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Polyclonal Antibody That Recognizes Calcium-Dependent Determinants in *Tetrahymena* Calmodulin†

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ABSTRACT: We report here that a precipitating antibody prepared against *Tetrahymena pyriformis* calmodulin recognizes calcium-dependent determinants in the native protein. The ability of the antibody to precipitate ^{35}S-labeled *Tetrahymena* calmodulin in direct radioimmunoassays was enhanced at least 3-fold in the presence of calcium. Competitive radioimmunoassay using homogeneous preparations of endogenously ^{35}S-labeled *Tetrahymena* calmodulin and protein A–Sepharose-purified immunoglobulin G demonstrated that this antibody preparation is specific for protozoan calmodulin. Homogeneous vertebrate, invertebrate, and plant calmodulins, as well as rabbit skeletal muscle troponin C, did not show significant competition with the ^{35}S-labeled *Tetrahymena* protein at concentrations 100-fold greater than that at which the homologous unlabeled *Tetrahymena* calmodulin produced 50% competition. A cyanogen bromide digest of *Tetrahymena* calmodulin also showed partial competition with the intact ^{35}S-labeled protein, but only in the presence of calcium. The *major* antigenic determinants were localized to the carboxyl-terminal half of the molecule by immunoassay of limited trypsin fragments of *Tetrahymena* calmodulin. The antibody bound native calmodulin complexed to bovine brain phosphodiesterase (EC 3.1.4.17) but failed to recognize the *Tetrahymena* calmodulin carboxyl-terminal fragment (76–147) when complexed to the enzyme.

Calmodulin is a widely distributed, highly conserved protein that mediates the calcium-dependent regulation of many enzymes [for a review, see Klee & Vanaman (1982)]. Despite this high degree of sequence homology, several laboratories have succeeded in producing antibodies against mammalian calmodulin (Andersen et al., 1978; Dedman et al., 1978; Wallace & Cheung, 1979; Van Eldik & Watterson, 1981; Hansen & Beavo, 1983; Pardue et al., 1983). Mammalian calmodulin has been successfully employed as an antigen as the native protein (Andersen et al., 1978; Dedman et al., 1978), in a chemically modified form (Wallace & Cheung, 1979; Van Eldik & Watterson, 1981), or in enzyme complexes (Hansen & Beavo, 1983). Recently, in vitro immunization also has been used to produce monoclonal antibody of usable titer (Pardue et al., 1983). These antibodies show little specificity for calmodulin from any particular source, and only in limited instances [e.g., see Andersen et al. (1978) and Chafouleas et al. (1979)] do they appear to form precipitating antigen–antibody complexes. By contrast, antibody against native calmodulin from the ciliated protozoan *Tetrahymena pyriformis* is both precipitating and highly specific for protozoan calmodulin (Suzuki et al., 1982; present study). In this paper, we report the characterization of an anti-*Tetrahymena* calmodulin antibody and the ability of this antibody to recognize calcium-dependent determinants on calmodulin and calmodulin–enzyme (bovine brain phosphodiesterase, EC 3.1.4.17) complexes. The localization of the major *Tetrahymena* calmodulin antigenic determinants to the carboxyl-terminal half of the molecule increases the usefulness of the antibody as a tool for studying the interactions of calmodulin with calmodulin binding proteins, both in vitro and in situ. a preliminary report of portions of this work has been presented (McCartney et al., 1982).

Materials and Methods

Materials. Pansorbin (buffered suspension of pickled *Staphylococcus aureus* cells) was purchased from Calbiochem-Behring Corp., La Jolla, CA. Protein A and *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton–Hunter reagent) were purchased from Sigma Chemical Co., St. Louis, MO. Cyanogen bromide (CNBr)[1] and methyl *p*-hydroxybenzimidate (Wood's reagent) were purchased from Pierce Chemical Co., Rockford, IL. Noble agar was purchased from Difco Laboratories, Detroit, MI. $Na^{125}I$ and $H_2{}^{35}SO_4$ were

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[1] Abbreviations: CNBr, cyanogen bromide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; RIPA, radioimmunoprecipitation assay; PDE, bovine brain 3′,5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.17); CaM, calmodulin; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; DPT, diazophenyl thio ester; APT, aminophenyl thio ester; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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purchased from New England Nuclear, Boston, MA. Trypsin and soybean trypsin inhibitor were obtained from Millipore Corp., Bedford, MA. All other reagents were standard laboratory reagent grade.

Protein Preparations. Calmodulin was purified to homogeneity from bovine brain, mung bean, peanut, *Renilla reniformis*, and *Tetrahymena pyriformis* extracts by modifications of published procedures (Watterson et al., 1976; Jamieson & Vanaman, 1979; Jones et al., 1979; Kakiuchi et al., 1981a; Charbonneau et al., 1983). *Dictyostelium discoideum* and *Paramecium tetraurelia* calmodulins were the generous gifts of Dr. G. A. Jamieson, Jr. (Washington University Medical School), and Dr. J. E. Schultz (Institute of Pharmacy, University of Tubingen, Tubingen, FRG), respectively. The purity and integrity of each calmodulin were checked both by nondenaturing and denaturing polyacrylamide gel electrophoresis, UV spectral analysis, and amino acid composition and by its ability to activate bovine brain 3′,5′-cyclic nucleotide phosphodiesterase (PDE, EC 3.1.4.17).

Troponin C was purified from rabbit skeletal muscle as described by Perry & Cole (1974). *Acanthamoeba castellani* cells were the gift of Dr. Edward Korn (National Heart, Lung and Blood Institute, of Health, Behtesda, MD). Calmodulin-deficient PDE was partially purified from bovine brain as described by Klee & Krinks (1978). PDE activity was assayed by a modification of the procedure of Cheung & Lin (1974).

