Characterization of Calmodulin and Calmodulin Isotypes from Sea Urchin Gametes*

Wilson H. Burgess‡
From the Department of Biology, University of Virginia, Charlottesville, Virginia 22021

Procedures were developed for the purification of calmodulin from the eggs and from the sperm of the sea urchin Strongylocentrotus purpuratus and Arbacia punctulata. Two forms of calmodulin were isolated from A. punctulata eggs, one from each of the other three sources. All five calmodulins are similar to vertebrate calmodulin as judged by their activation of bovine brain cyclic nucleotide phosphodiesterase, calcium-dependent interaction with troponin I and with chlorpromazine, increased anode mobility on sodium dodecyl sulfate gels in the presence of calcium, cross-reactivity with anti-vertebrate calmodulin antibodies and amino acid compositions including the presence of a single residue of trimethyllysine. The two forms of calmodulin from A. punctulata eggs, calmodulin A and calmodulin B, can be distinguished from one another on the basis of DEAE-cellulose chromatography, amino acid analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence or absence of calcium and competitive radioimmunoassays. There is no evidence that either form is a proteolytic product of the other.

Calmodulin is the prototype of calcium-modulated proteins. It is present in the cytosol of all (or most) cells of all (or most) eukaryotes (see review by Klee et al., 1980; New York Academy of Sciences, Vol. 356, 1980). Unlike other calcium-modulated proteins such as the myosin light chains, troponin Cs, and parvalbumins, neither tissue-specific nor species-specific isotypes of calmodulin have been found. Although differences in the properties of calmodulin from vertebrates, plants, or invertebrates have been reported, only a single calmodulin from one another on the basis of DEAE-cellulose chromatography, amino acid analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence or absence of calcium and competitive radioimmunoassays. There is no evidence that either form is a proteolytic product of the other.

Calmodulin has evolved very slowly. Of 148 residues, there are at most only seven differences in amino acid sequence between calmodulin from bovine brain (Watterson 1980b) and that from the marine invertebrate, Renilla reinfermns (Jamieson et al., 1980). This is consistent with the fact that in contrast to the specialized roles of other calcium-modulated proteins, calmodulin interacts in a calcium-dependent and -specific manner with at least 15 different proteins.

We are studying the distribution and the regulatory functions of calmodulin during fertilization and early development in the sea urchins, Strongylocentrotus purpuratus and Arbacia punctulata.

It was reported previously (Burgess et al., 1980) that calmodulin isolated from S. purpuratus sperm is very similar to bovine brain calmodulin as judged by its interaction with troponin I, activation of bovine brain cyclic nucleotide phosphodiesterase and its electrophoretic mobility on SDS gels in the presence of 0.1 mM CaCl₂ or in the presence of 0.1 mM EDTA. More recently, calmodulin from the eggs and from the sperm of S. purpuratus and A. punctulata has been isolated and further characterized. During the course of this work, two forms of calmodulin were identified in the unfertilized eggs of A. punctulata. The isolation and the characterization of these proteins are described here.

MATERIALS AND METHODS

Preparation of Troponin I-Sepharose and CAPP-Sepharose

Troponin I was purified from rabbit skeletal muscle and coupled to Sepharose as described by Needleman and Muallem (1976). Bovine serum albumin was used as the standard. The protein concentrations used in the competitive radioimmunoassays were determined by amino acid analysis.

Polyacrylamide Gel Electrophoresis

Electrophoresis in the presence of 0.1% SDS (w/v) was performed on 12% or on 5% (w/v) acrylamide gels using the method of Laemmli (1970). When electrophoresis was carried out in the presence of calcium or EDTA, the sample buffer, running buffers and the gels were all made 0.1 mM in CaCl₂ or EDTA respectively, as previously described (Burgess et al., 1980a). Autoradiographs of the acrylamide gels were developed by autoradiography and exposure (Jenkin 1975) was used. Following electrophoresis at 20 V, the gels were stained for one hour in 0.1% (w/v) Coomassie brilliant blue R250, 50% (v/v) methanol, 10% (v/v) acetic acid and destained in 10% (v/v) methanol, 1% (v/v) acetic acid.

The abbreviations used are: SDS, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; CAPP, 2-chloro-10-(3-aminopropl) phenothiazine hydrochloride; EGTA, [ethylenbis(oxyethyl)eninitrilo]tetraacetic acid.

1 Portions of this paper (including "Materials and Methods," some data for "Results," Figs. 1 and 2) are presented in miniprint as prepared by the author. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. B1M-718, cite author, and include a check for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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‡ Present address, Howard Hughes Medical Institute, Vanderbilt University School of Medicine, Nashville, TN 37232.

