Similarities and dissimilarities between calmodulin and a *Chlamydomonas* flagellar protein

(motility/phenothiazines/immunochemistry/calcium modulated proteins/phosphodiesterase activation)

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ABSTRACT   A protein that resembles vertebrate calmodulins and troponin C has been isolated from *Chlamydomonas* flagella by using a calmodulin purification protocol that included calcium-dependent affinity-based adsorption chromatography on phenothiazine-Sepharose conjugates. The flagellar protein resembled calmodulin in elution from reverse-phase columns, had a peptide map similar to that of calmodulin, and competed with vertebrate calmodulin in a radioimmunoassay using antisera against vertebrate calmodulin. However, this flagellar protein did not activate phosphodiesterase, lacked N^ϵ-trimethyllysine, and had an isoelectric point approximately 0.3 pH unit higher than that of vertebrate calmodulin. When analyzed by polyacrylamide gel electrophoresis under various conditions, the *Chlamydomonas* protein migrated between vertebrate calmodulins and rabbit skeletal muscle troponin C and did not manifest a large calcium-dependent mobility shift. This calmodulin-like protein was identified as one of the approximately 200 ^{35}S-labeled components in *Chlamydomonas* flagella resolved by two-dimensional gel electrophoresis. These studies indicate that calmodulin and a structurally and functionally homologous protein are present in the same cell. These studies also demonstrate that caution is necessary: (*i*) in identifying a protein as a calmodulin, (*ii*) in using phenothiazines or antisera directed against vertebrate calmodulins as specific probes for calmodulin, and (*iii*) in the interpretation of experiments on biological systems in which calmodulin is substituted for the homologous calmodulin-like protein.

Calcium has been implicated in the regulation of metabolic and mechanochemical processes in a number of biological systems (for a review, see ref. 1). Thermodynamic, kinetic, and crystallographic data strongly suggest that the targets of calcium acting as a biological signal transducer in the cytoplasm are calcium-modulated proteins (2). Two examples of this class of calcium-binding proteins are troponin C and calmodulin (3–6). The most extensively studied calcium-dependent mechanochemical system is vertebrate striated muscle and its regulation by troponin C (7). Other mechanochemical systems in which calcium regulation has been implicated are eukaryotic cilia and flagella (8–10). However, in these structures the molecular targets of calcium have not been described.

In *Chlamydomonas reinhardtii* the effect of calcium on flagellar movement has been observed in isolated flagellar apparatus and in whole cells subjected to photostimulation (11, 12). Both studies indicated that above a calcium concentration of 1 μM, swimming changes from a forward to a backward motion. Besides the physiological dependence of motility on calcium, use of *C. reinhardtii* provides some unique advantages for the study of regulation of flagellar motility by calcium. Polypeptide components can be labeled to a high specific activity by growth in a medium containing [^{35}S]sulfuric acid (13).

This allows for the reproducible resolution of complex mixtures of flagellar polypeptides by two-dimensional gel electrophoresis (13). In addition, procedures for the isolation of motility mutants have been described (14). The combined use of mutant analysis, differential extraction, two-dimensional polyacrylamide gel analysis, and electron microscopic analysis has resulted in the identification of several axonemal polypeptides as components of different flagellar substructures (13, 15, 16). Recently, an actin-like protein has been isolated from *Chlamydomonas* flagella and shown to be one of the polypeptides assembled in the axoneme (17). The isolation of calcium-modulated proteins from *C. reinhardtii* flagella and the correlation of these proteins with specific sets of flagellar components analyzed by two-dimensional gel electrophoresis would enhance our knowledge of flagellar substructures and provide a firm basis for future studies of calcium regulation.