Concentrations of stock solutions of purified calmodulin and troponin C preparations were determined following acid hydrolysis by amino acid analysis on either a Beckman Model 121 or a Beckman Model 6300 amino acid analyzer. All other protein concentrations were determined by the method of Bradford (1976).

Radiolabeled Proteins. In vivo ^{35}S-labeled *Tetrahymena* calmodulin (specific activity 0.038 μCi/μg) was prepared as previously described (Blum et al., 1980). ^{125}I-labeled *Tetrahymena* calmodulin (specific activity 0.032 μCi/μg) was prepared by a modification of the method of Praissman et al. (1982). *Tetrahymena* calmodulin (3 nmol) was reacted with ^{125}I-labeled methyl *p*-hydroxybenzimidate (2 nmoles, 1 mCi/nmol) in 50 μL of 50 mM sodium borate–5 mM calcium chloride, pH 8.5, for 20 h at room temperature. Unreacted reagent was removed from the ^{125}I-calmodulin by repeated dialysis against 10 mM ammonium bicarbonate. ^{125}I-labeled protein A (specific activity 10 μCi/μg) was prepared by the method of Bolton & Hunter (1973).

Affinity Chromatography. Protein A–Sepharose CL-4B, obtained from Pharmacia Fine Chemicals, Piscataway, NJ, was used as supplied for affinity chromatography of both preimmune and hyperimmune antisera. Serum (1.0 mL) was applied to a 1 × 3 cm bed of protein A–Sepharose equilibrated in 50 mM Tris (pH 7.4)–150 mM NaCl (RIPA buffer). The column was washed to the A_{280nm} base line with RIPA buffer and then eluted with 100 mM CH_3COOH–150 mM NaCl, pH 3.8. The eluted material, containing all the anti-*Tetrahymena* calmodulin activity, was dialyzed against 0.1× RIPA buffer, lyophilized, and resuspended in one-tenth the final dialyzed sample volume of deionized water. Aliquots were stored at −70 °C.

For calmodulin–Sepharose 4B affinity chromatography, partially purified IgG, obtained by precipitation from 15 mL of immune serum with 18% (w/v) Na_2SO_4, was applied to a 1 × 8.5 cm bed of *Tetrahymena* calmodulin–Sepharose [prepared as described by Watterson & Vanaman (1976)] and equilibrated in RIPA buffer + 1 mM $CaCl_2$. Fractions (4.0 mL) were collected until a base line of A_{235nm}-absorbing material was reached. Then the column was eluted with RIPA buffer + 2 mM EGTA, followed by a 3.5 M $MgCl_2$ strip. Peak fractions were pooled and dialyzed against 10 mM NH_4HCO_3, lyophilized, resuspended in RIPA buffer, and assayed for cross-reactivity with *Tetrahymena* calmodulin.

Cleavage of Tetrahymena Calmodulin. Cyanogen bromide (CNBr) cleavage of *Tetrahymena* calmodulin was performed as previously described (Jamieson et al., 1979). The products were characterized by electrophoresis on 15% alkaline urea–polyacrylamide gels (Grand et al., 1979) and by amino acid analysis following hydrolysis. Limited (15-min) trypsin digestion of calmodulin was performed in the presence of 1 mM $CaCl_2$ by modification of the procedure of Walsh et al. (1977). *Tetrahymena* calmodulin (3.0 mg) was dissolved in 1.31 mL of 10 mM NH_4HCO_3–1 mM $CaCl_2$, pH 7.9, and 0.19 mL of 0.5 mg/mL trypsin was added. Digestion was allowed to proceed for 15 min at room temperature. Proteolysis was stopped by the addition of 30 μL of a 1 mg/mL stock solution of soybean trypsin inhibitor (0.32 mg required to inhibit 1 mg of trypsin) solution. The reaction mixture was lyophilized, and the calmodulin fragments were purified by reverse-phase high-performance liquid chromatography (HPLC) on a preparative Waters μBondapak phenyl column (Newton et al., 1984) as described in the legend to Figure 3. The identity of each fragment was determined by comparison of its amino acid composition (see paragraph at end of paper regarding supplementary material) with that expected from the published sequence of *Tetrahymena* calmodulin (Yazawa et al., 1981).

Production of Anti-Tetrahymena Calmodulin Sera. Electrophoretically homogeneous *Tetrahymena* calmodulin (50 μg) was emulsified in Freund's complete adjuvant and injected subcutaneously into a female New Zealand white rabbit. Booster injections of calmodulin in Freund's incomplete adjuvant were given at days 18 (50 μg), 22 (150 μg), 36 (100 μg), and 63 (100 μg). The rabbit was bled from the ear vein approximately every 7 days from day 30 to day 142 when the rabbit was killed. Blood was allowed to clot at 4 °C overnight. Serum was collected by centrifugation at 900g for 15 min and stored at −70 °C. Initially, sera were assayed against *Tetrahymena* calmodulin in Ouchterlony double-diffusion assays.

Ouchterlony Double-Diffusion Assay. Gel mixtures consisted of 1.5% (w/v) Noble agar, 50 mM Tris (pH 7.4), 150 mM NaCl, 0.02% (w/v) NaN_3, and either 5 mM $CaCl_2$ or 5 mM EGTA. Gels were formed by pouring 2.5 mL of this mixture onto 1 × 3 in. microscope slides. After solidification, 4-mm wells were cut, with 6 mm separating each well, by using a standard template. Immune serum was preincubated in its well 3 h before the addition of test antigen (10 μg in 10 μL or gel buffer) to the gel. Assays were incubated 24 h at room temperature and then soaked in 1% (w/v) NaCl for 48 h followed by deionized water for 24 h. The washed gels were dried and stained with Coomassie Blue.