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Purification of Calmodulin from S. purpuratus Eggs

Studies of eggs from S. purpuratus were initiated by injecting 0.3 M of 0.3 M KCl into the perivitelline cavity with a micropipette. The eggs were washed twice with calcium-free sea water (Marine Biological Laboratories, 1961), homogenized at 4°C in three volumes of 0.6 M Tris, pH 8.0, 0.1 M l-mercaptoethanol, and 0.1% Nonidet P-40, and centrifuged for 10 min at 10,000 g. The supernatant was adjusted to pH 6.0 and centrifuged at 10,000 g for 20 min. The precipitated material was resuspended in 100 mM Tris, pH 8.0, 0.1 M l-mercaptoethanol, 0.1% Nonidet P-40, and centrifuged overnight against two changes of the same buffer. The dialysed solution was clarified by centrifugation for 10 min at 15,000 g and applied to a column (1.6 cm x 60 cm) of CAPP-Sepharose that was equilibrated in the same buffer. The column was washed with five column volumes of the same buffer made 0.5 M as well. Calmodulin containing fractions were eluted with the same buffer made 0.5 M in l-mercaptoethanol. The calmodulin contained fractions were pooled and dialysed against three changes of 100 mM Tris, pH 8.0, 0.1 M l-mercaptoethanol. The protein was resuspended in about one ml of an appropriate buffer and applied to a SDS-polyacrylamide gel electrophoresis system, as described previously (Sommer and Waterman, 1984).

RESULTS

Purification of Calmodulin from S. purpuratus Eggs—Purification schemes for calmodulin from S. purpuratus eggs similar to that one described by Head et al. (1979) or that described under "Materials and Methods" of this paper for A. punctulata eggs were used initially. However, neither procedure was satisfactory because a large amount of lipid or lipoprotein material came to the surface during centrifugation following the 70% saturated ammonium sulfate step or the 70% saturated ammonium sulfate, pH 4.1, step in lieu of a protein pellet. It is believed this occurred as a result of the increase in the density of the solution as the ammonium sulfate concentration was increased from 50% to 70% saturation. The ethanol precipitation procedure described by Charbonneau and Cormier (1979) was tried because the density of the protein solution decreases as the concentration of precipitant, ethanol, is increased. This procedure proved to be satisfactory for S. purpuratus eggs. The 0 to 50% ethanol cut yields a large pellet, which was discarded. The 50 to 80% ethanol fraction yields a small white pellet, which contains calmodulin and other proteins.

Purification of Calmodulin from A. punctulata Sperm—The purification of calmodulin from A. punctulata eggs was carried out using the method described by Head et al. (1979) for the purification of calmodulin from A. punctulata eggs. The eggs were washed in artificial sea water three times with NaClO, homogenized in calcium-free sea water, and centrifuged at 10,000 g. The supernatant was adjusted to pH 6.0 and centrifuged at 10,000 g for 20 min. The precipitated material was resuspended in 100 mM Tris, pH 8.0, 0.1 M l-mercaptoethanol, and 0.1% Nonidet P-40. The homogenate was made 1 M in l-mercaptoethanol, adjusted to pH 4.1, and centrifuged for 1 h at 15,000 g. The supernatant was made 0.5 M in calcium chloride and centrifuged for 1 h at 15,000 g. The supernatant was adjusted to pH 6.0 and 0.1 M in calcium chloride and centrifuged for 1 h at 15,000 g. The supernatant contained calmodulin, which was precipitated by ethanol. The protein was resuspended in about one ml of an appropriate buffer and applied to a SDS-polyacrylamide gel electrophoresis system, as described previously (Sommer and Waterman, 1984).
purpuratus sperm using a modification of the procedure of Head et al. (1979) was reported previously (Burgess et al., 1980). More recently, the procedure described under "Materials and Methods" to isolate calmodulin from S. purpuratus or from A. punctulata sperm has been used. Fig. 1b shows the electrophoretic pattern of the proteins from the 50 to 80% ethanol precipitate of S. purpuratus sperm that are eluted from CAPP-Sepharose by EGTA. The major protein present is calmodulin. Several higher and lower apparent molecular weight contaminants are present; they are removed by chromatography on troponin I-Sepharose (Fig. 2, a and b). The sperm calmodulin migrates as a single band on SDS gels with an apparent molecular weight of 20,000 to 21,000 in the presence of 0.1 mM EDTA or with an apparent molecular weight of 14,000 to 15,000 in the presence of 0.1 mM CaCl₂. Similar results were obtained using A. punctulata sperm (Fig. 2, a and b, "Discussion").