We describe here the isolation of a protein from *Chlamydomonas* flagella that resembles calmodulin and troponin C. "Calmodulin" is the name proposed (4) for a brain phosphodiesterase activator protein. This activator protein has been shown (5, 18) to be indistinguishable from the unique chemical structure termed "modulator protein" (5, 6). Bovine brain calmodulin is a multifunctional, calcium-modulated protein that has a calculated molecular mass of 16,680 g/mol and is structurally homologous to troponin C (6). As previously discussed (5, 6, 19, 20), calcium-modulated proteins have extremely-similar physical, chemical, and sometimes functional properties. The flagellar protein described in this report clearly possesses many of the characteristic properties of this class of proteins, especially those features previously thought to be unique features of calmodulin (3, 4). However, the flagellar protein does not appear to be a calmodulin.

MATERIALS AND METHODS

Culture of cells, labeling with [^{35}S]sulfuric acid, preparation of flagella, and two-dimensional electrophoresis of polypeptides were performed as described (15, 16). Silver staining of unlabeled flagellar polypeptides in two-dimensional gels was as described elsewhere (21). Calmodulin was isolated from bovine brain and chicken gizzard by using described procedures (20) and phenothiazine-Sepharose [2-chloro-10-(3-aminopropyl)phenothiazine-Sepharose] chromatography (22). Parvalbumin and S100b were generous gifts of R. Kretsinger (Charlottesville, VA). Flagellar preparations were sonicated (Branson model W185 with microtip) for 30 sec at 4°C (approximately 25 W) in 2 vol of buffer I (0.1 M Tris·HCl/2 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA)/2 mM 2-mercaptoethanol, pH 8.0), and centrifuged at 10,000 × *g* for

Abbreviation: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

30 min. The supernatant fraction was prepared for chromatography on phenothiazine-Sepharose conjugates as described (22).

Troponin was isolated from rabbit skeletal muscle by the procedure of Ebashi *et al.* (23), and troponin C was subsequently purified by the method of Greaser and Gergely (24). Trypsin digestions were performed in the presence of EGTA as described (20). Column peptide maps of trypsin digests were obtained on a Whatman Partisil M9 ODS-2 column (9.4 × 250 mm) with a modified (25) Hewlett–Packard 1084B liquid chromatography system. Solvent A was 0.1% hydrochloric acid (Baker, Ultrex) and solvent B was acetonitrile (Burdick & Jackson, Muskegon, MI, UV grade). The microprocessor-controlled elution gradient consisted of the following time program: 0 min, %B = 5; 5.0 min, %B = 5; 5.5 min, %B = 10; 10.0 min, %B = 10; 40.0 min, %B = 50; 45.0 min, %B = 50. Amino acid and protein analyses were done as described (20, 22). Fluorography was done according to the procedure of Bonner and Laskey (26). Polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate was performed essentially as described (20, 22); details are given where appropriate in the text. Phosphodiesterase was prepared and activator assays were done as described (25). Antibody against calmodulin was prepared and radioimmunoassays were performed as described elsewhere (27).

RESULTS

When a *Chlamydomonas* flagellar preparation was fractionated according to a protocol for the purification of calmodulin, the elution profile shown in Fig. 1 was obtained at the phenothiazine-Sepharose chromatography step. The flagellar preparation was applied to a phenothiazine-Sepharose column in the presence of calcium, and a polypeptide was step-eluted with buffer containing EGTA instead of calcium. Bovine brain calmodulin, chicken gizzard calmodulin, and spinach leaf calmodulin were eluted under identical conditions from the same column.