Radioimmunoassays. Direct radioimmunoassays were performed in 50 mM Tris (pH 7.4)–150 mM NaCl (RIPA buffer) containing 0.2% (w/v) bovine serum albumin (BSA) and in the presence (5 mM $CaCl_2$) or absence (5 mM EGTA) of calcium. Assays were performed in duplicate in 1.5-mL microfuge tubes containing 4000 cpm of ^{35}S-labeled *Tetrahymena* calmodulin (specific activity 0.038 μCi/μg) or 16 000 cpm of ^{125}I-labeled *Tetrahymena* calmodulin (specific activity 0.032 μCi/μg) and varying amounts of preimmune and anti-*Tetrahymena* calmodulin IgG purified on protein A–Sepharose. The total volume was brought to 50 μL with RIPA buffer, and the tubes were incubated at 4 °C for 30 min. Five hundred microliters of RIPA buffer washed Pansorbin was

added to each tube. Following a 15-min incubation at 4 °C, the tubes were centrifuged for 2 min in a microfuge and the supernatants discarded. The pellets were washed once with RIPA buffer and resuspended in 500 μL of deionized water. Each sample was counted for 2 min, and the values for cpm precipitated were converted to the percent total cpm in each assay.

Competitive radioimmunoassays were carried out by using an amount of IgG determined by direct radioimmunoassay to precipitate 50% of the added radiolabeled *Tetrahymena* calmodulin. ^{35}S- or ^{125}I-labeled *Tetrahymena* calmodulin, competing antigen, and anti-*Tetrahymena* calmodulin IgG were incubated together in microfuge tubes at 4 °C for 30 min. The assay mixtures were treated with RIPA buffer washed Pansorbin for 15 min at 4 °C. These were then pelleted, washed, and counted as described for the direct radioimmunoassay. Values for CPM precipitated were corrected for background and converted to percent CPM precipitated relative to control mixtures containing no added competing antigen.

Diazophenyl Thio Ester (DPT) Paper Assay. Aminophenyl thio ester (APT) paper was prepared as described by Reiser & Wardale (1981). The paper was stored over silica gel in a sealed container at 4 °C until ready to use. Diazotization (APT → DPT) of paper was performed by treatment in 100 mL of ice-cold 1.2 N HCl followed by the addition of 2.85 mL of 10 mg/mL $NaNO_2$ (Alwine et al., 1979). After 30 min, the strips were rinsed with deionized water, and the paper was placed on a clean sheet of Whatman 3MM paper. Two-microliter aliquots of test antigen (0.5 nmol in 10 mM sodium borate, pH 9.8) was spotted in duplicate on the paper and allowed to react for 30 min. The paper was rinsed with 10 mM sodium borate, pH 9.8, and then placed in 1 M glycine, 0.2% (w/v) bovine serum albumin, 50 mM Tris, pH 7.4, and 150 mM NaCl overnight to destroy the reactivity of the paper.

For use in assays, quenched, antigen-coated paper was preequilibrated 3 × 30 min in 50 mM Tris, pH 7.4, 150 mM NaCl, and either 5 mM $CaCl_2$ or 5 mM EGTA (RIPA buffer + $CaCl_2$; RIPA buffer + EGTA). Wet papers were incubated for 5 h with 100 μL of immune serum diluted to 2.5 mL with RIPA buffer + $CaCl_2$ or EGTA in sealed freezer bags with gentle agitation. Papers were removed from the bags, rinsed 3 × 30 min with the appropriate RIPA buffer, and then reacted overnight with 0.5 μCi of ^{125}I-protein A in 2.5 mL of RIPA buffer. The resulting immunoblots were washed 3 × 30 min in RIPA buffer, air-dried, and placed against Kodak XAR-5 X-ray film at −70 °C.

Results

Purification and Characterization of Anti-Tetrahymena Antibody. Figure 1 shows the results of initial characterization of the rabbit antiserum prepared against purified *Tetrahymena* calmodulin as described under Materials and Methods. In an Ouchterlony double-diffusion assay (Figure 1 inset), purified *Tetrahymena* and *Paramecium* calmodulins gave rise to a single fused precipitin line, while calmodulins from less related organisms did not cross-react with the antiserum. In addition, the intensity of the precipitin lines formed with the protozoan calmodulins were considerably greater in the presence of 5 mM $CaCl_2$ than with 5 mM EGTA, indicating a calcium-dependent specificity in the interaction between calmodulin and the antibody.

A further assessment of the specificity of this anti-*Tetrahymena* antibody preparation was obtained by competitive radioimmunoassays also shown in Figure 1. Fixed amounts of endogenously labeled ^{35}S-labeled *Tetrahymena* calmodulin and

