protein preparations obtained from *E. coli* and yeast were added to the enzyme assays under the conditions used for the experiments illustrated in Fig. 3 at protein concentrations up to 110 and 30 μ g/ml respectively.

Phosphodiesterase. Bovine brain phosphodiesterase, in common with myosin light-chain kinase, can be activated by calmodulins from many sources. In this respect the calmodulins from mammalian tissues are very similar in their effectiveness. Fig. 4 shows a comparison of the activating effect of calmodulins from bovine brain, barley and basidiomycetes on the cyclic nucleotide phosphodiesterase and indicates that the latter two calmodulins are clearly less effective than the mammalian protein in activating the bovine brain phosphodiesterase.

Discussion

In their behaviour on electrophoresis in the presence of troponin I and on troponin I-Sepharose affinity columns the calmodulins from barley and basidiomycetes are indistinguishable from the protein from bovine brain and other mammalian tissues. The marked differences of amino acid composition distinguish the plant and fungal calmodulins from the mammalian proteins, which as a group are remarkably similar in amino acid sequence. It indicates that significant divergence has occurred during evolution in this widely distributed protein. Particularly significant is the absence of trimethyllysine, residues of which have so far been reported to be a constant feature of calmodulin from animals.

The differences in primary sequence implied by the amino acid composition is reflected in the differences shown by the plant and fungal proteins in activating the two calmodulin-requiring enzymes of mammalian origin. On a molar basis the barley and basidiomycete calmodulins are between one and two orders of magnitude less effective than mammalian calmodulin in activating cyclic nucleotide phosphodiesterase and myosin light-chain kinase.

Thus it can be concluded that, although a number of differences exist, the proteins from barley and basidiomycetes possess the same general properties as mammalian calmodulin.

Some of the mammalian enzymes shown to be activated by calmodulin are present in plants and fungi. For example, some workers claim to have identified 3':5'-cyclic AMP in plants (Ashton & Polya, 1978). Also, processes that are Ca²⁺-regulated, and might, therefore, be expected to involve a Ca²⁺-binding protein similar to calmodulin, have

been demonstrated. Anderson & Cormier (1978) have shown that a protein is present in pea (*Pisum sativum*) that activates the endogenous NAD⁺ kinase as well as porcine brain phosphodiesterase. Although not purified to homogeneity, the protein was shown to be very similar in its properties to mammalian calmodulin.

In addition to interacting with the enzymes such as myosin light-chain kinase (Nairn & Perry, 1979), calmodulin in higher animals forms strong complexes with a number of basic proteins present in the cell, such as the calmodulin-binding proteins that have been identified by several groups (Grand & Perry, 1979; Klee & Krinks, 1978; Wang & Desai, 1977). As yet, no enzymic properties have been ascribed to these proteins, which possess unusual properties in that they form complexes with calmodulin that are stable in 6M-urea and are Ca²⁺-dependent. As judged by the behaviour of extracts of barley and basidiomycetes on electrophoresis, similar calmodulin-binding proteins are probably present in these organisms also.

If calmodulin is present in yeast or *E. coli*, the results suggest that it possesses a number of properties that are different from those of the barley or basidiomycete proteins. In the case of *E. coli*, the electrophoretic studies on extracts suggest that proteins similar to calmodulin-binding proteins may be present in prokaryotes. Nevertheless, if a calmodulin-type protein is present, it certainly does not behave normally in the preparative procedure. It is possible that the Ca²⁺-binding protein in prokaryotes is more primitive and smaller than the animal protein, comprising only one 'EF hand' as has been suggested by Kretsinger (1975) for an ancestral form of calmodulin. Alternatively the calmodulin-like protein may form part of the primary sequence of a much larger protein. Nevertheless, we have not yet obtained extracts from yeast or *E. coli* that activate either myosin light-chain kinase or cyclic nucleotide phosphodiesterase.

We thank Dr. D. J. Candy, Dr. J. A. Cole and Ms. M. M. Cooke for gifts of the barley, *E. coli* and yeast samples respectively. We are grateful to Ms. Sue Brewer for performing the amino acid analyses, to the Medical Research Council for financial support and to CIBA Laboratories Ltd., Horsham, West Sussex, U.K., for providing A. C. N. with a Studentship and research expenses.

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