The flagellar polypeptide that was step-eluted from phenothiazine-Sepharose with EGTA was analyzed by polyacrylamide gel electrophoresis. In the presence (Fig. 2A) and absence (Fig. 2B) of sodium dodecyl sulfate, the *Chlamydomonas* flagellar protein migrated between chicken gizzard calmodulin and rabbit skeletal muscle troponin C.
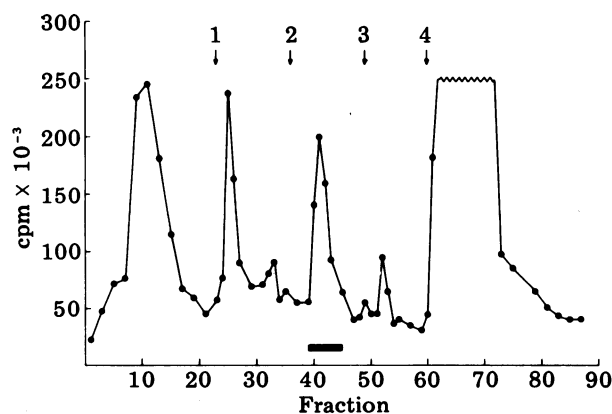
FIG. 1. Affinity-based adsorption chromatography of *Chlamydomonas* flagellar extract on phenothiazine-Sepharose. Samples were applied in buffer F (10 mM Tris·HCl/1 mM MgCl₂/1 mM 2-mercaptoethanol/2 mM CaCl₂, pH 8.0) and elution was with buffer F or buffer E (10 mM Tris·HCl/1 mM MgCl₂/1 mM 2-mercaptoethanol/2 mM EGTA, pH 8.0) as shown: 1, buffer F + 0.2 M NaCl; 2, buffer E + 0.2 M NaCl; 3, buffer E + 1 M NaCl; 4, buffer E + 8 M urea. Bar indicates pooled fractions containing the calmodulin-like protein.
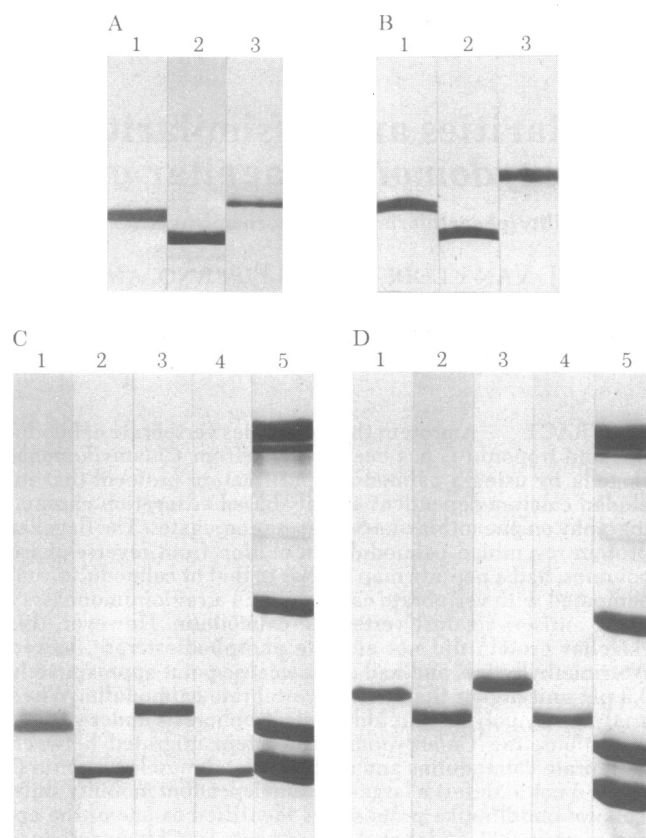
FIG. 2. Electrophoretic analysis of calmodulin, troponin C, and a calmodulin-like protein from *Chlamydomonas* flagella. The anode is at the bottom of all gels shown. In all panels, lane 1 is an autoradiogram and lanes 2–5 were stained with Coomassie blue. Lane 1, *Chlamydomonas* calmodulin-like protein; lane 2, chicken gizzard calmodulin (10 μg); lane 3, rabbit skeletal muscle troponin C (10 μg). (*A*) Electrophoresis in the presence of sodium dodecyl sulfate and utilizing a gradient [8–18% (wt/vol)] of acrylamide. Samples were incubated at 100°C for 3 min before analysis. (*B*) Electrophoresis in the absence of sodium dodecyl sulfate on a 12.5% (wt/vol) acrylamide gel. (*C*) Electrophoresis in the presence of sodium dodecyl sulfate on a 12.5% acrylamide gel containing 1 mM Ca²⁺. All samples and gel buffer also contained 1 mM Ca²⁺. Samples were incubated at 100°C for 3 min before analysis. Lane 4, bovine brain calmodulin (10 μg); lane 5, 10 μg of each molecular weight standard (from top to bottom, bovine serum albumin, catalase, chymotrypsinogen, myoglobin, and lysozyme). (*D*) As in *C* except that the gel, gel buffer, and samples contained 1 mM EGTA instead of Ca²⁺.