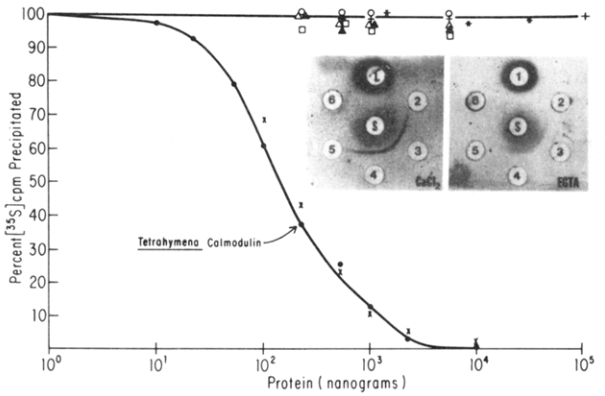

FIGURE 1: Species specificity of rabbit anti-*Tetrahymena* calmodulin IgG. The main panel shows competitive radioimmunoassays with homogeneous calmodulins from a variety of sources. All reactions were carried out in the presence of 5 mM $CaCl_2$ using an amount of protein A–Sepharose-purified IgG determined by direct radioimmunoassay (Figure 2 left panel) to precipitate 50% of the added ^{35}S-labeled *Tetrahymena* calmodulin. Assays were performed as described under Materials and Methods. (●) *Tetrahymena* calmodulin; (×) *Paramecium* calmodulin; (+) bovine brain calmodulin; (○) *Renilla* calmodulin; (□) mung bean calmodulin; (△) peanut calmodulin; (▲) rabbit skeletal muscle troponin C; (*) *Dictyostelium* calmodulin. Inset: Agar double diffusion performed as described under Materials and Methods. $CaCl_2$, gel contained 5 mM $CaCl_2$; EGTA, gel contained 5 mM EGTA: (S) immune serum raised against *Tetrahymena* calmodulin; (1) crude *Acanthamoeba* cell extract (10 μL); (2) *Renilla* calmodulin (10 μg); (3) *Tetrahymena* calmodulin (10 μG); (4) *Paramecium* calmodulin (10 μg); (5) bovine brain calmodulin (10 μg); (6) mung bean calmodulin (10 μg).

protein A–Sepharose-purified anti-*Tetrahymena* calmodulin IgG (see Materials and Methods) were reacted with various unlabeled purified calmodulins as well as the related Ca^{2+}-binding protein troponin C. The only proteins that effectively competed with the ^{35}S-labeled *Tetrahymena* calmodulin for antibody binding were the *Tetrahymena* and *Paramecium* calmodulins, confirming the specificity of the antibody for protozoan calmodulin.

Effect of Calcium on Antibody Reactivity. The effect of calcium on the interaction between antibody and *Tetrahymena* calmodulin, noted in the double-diffusion assay, was examined in a more quantitative manner by radioimmunoassay as shown in Figure 2. The ability of antibody to bind intact *Tetrahymena* calmodulin in the presence and absence of calcium was measured in a direct precipitation assay by reacting ^{35}S-labeled *Tetrahymena* calmodulin with purified IgG in buffer containing 5 mM $CaCl_2$ or 5 mM EGTA, followed by precipitation of the IgG and IgG–calmodulin complexes with Pansorbin. As shown in the left panel of Figure 2, the immune IgG exhibited greater avidity for *Tetrahymena* calmodulin in the presence of calcium, as precipitation of 50% of the ^{35}S-labeled *Tetrahymena* calmodulin in EGTA buffer required at least a 3-fold increase in IgG concentration over that required in the presence of calcium.

This differential antibody reactivity toward *Tetrahymena* calmodulin was accentuated when the protein was cleaved with cyanogen bromide (CNBr). The right panel of Figure 2 shows the results of a competitive radioimmunoassay in which the ability of the *Tetrahymena* calmodulin CNBr fragments to compete with whole ^{35}S-labeled *Tetrahymena* calmodulin for antibody in the presence or absence of calcium was compared to unlabeled *Tetrahymena* and bovine brain calmodulins. The CNBr-digested *Tetrahymena* calmodulin retained a majority of the native calmodulin's ability to compete, but only in the presence of calcium. In the absence of calcium, the digest was unable to compete with the native antigen.

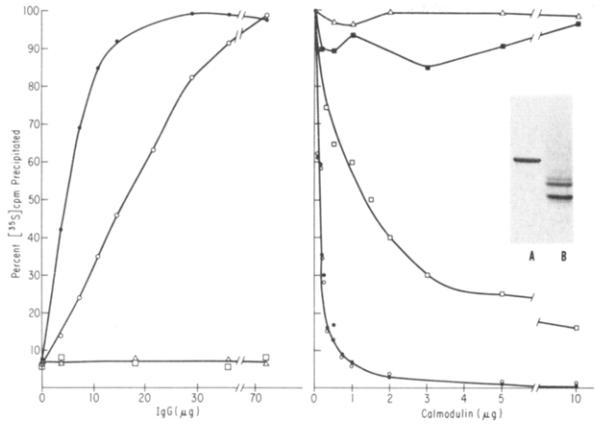

FIGURE 2: Calcium dependence of immune complex formation as measured by direct radioimmunoassay. (Left panel) Direct radioimmunoassay of unmodified *Tetrahymena* calmodulin. Assays were performed as described under Materials and Methods. Each assay mixture contained 4000 cpm of ^{35}S-labeled *Tetrahymena* calmodulin (specific activity 0.038 μCi/μg). Preimmune IgG and immune IgG were purified by protein A–Sepharose affinity chromatography as described under Materials and Methods. (●) Immune IgG, RIPA buffer + 5 mM $CaCl_2$; (○) immune IgG, RIPA buffer + 5 mM EGTA; (△) preimmune IgG, RIPA buffer + 5 mM $CaCl_2$; (□) preimmune IgG, RIPA buffer + 5 mM EGTA. (Right panel) Competitive radioimmunoassays of cyanogen bromide digested *Tetrahymena* calmodulin. Assays were performed as described under Materials and Methods. All samples contained 4000 cpm of ^{35}S-labeled *Tetrahymena* calmodulin (specific activity 0.038 μCi/μg). (Circles) *Tetrahymena* calmodulin; (squares) CNBr-digested *Tetrahymena* calmodulin; (triangles) bovine brain calmodulin; (open symbols) assays were performed in RIPA buffer + 5 mM $CaCl_2$; (closed symbols) assays were performed in RIPA buffer + 5 mM EGTA. Values of ^{35}S cpm precipitated were converted to percent ^{35}S cpm precipitated relative to the total ^{35}S cpm precipitated with no competing antigen. Total counts precipitated in the presence of EGTA with no competing antigen (1000 cpm) were 40% of the total counts precipitated in the presence of calcium (2500 cpm). Inset: Fifteen percent alkaline urea–polyacrylamide gel electrophoresis of whole and CNBr-digested *Tetrahymena* calmodulin. (A) *Tetrahymena* calmodulin (10 μg); (B) *Tetrahymena* calmodulin CNBr digest (50 μg).