**Figure 1.** Electrophoretic analysis of calmodulin A and calmodulin B from A. punctulata eggs after DEAE cellulose chromatography. Electrophoresis was performed on a 12.5% polyacrylamide slab gel in the presence of SDS and 0.1 M EDTA. Panel a, analysis of the eluent from the first DE-52 column used in the purification procedure. Samples from every tenth 10 ml fraction from the gradient beginning with lane A were electrophoresed with the exception of lane 1 which contains molecular weight standards, and lanes K, L, M, N, O, and P, which contained fractions 95, 96, 97, 98, 99 and 100 respectively. Both calmodulin A and calmodulin B were found in these fractions. Panel b, separation of calmodulin A from calmodulin B by DE-52 chromatography. The mixture of calmodulin A and B obtained from troponin I-Sepharose was applied to a DE-52 column and separated using a more shallow NaCl gradient than that used in figure 1a.

**Figure 4.** Electrophoretic analysis of the EGTA eluted fractions from troponin I-Sepharose during the purification of calmodulin A and calmodulin B from troponin I-Sepharose. Portions of the fractions shown in Fig. 3 were pooled and then rechromatographed on the same column in order to obtain additional pure calmodulin A and pure calmodulin B. The electrophoretic mobilities of calmodulin A and of calmodulin B in the presence of 0.1 mM EDTA are compared with those of protozoan calmodulin from Tetrahymena pyriformis, vertebrate calmodulin, and spinach leaf calmodulin in Fig. 4c. Calmodulin A co-migrates with the vertebrate calmodulin as do the other urchin calmodulins, whereas the electrophoretic mobility of calmodulin B is intermediate to that observed for the protozoan or the plant calmodulins. In one preparation soybean trypsin inhibitor, phenylmethylsulfonyl fluoride and p-tosyl-L-arginine methyl ester were included to inhibit proteolysis. There was no detectable difference in the yield of the A or of the B form of calmodulin in this preparation.

There is a variable amount of non-protein material associated with the final lyophilized solid from all of the urchin calmodulins we have purified. This material is present even after exhaustive dialysis and high speed centrifugation, yet can be removed from the protein by passing the sample over a PD-10 (Pharmacia) desalting column equilibrated with 10 mM CaCl₂.
mm ammonium bicarbonate. The excluded volume contains pure protein, the included volume contains a variable amount of UV absorbing material. This result is similar to that first reported by Watterson et al. (1980a) during the purification of spinach leaf calmodulin.

Phosphodiesterase Stimulatory Activity—The ability of the urchin calmodulins to stimulate bovine brain cyclic nucleotide phosphodiesterase was assayed as described under "Materials and Methods." The stimulation of the enzyme by these proteins including both the A and the B form of calmodulin from A. punctulata eggs is both qualitatively and quantitatively similar to the stimulation by bovine brain calmodulin (data not shown).

Radioimmunoassays—The results of a competition radioimmunoassay are shown in Fig. 5. Calmodulin from S. purpuratus eggs or sperm, calmodulin from A. punctulata sperm, and calmodulin B from A. punctulata eggs are similar in their ability to compete with chicken gizzard calmodulin in the assays. Calmodulin A from A. punctulata eggs also competes for the antiserum; however, 10 to 20 times more protein is required for 50% competition.

Chloropromazine Binding of Calmodulin A and Calmodulin B from A. punctulata Eggs—As discussed under "Materials and Methods," the CAPP-Sepharose chromatography step was not used in the purification of calmodulin A and B from A. punctulata eggs. After the troponin I-Sepharose column step the mixture of the two proteins was applied to a CAPP-Sepharose column in order to determine whether this step might be used to separate the two proteins. Both proteins bind to CAPP-Sepharose in a calcium-dependent manner even in the presence of 0.5 M NaCl.

Amino Acid Analysis—The amino acid compositions of the five urchin calmodulins that were isolated are given in Table I along with the published compositions of calmodulins from other sources.

Several of the features of the amino acid composition of vertebrate calmodulin are shared by all five urchin calmodulins including: a high aspartic acid and glutamic acid content, the absence of tryptophan and cysteine, and the presence of a single residue of trimethyllysine. There are several differences in the compositions as can be seen in the table.

The two most important points to make from the composition data are 1) that the amino acid compositions of the urchin calmodulins are very similar to the compositions of bovine brain calmodulin and of Renilla reniformis calmodulin and 2) that the amino acid compositions of the A and of the B forms of calmodulin from A. punctulata eggs do not indicate that one is a breakdown product of the other.