It has been reported (22, 28–30) that the mobility of calmodulin during sodium dodecyl sulfate/polyacrylamide gel electrophoresis is altered by the presence of calcium or chelator in the sample. Burgess *et al.* (28) have reported that other proteins in the same family of calcium-modulated proteins, such as troponin C, parvalbumin, and S-100b, do not undergo this large mobility shift. We have routinely observed a slight mobility shift in troponin C in the gel system shown in Fig. 2 but it is not nearly as large as that observed with calmodulin (Fig. 2). The ³⁵S-labeled flagellar protein (Fig. 2 *C* and *D*, lane 1) showed a slight calcium-dependent mobility shift in the presence of sodium dodecyl sulfate. The mobility shift of the flagellar protein was not as great as that of the chicken gizzard and bovine brain calmodulins (Fig. 2 *C* and *D*, lanes 2 and 4) but was slightly greater than that seen with rabbit skeletal muscle troponin C (Fig. 2 *C* and *D*, lane 3). In the presence or absence of calcium, the flagellar protein migrated between the vertebrate calmodulins and troponin C. Experiments in which calmodulin and the flagellar protein were mixed before electrophoresis also demonstrated the difference in mobility. We have

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previously reported (22) that plant calmodulin and calmodulin lacking N^{ϵ}-trimethyllysine also undergo this shift.

Because comparative tryptic peptide maps have been shown to distinguish vertebrate calmodulins from troponin C (5, 18), comparative tryptic peptide mapping of calmodulin, the flagellar protein, and troponin C was done. The ^{35}S-labeled *Chlamydomonas* protein and unlabeled chicken gizzard calmodulin were mixed, digested with trypsin, and analyzed by column peptide mapping. Six ^{35}S-labeled peaks were readily detected, of which two migrated as a doublet and three isotopic peaks comigrated exactly with calmodulin tryptic peptides (Fig. 3). Amino acid analysis of an acid hydrolysate of the flagellar protein demonstrated that the majority of the ^{35}S label comigrated with methionine. The approximate number of methionine-containing tryptic peptides expected for a calmodulin digest (20) were found in this analysis of the flagellar protein. Furthermore, methionine was detected in acid hydrolysates of the three calmodulin peptide pools that comigrated with the labeled flagellar peptides. When a trypsin digest of troponin C was analyzed in an identical manner, the profile obtained had little similarity to the one shown. It is possible that the second peak of the doublet at 42–43 min is undigested flagellar protein. In replicate analyses, calmodulin had a retention time of 40–41 min and the flagellar protein had a retention time of 41–43 min. Therefore, the flagellar protein and its methionine-containing tryptic peptides had retention times on reverse-phase columns similar to, but distinct from, the calmodulin retention time.

Because isolated flagella are the starting material in the purifications described above, severe restrictions are placed on the total amount of protein that can be purified from a single flagella preparation. Therefore, flagella preparations were accumulated over several weeks, stored at $-20°C$, and then subjected to the purification protocol used for the high-specific activity/low-protein preparations described above. The purified protein was: (*i*) analyzed by polyacrylamide gel electrophoresis, (*ii*) tested for phosphodiesterase activator activity, (*iii*) tested for calmodulin immunoreactivity, and (*iv*) analyzed for the presence of N^{ϵ}-trimethyllysine.