No intact calmodulin was detectable in this CNBr digest by gel electrophoretic analysis (Figure 2, right panel, inset), and amino acid analysis revealed no detectable methionine residues. It should be noted that the apparent lack of calcium-dependent antibody reactivity with native *Tetrahymena* calmodulin in this competition experiment is due to the condition of antigen excess in the assays and to the normalization of the data relative to the amount of ^{35}S-calmodulin precipitated with no added competing antigen.

The calcium-dependent nature of *Tetrahymena* calmodulin antibody complex formation was further demonstrated by *Tetrahymena* calmodulin–Sepharose affinity chromatography. Three separate peaks of A_{235nm}-absorbing material were obtained when partially purified hyperimmune IgG was further resolved on such a column as described under Matierals and Methods (data not shown). When assayed for the ability to precipitate ^{35}S-labeled *Tetrahymena* calmodulin in a direct radioimmunoassay (see Materials and Methods), all the anti-*Tetrahymena* calmodulin activity was bound by the calmodulin–Sepharose column in the presence of Ca^{2+} and subsequently eluted by EGTA. The relative calcium- and EGTA-specific responses of the eluted antibody pools, however, were the same as that for the nonfractionated IgG shown in the left panel of Figure 2 (data not shown). The most likely explanation of these observations is that the differential responses of the antibody to calmodulin in calcium and EGTA are due to differential antibody avidities for the same set of

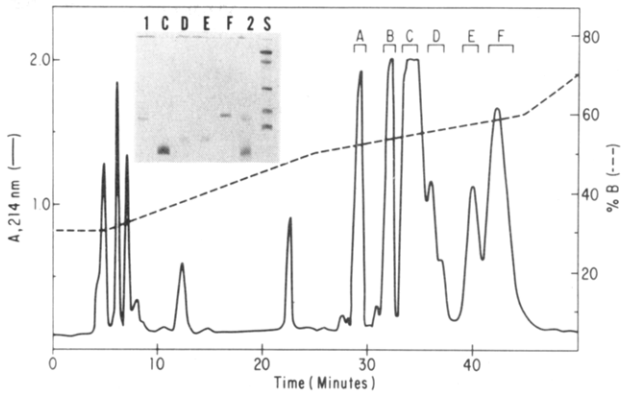

FIGURE 3: HPLC purification of *Tetrahymena* calmodulin tryptic fragments. Trypsin digestion of *Tetrahymena* calmodulin was performed as described under Materials and Methods. Lyophilized digest (1.4 mg) was dissolved in 70 μL of buffer A (10 mM Na_2HPO_4 and 2 mM EGTA, pH 6.1) and injected onto a 0.79 × 30 cm μBondapak phenyl (Waters Corp., Millford, MA) column equilibrated in 70% buffer A and 30% buffer B (50% CH_3CN in 5 mM Na_2HPO_4 and 2 mM EGTA, pH 6.1). The column was eluted at a flow rate of 0.8 mL/min with the gradient shown above the trace (%B). Eluted peaks were pooled as indicated (A–F). Inset: Fifteen percent SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) of HPLC-purified *Tetrahymena* calmodulin fragments. (1) *Tetrahymena* calmodulin (2 μg); (2) trypsin-digested *Tetrahymena* calmodulin (HPLC sample, 10 μg); (C–F) 25 μL of HPLC pools C–F, respectively [materials in HPLC pools A and B migrated too close to the dye front to be resolved (data not shown)]; (S) molecular weight standards: 66 000 (BSA), 45 000 (ovalbumin), 24 000 (trypsinogen), 18 000 (β-lactoglobin), and 14 000 (lysozyme). Fragments were identified by amino acid analysis as (A) residues 1–30, (B) residues 38–74, (C) residues 76–147, (D) unresolved mixture, (E) residues 1–75, and (F) residues 1–147 (whole calmodulin).

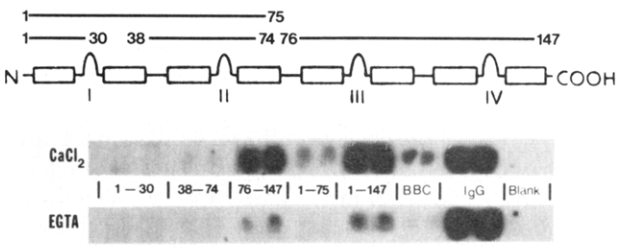

FIGURE 4: Diazophenyl thio ester paper immunoassay. (Top) Schematic representation of the calmodulin molecule showing the four Ca^{2+} binding loops (I–IV) flanked by α-helical segments (open rectangles). The four major tryptic fragments generated during the 15-min trypsin digestion of *Tetrahymena* calmodulin are depicted above the calmodulin molecule. (Bottom) Autoradiograms of 0.5-nmol aliquots of the indicated peptides and proteins covalently attached to DPT paper probed with anti-*Tetrahymena* calmodulin antiserum and ^{125}I-protein A as described under Materials and Methods. Duplicate pairs of each sample were assayed, one in the presence of 5 mM $CaCl_2$, and the other in the presence of 5 mM EGTA. 1–147, native *Tetrahymena* calmodulin; B.B.C., bovine brain calmodulin; IgG, anti-*Tetrahymena* calmodulin IgG (positive ^{125}I-protein A binding control); Blank, 10 mM sodium borate, pH 9.8 (background control).

sequence-specified antigenic determinants.