**DISCUSSION**

Calmodulin from the eggs and the sperm of the sea urchins, S. purpuratus and A. punctulata, was isolated and characterized. A. punctulata eggs contain what appears to be two different calmodulins. Only one calmodulin was identified in each of the other three sources. All five calmodulins are similar to vertebrate calmodulin as judged by their activation of bovine brain cyclic nucleotide phosphodiesterase, calcium-dependent interaction with troponin I and with chlorpromazine, increased anode mobility on SDS gels in the presence of calcium, cross-reactivity with anti-vertebrate calmodulin antibodies and amino acid compositions. In addition, calmodulin...
from *S. purpuratus* eggs increases the calcium sensitivity of microtubules purified from bovine brain by cycles of temperature-dependent polymerization and depolymerization to the same extent as does bovine brain calmodulin.\(^3\)

These results on the properties of urchin calmodulins are consistent with those of Jamieson et al. (1980), Seamon and Moore (1980), Yazawa et al. (1980), and others who showed only minor differences between the properties of calmodulin isolated from marine invertebrates and those of vertebrate calmodulin.

The procedures described under “Materials and Methods” for the isolation of calmodulin from the different sources should be useful, particularly to those who have encountered difficulties using ammonium sulfate fractionation to isolate calmodulin from *S. purpuratus* eggs and to those interested in studying the functional role of the two forms of calmodulin in *A. punctulata* eggs.

Jamieson et al. (1980) reported the isolation and characterization of calmodulin-like protein from *Chlamydomonas reinhardtii*. Unlike the second form of calmodulin we isolated from *A. punctulata* eggs, the proteins characterized by these two groups can be distinguished from calmodulin on the basis of cyclic nucleotide phosphodiesterase activation as well as by amino acid analysis or electrophoretic mobility on SDS-gels.

The two forms of calmodulin from *A. punctulata* eggs exhibit similar troponin I binding, chloropromazine binding, and cyclic nucleotide phosphodiesterase activation. Both contain 1 eq of trimethyllysine.

The two proteins can be distinguished by DEAE-cellulose chromatography, amino acid analysis, ultraviolet absorption spectra, and by SDS-polyacrylamide gel electrophoresis in the presence or the absence of calcium although both proteins undergo a large mobility shift. One form, calmodulin A, co-migrates with vertebrate calmodulin and the other urchin calmodulins in the presence of 1 mM EDTA (apparent molecular weight 20,000 to 21,000) whereas the other form, calmodulin B, migrates with an apparent molecular weight closer to that of plant protozoan calmodulin (17,000 to 18,000) under the same conditions. The two forms migrate as a tight doublet with an apparent molecular weight similar to that of vertebrate and plant calmodulin (14,000 to 15,000) when electrophoresis is conducted on SDS gels in the presence of 0.1 mM CaCl\(_2\). The electrophoretic mobility of calmodulin on SDS gels is itself anomalous. For example, Van Eldik et al. (1980a) showed that spinach calmodulin does not co-migrate with vertebrate calmodulin on SDS gels in the presence of EDTA (apparent molecular weight difference 1000 to 2000), yet amino acid sequence analysis reveals little or no difference between spinach and vertebrate calmodulin with respect to the number of amino acid residues (Iverson et al., 1981).

In addition, calmodulin A can be distinguished from calmodulin B by competition radioimmunoassays using anti-vertebrate calmodulin antibodies. Both proteins compete quantitatively for the antiserum; however, 10 to 20 times more calmodulin A than calmodulin B or the other urchin calmodulins is required for 50% competition.

The antibodies used in these experiments have been characterized by Van Eldik and Watterson (1981). They showed that all of the immunoreactivity of vertebrate calmodulin to these antibodies is contained in a peptide consisting of residues 127 to 144, the calcium-binding loop of the fourth domain. The reactivity with this particular antisera can be affected by amino acid sequence differences in this region (Fok et al., 1981). For example, one of the differences between the amino acid sequences of *R. reinformis* and spinach calmodulin and the sequence of bovine brain calmodulin occurs at residue 143, glutamine in bovine brain, lysine in *R. reinformis* and spinach (Jamieson et al., 1980, and Iverson et al., 1981). The reactivity of spinach calmodulin with these antibodies is similar to the reactivity observed with calmodulin A from *A. punctulata* eggs.\(^4\)

Further studies are necessary to determine the differences between the A and B forms of calmodulin that we isolate from *A. punctulata* eggs. As many as six putative calmodulin-binding proteins of apparent molecular weights 33,000 to 150,000 in various fractions of unfertilized *A. punctulata* eggs have been identified using the \(^{125}\)I-calmodulin gel overlay technique of Carlin et al. (1980). This procedure is being used to determine whether we can detect a difference in the number, type, compartmentalization, or affinity of proteins that bind to calmodulin A and those that bind to calmodulin B.

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REFERENCES


\(^3\) T. C. Keller, personal communication.

\(^4\) L. J. Van Eldik, personal communication.
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W H Burgess


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