As summarized below, the larger-scale preparation yielded the same protein and allowed a further demonstration of the similarities and dissimilarities between calmodulin and the flagellar protein. First, upon electrophoretic analysis all Coomassie blue-staining material comigrated exactly with the
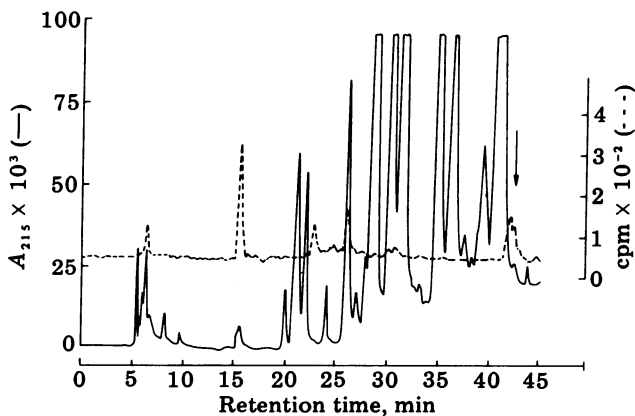
FIG. 3. Peptide maps of calmodulin and *Chlamydomonas* protein on reverse-phase column. Chicken gizzard calmodulin and ^{35}S-labeled *Chlamydomonas* protein were mixed, treated with trypsin, and prepared for analysis. A 100-μl sample of the digest containing 5 nmol of calmodulin and 2600 cpm of radioactivity was injected at zero time. Gradient elution from a Whatman Partisil ODS-2 column was performed. Arrow, retention time of undigested *Chlamydomonas* protein.
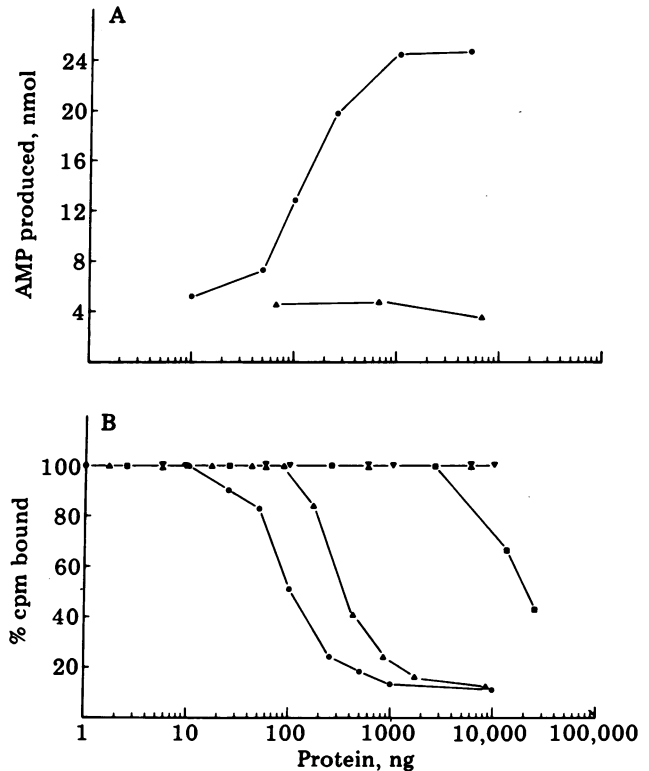
FIG. 4. Activator activity and immunoreactivity of *Chlamydomonas* flagellar protein. (*A*) The ability of bovine brain calmodulin (●) and *Chlamydomonas* flagellar protein (▲) to stimulate "activator-depleted" bovine brain 3',5'-cyclic nucleotide phosphodiesterase was determined. A reaction volume of 250 μl containing 2 mM cyclic AMP was used in the experiment shown. A 50-μl aliquot of each reaction mixture was analyzed by quantitative liquid chromatography for AMP production and cyclic AMP degradation. The ordinate shows the amount of product formed in 250 μl during a 5-min incubation at 30°C. Points are the mean of duplicate determinations; SEM for all points was less than 4%. (*B*) Various concentrations of bovine brain calmodulin (●), *Chlamydomonas* flagellar protein (▲), rabbit skeletal muscle troponin C (■), parvalbumin (▼), and S100b (✕) were mixed with anticalmodulin antiserum (1:30 dilution) and incubated overnight at 4°C. A constant amount of ^{125}I-labeled bovine brain calmodulin (1 ng; 50,000 cpm) was added to each assay tube, and the mixture was incubated overnight at 4°C and processed as summarized in the text. The degree of competition is expressed as a percentage of the cpm bound in the absence of competing antigen. The percentage of the cpm bound in the absence of antiserum was always less than 5%. SEM for all points was less than 5%.