Localization of Antigenic Determinants. In order to identify the major antigenic regions of the molecule, we subjected *Tetrahymena* calmodulin to limited trypsin digestion as described under Materials and Methods. This digestion procedure yielded primarily four large fragments which could be more readily purified to homogeneity in reasonable quantities than the CNBr fragments. The trypsin digest was resolved by reverse-phase HPLC as shown in Figure 3 to yield homogeneous fragments as judged by SDS–PAGE (Figure 3 inset). The relative position of each fragment in the calmodulin sequence, determined by amino acid analysis, is noted in the legend to Figure 3 and shown diagrammatically in the upper

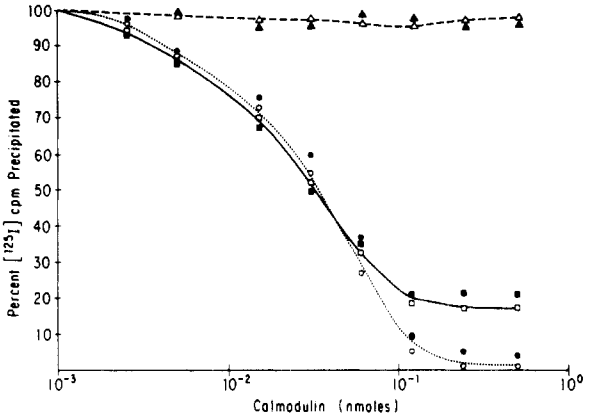

FIGURE 5: Competitive radioimmunoassays of *Tetrahymena* calmodulin tryptic fragments. Assays were performed as described under Materials and Methods. All reactions contained 16000 cpm of ^{125}I-labeled *Tetrahymena* calmodulin (specific activity 0.032 μCi/μg). (Triangles) *Tetrahymena* calmodulin fragment 1–75; (squares) *Tetrahymena* calmodulin fragment 76–147; (circles) undigested *Tetrahymena* calmodulin; (open symbols) assays were performed in RIPA buffer + 5 mM $CaCl_2$; (closed symbols) assays were performed in RIPA buffer + 5 mM EGTA. *Tetrahymena* calmodulin tryptic fragments 1–30 and 38–74 gave essentially the same results as fragment 1–75 (data not shown). Values for ^{125}I cpm precipitated were converted to percent ^{125}I cpm precipitated relative to the total counts precipitated with no competing antigen. Total counts precipitated in the presence of EGTA (6800) with no added competing antigen were 45% of the total counts precipitated in the presence of calcium (15000).

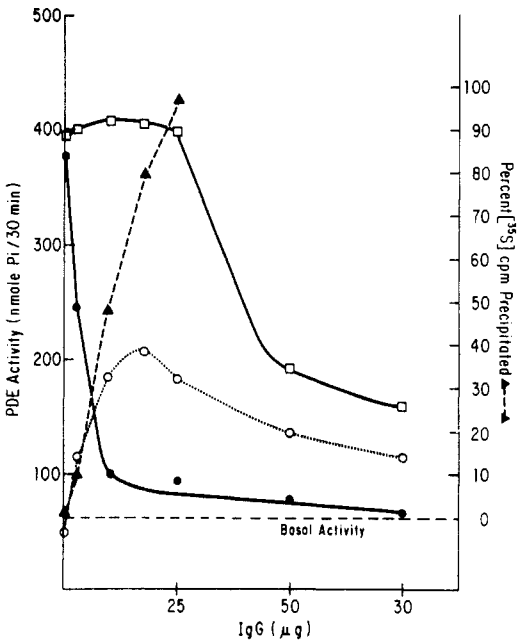

FIGURE 6: Phosphodiesterase activity + IgG ± Pansorbin. (□) PDE activity minus Pansorbin treatment: PDE (500 μg of total protein), 0.2 μg of *Tetrahymena* calmodulin, 0.05 μg of ^{35}S-labeled *Tetrahymena* calmodulin (1000 cpm), and varying amounts of anti-*Tetrahymena* calmodulin IgG were incubated together and assayed for PDE activity. A total of 0.25 μg of ^{35}S-labeled plus unlabeled calmodulin resulted in 80% activation of PDE. (●) PDE activity plus Pansorbin treatment: This assay was performed as described above, except that after 30 min the intitial PDE, IgG, and calmodulin mixture was treated with 300 μL of prewashed Pansorbin. Following a 15-min incubation, the Pansorbin was pelleted and the supernatant assayed for PDE activity. The Pansorbin pellet was assayed for PDE activity and total ^{35}S cpm as described below. (○) PDE activity in Pansorbin pellet: Pansorbin pellets were resuspended in 250 μL of RIPA buffer + 1.0 mM EGTA and incubated 5 min. The supernatants were collected after centrifugation (8730*g*, 2 min) and assayed for PDE activity in the presence of $CaCl_2$ (1 mM) and added calmodulin (0.2 μg). (▲) *Percent* ^{35}S cpm precipitated: EGTA-washed Pansorbin pellets were resuspended in 0.5 mL of deionized water and counted. cpm values were normalized relative to the total cpm added to each assay.

portion of Figure 4. The lower portion of Figure 4 shows the results of an experiment in which the four *Tetrahymena* calmodulin tryptic fragments, undigested *Tetrahymena* calmodulin, and undigested bovine brain calmodulin were covalently linked to diazotized filter paper and probed with anti-*Tetrahymena* calmodulin serum and ^{125}I-protein A as described under Materials and Methods. The majority of the immunoreactivity of the *Tetrahymena* calmodulin molecule existed in the carboxyl-terminal half of the molecule, fragment 76–147. The reactivity of this fragment was only slightly less than that of whole *Tetrahymena* calmodulin (1–147), and in both cases, the reactivity in calcium was considerably greater than in EGTA. Both bovine brain calmodulin and fragment 1–75 also exhibited some calcium-specific reactivity, which was unexpected because of the inability of these molecules to compete for antibody binding in competitive radioimmunoassays (Figures 1 and 5, respectively).

Figure 5 shows the results of competitive radioimmunoassay in which the abilities of fragments 1–75 and 76–147 to compete with ^{125}I-labeled *Tetrahymena* calmodulin for antibody were compared quantitatively with unlabeled whole *Tetrahymena* calmodulin (1–147). The response of fragment 76–147 was almost identical with that of undigested calmodulin, confirming the observation using the DPT paper assay that the major antigenic determinants are in this portion of the *Tetrahymena* calmodulin molecule. Fragment 1–75 showed no ability to compete in this assay.

Effect of Antibody on Calmodulin-Stimulated Phosphodiesterase Activity. Jamieson et al. (1979) demonstrated that the degree of bovine brain PDE stimulation by *Tetrahymena* calmodulin was equal to that of bovine brain calmodulin. It was therefore possible to determine whether or not antibody competed with PDE for *Tetrahymena* calmodulin binding and if the formation of enzymatically active PDE–CaM–IgG complexes was possible.

Figure 6 depicts the results of an experiment in which equal aliquots of PDE were incubated with *Tetrahymena* calmodulin reacted with increasing amounts of antibody and then assayed for PDE activity. The addition of greater than 25–30 μg of immune IgG resulted in a sharp decrease in PDE activity, whereas preimmune IgG had no effect.

Two possible explanations for this inhibitory effect of antibody were (1) that immune IgG competed with PDE for calmodulin binding, resulting in the loss of calmodulin-stimulated activity, or (2) that PDE–CaM–IgG complexes were precipitated at high IgG concentrations. To distinguish between these two possibilities, the experiment described above was repeated with the addition of a Pansorbin precipitation step to remove the IgG–calmodulin complexes from the reaction before assaying for PDE activity. The addition of ^{35}S-labeled *Tetrahymena* calmodulin allowed parallel determination of its distribution in the assays.

Pansorbin precipitation resulted in a much sharper decrease in the PDE activity remaining in solution than that observed with IgG alone. Removal of activity was essentially complete at 10 μg of IgG with pansorbin while PDE activity remained in the supernatant at up to 25 μg of added IgG without Pansorbin. This indicates that the rapid loss of PDE activity obtained with Pansorbin precipitation was not due merely to sequestration of the calmodulin by the antibody but rather precipitation of active PDE–CaM–IgG complexes. This conclusion was further supported by the demonstration that PDE activity was released from the Pansorbin pellet by EGTA

Table I: Precipitation of PDE–Calmodulin Complexes[a]

	PDE act. (nmol of P_i/30 min)	
	supernatant	pellet
PDE (basal activity)		
+buffer, Pansorbin	78	5
+IgG, Pansorbin	81	6
PDE + bovine brain calmodulin		
+buffer, Pansorbin	310	30
+IgG, Pansorbin	305	31
PDE + *Tetrahymena* calmodulin		
+buffer, Pansorbin	303	24
+IgG, Pansorbin	108	155
PDE + *Tetrahymena* calmodulin 76–147		
+buffer, Pansorbin	172	8
+IgG, Pansorbin	172	6

[a] Crude PDE (750 μg), alone or combined with either bovine brain calmodulin (0.02 nmol), *Tetrahymena* calmodulin (0.02 nmol), or *Tetrahymena* calmodulin tryptic fragment 76–147 (1.5 nmol), was assayed for PDE activity in a reaction volume of 0.5 mL. Under these conditions, PDE was fully activated by bovine brain calmodulin, while fragment 76–147 was present at a concentration sufficient to partially inhibit the activation of PDE by calmodulin. PDE activity: Nanomoles of P_i released in 30 min. Assay conditions: Fifty microliters of anti-*Tetrahymena* calmodulin IgG or RIPA buffer (no IgG control) was added to the reaction after a 15-min preincubation of PDE with the designated calmodulin or calmodulin fragment. Pansorbin in 200 μL of RIPA buffer was added after 15 min, incubation was continued for an additional 15 min, and then reaction mixtures were centrifuged and the supernatants removed. Supernatant PDE activity: Four hundred fifty microliters of supernatant was combined with 0.6 nmol of bovine brain calmodulin (3× required for maximal PDE activation) and assayed for PDE activity. Pellet PDE activity: Pansorbin pellets were resuspended twice in 200 μL of 1 mM EGTA. The two EGTA supernatants were adjusted to 1.5 mM $CaCl_2$, combined with 0.6 nmol of bovine brain calmodulin, and assayed for PDE activity. PDE (basal activity): Assayed as above except no calmodulin was added.

treatment, presumably as a result of dissociating the PDE–CaM complex.

The ^{35}S-labeled *Tetrahymena* calmodulin precipitation curve indicated that by 25 μg of IgG all of the added calmodulin was precipitated, even in the presence of EGTA, as calmodulin cpm values were determined in the EGTA-washed Pansorbin pellets. The actual point in the PDE assay mixture (+calcium) at which calmodulin was completely bound to IgG is probably closer to 10 μg, corresponding to the point at which maximum inhibition of calmodulin-stimulated PDE activity first occurred.