^{35}S-labeled protein (data not shown). Second, as shown in Fig. 4*A*, the flagellar protein did not stimulate bovine brain phosphodiesterase under the conditions used. Third, the flagellar protein quantitatively competed with bovine brain calmodulin in radioimmunoassay (Fig. 4*B*). All vertebrate and plant calmodulins tested showed competition curves indistinguishable from that of bovine brain calmodulin curve (data not shown), but other structurally and functionally related proteins such as parvalbumin and S100b did not compete. Skeletal muscle troponin C competed only at relatively high protein concentrations. This level of competition may be due to: (*i*) the high degree of structural similarity between calmodulin and troponin C (6), of (*ii*) a minor calmodulin contamination in the troponin C preparation analogous to that found in parvalbumin preparations (31). Fourth, no N^{ϵ}-trimethyllysine was detected in an acid-hydrolyzed aliquot of the flagellar protein (data not shown).

A two-dimensional map of ^{35}S-labeled flagellar polypeptides contained more than 200 components in the molecular weight
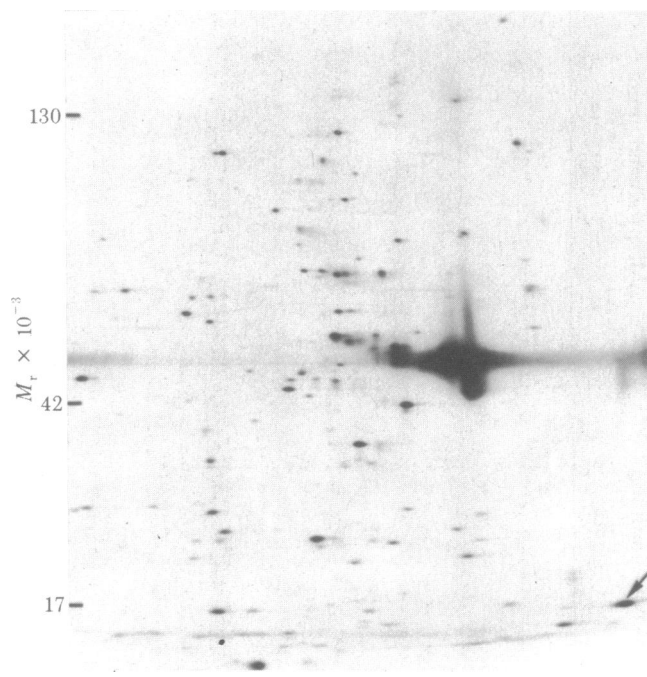
FIG. 5. Autoradiogram of the polyacrylamide slab gel used for the two-dimensional separation of ^{35}S-labeled polypeptides forming *Chlamydomonas* flagella. The sample was applied at the anode; basic polypeptides are on the left. Only part of the original map is shown; polypeptides having isoelectric points higher than 8.5 or molecular weights higher than 150,000 are not present. Tubulin subunits were not resolved and formed streaks as a consequence of overloading. Arrow, component that was purified and characterized as described in the text.

range 15,000–150,000 and in the isoelectric point range 4–8.5 (Fig. 5). The arrow denotes the position of a radioactive spot that comigrated with the flagellar protein isolated as described above. The identification of the flagellar protein in two-dimensional maps of flagellar polypeptides was done by mixing unlabeled flagella with the purified, ^{35}S-labeled flagellar protein and then directly comparing the resultant autoradiogram of the gel with the pattern revealed by silver staining. In experiments in which bovine brain calmodulin and ^{35}S-labeled flagella were mixed and the pH of the isoelectric focusing gel was measured, it was found that bovine brain calmodulin was 0.3 pH unit more acidic than the *Chlamydomonas* flagellar protein.

DISCUSSION

We conclude from these studies that *Chlamydomonas* flagella contain a protein that is structurally, functionally, and immunologically similar to, but not identical to, calmodulin. *Chlamydomonas* flagella may also contain calmodulin. However, under the conditions used in this study, a calmodulin-like protein, but not calmodulin, was isolated from flagella.

In a preliminary experiment, when *Chlamydomonas* cell bodies were subjected to an identical purification protocol, a polypeptide with electrophoretic properties similar to those of calmodulin was isolated as well as a polypeptide resembling the flagellar protein. This mixture of polypeptides stimulated phosphodiesterase.

It is possible that flagellar motility may be regulated by a protein such as the one described in this report. The calmodulin-like protein described here is certainly a major flagellar polypeptide because: (*i*) it is one of the major ^{35}S-labeled components resolved by two-dimensional gel electrophoresis,

(*ii*) the flagellar preparations used in these studies are not contaminated by cellular debris (16), and (*iii*) based on isotopic recovery the flagellar protein constitutes 0.6–1.0% of the extracted flagellar protein. However, it should be emphasized that the localization and function of this protein in *Chlamydomonas* flagella are not presently known. Careful, quantitative, suborganellar localization studies will be required in order to establish the location of this protein in the flagella and to identify any proteins which it might regulate.

During the course of these studies, Chafouleas *et al.* (32) reported the presence of a heat-stable compound in extracts of whole *Chlamydomonas* that quantitatively competed with calmodulin in radioimmunoassay but did not activate phosphodiesterase. The assumption was made (32) that this compound was calmodulin but not enough was present for detection by phosphodiesterase activator assay or that a heat-stable inhibitor was present. It is not known if the immunoreactive material of Chafouleas *et al.* (32) is calmodulin, the calmodulin-like protein described here, or a mixture of the two. The demonstration herein that a protein that is distinct from calmodulin can compete with calmodulin in radioimmunoassays indicates the caution necessary in the interpretation of radioimmunoassay and immunocytologic studies.

Recently, Jamieson *et al.* (33) isolated calmodulin from whole *Tetrahymena* cells. These investigators (33) suggested that the same molecule may be involved in the regulation of ciliary and flagellar function and achieved the purification of a calmodulin-like protein from demembranated cilia. Calcium-dependent interaction with phenothiazine-Sepharose and comigration with calmodulin in one gel system were used as criteria for the identification of the ciliary protein as a calmodulin. In light of the data summarized here, it is not known if the ciliary protein is calmodulin or a calmodulin-like protein. Clearly, the direct demonstration in this report that *Chlamydomonas* flagella contain a protein that is distinct from calmodulin indicates that studies on the biological function of calmodulin-like proteins should be performed with the protein purified from the homologous system.

Finally, this and other reports (5, 6, 19, 20, 22, 27) define more fully what is and is not a unique characteristic of calmodulins. Because of the multiple activities attributed to calmodulin, it becomes increasingly important that the characteristics that define calmodulin and distinguish it from structurally and functionally related proteins be elucidated. This information will, in turn, allow for the unambiguous interpretation of biological studies.

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