Recently, Newton et al. (1984) reported that a partial trypsin digest fragment of bovine brain calmodulin containing residues 78–148, while having lost its ability to activate PDE, could inhibit calmodulin activation of PDE ($K_i \sim 10^{-5}$ M). R. Klevit and T. C. Vanaman (unpublished results) have shown that this same fragment will inhibit the calcium-dependent cross-linking of azidocalmodulin to PDE. As *Tetrahymena* calmodulin fragment 76–147 appears to contain the major immunoreactive site in this calmodulin molecule, it was of interest to determine if (1) this fragment could inhibit calmodulin activation of PDE and (2) antibody could bind PDE–fragment complexes.

As shown in Table I, fragment 76–147 of *Tetrahymena* calmodulin did inhibit the activation of PDE. The presence of 1.5 nmol (3 μM) of this fragment significantly inhibited the activation of PDE by 0.6 nmol (1.2 μM) of bovine brain calmodulin (Table I, PDE + fragment 76–147, buffer + Pansorbin supernatant). [Addition of less than 1.0 nmol of fragment 76–147 to the assay had no inhibitory effect on PDE activation (data not shown).] However, PDE fragment 76–147 complexes were not precipitated by antibody and Pansorbin. The decreased PDE activity observed in the presence of fragment and antibody as shown in the table was due to inhibition of PDE activation by excess fragment rather than enzyme precipitation because (1) identical PDE activity (172 nmol of P_i/30 min) was obtained with and without antibody and (2) no PDE activity was released from the Pansorbin pellet, in contrast to the results obtained with native *Tetrahymena* calmodulin as shown in Figure 6.

Discussion

The polyclonal and highly specific nature of the anti-*Tetrahymena* calmodulin distinguishes it from the previously described antibodies against mammalian calmodulin (Andersen et al., 1978; Dedman et al., 1978; Chafouleas et al., 1979; Wallace & Cheung, 1979; Van Eldik & Watterson, 1981; Hansen & Beavo, 1983; Pardue et al., 1983). It should be noted that Suzuki et al. (1982) have reported the production of a similar anti-*Tetrahymena* calmodulin antibody.

The failure of the antibody to cross-react strongly with nonprotozoan calmodulin suggests that it is directed primarily against uniquely protozoan (perhaps uniquely ciliate) determinants. The primary structure of *Tetrahymena* calmodulin (Yazawa et al., 1981) differs from that of mammalian calmodulin (Watterson et al., 1980) at 15 positions—14 amino acid changes and 1 amino acid deletion. The majority of the differences (11 changes, 1 deletion) occur in the carboxyl-terminal half of the molecule, making this region the most likely to contain the species-specific antigenic determinants. This is in accord with the demonstration here that *Tetrahymena* calmodulin fragment 76–147 contains the majority of the immunoreactivity of the *Tetrahymena* calmodulin molecule. Van Eldik et al. (1981) reported that a major immunoreactive region of vertebrate calmodulin (residues 127–144) is found in the carboxyl-terminal portion of the molecule. Antiserum specific for vertebrate calmodulin residues 137–143 (Van Eldik et al., 1983a,b) reacted poorly with protozoan calmodulin (Watterson et al., 1984). It is possible that the lack of immunological cross-reactivity between mammalian and protozoan calmodulins is dictated by differences in this region of the sequence. *Tetrahymena* calmodulin has an arginine at position 143 instead of the glutamine found in vertebrate calmodulin. However, it must be noted that the antiserum to vertebrate calmodulin used in the studies of Watterson et al. (1974) was prepared with the performic acid oxidized protein. Thus, lack of cross-reactivity might result from differences between modified and unmodified calmodulins rather than species-specific differences in primary structure.

The *Tetrahymena* calmodulin antigenic determinants are at least partially dictated by protein conformation, as antibody avidity for calmodulin is greatest in the presence of calcium. The failure to isolate a totally calcium-specific antibody, even after Ca^{2+}-dependent affinity chromatography on a *Tetrahymena* calmodulin–Sepharose column, suggests, however, that the avidity differences are due not to a subpopulation of antibody molecules that only recognize calcium-specific determinants but rather to differential antibody avidity for the same set of linear deteminants. In other words, the strength with which the polyclonal antibody binds calmodulin (avidity) is influenced by the conformation of calmodulin, but the linear antigenic determinants are the same with and without calcium. This explanation is supported by the Ouchterlony double-diffusion assays, in which the ability of antibody to form precipitating complexes with protozoan calmodulin was diminished, but not destroyed, in the absence of calcium. The isolation of completely calcium-specific anti-calmodulin antibodies has been reported in preliminary form by Hansen & Beavo (1983) and Harper (1982, 1983). In the latter studies,

the antiserum prepared against dinitrophenylated mammalian calmodulin appears to recognize Ca^{2+}-dependent determinants even in plant calmodulins (Harper, 1983).

The totally calcium-dependent reactivity of the *Tetrahymena* calmodulin CNBr digest in the competitive radioimmunoassay can be explained by predicting that the calcium conformations of the antigenic determinant containing CNBr peptides and native *Tetrahymena* calmodulin are similar in the Ca^{2+}-liganded state, while in EGTA the peptides assume conformations very much different from that of native calmodulin.

The ability of the antibody to precipitate PDE–CaM complexes provides a probe for calmodulin–enzyme interactions. We have shown here that antibody-bound calmodulin complexed with PDE. In other studies (data not shown), this antibody preparation failed to interact with calmodulin bound to 14S dynein, a calmodulin-stimulated ATPase isolated from *Tetrahymena* cilia (Blum et al., 1980). These results indicate that conformational variability may exist in the interaction between calmodulin and different calmodulin binding proteins. Similar observations have been made by Hansen & Beavo (1983), who purified an antivertebrate calmodulin monoclonal antibody which recognized CaM–PDE complexes but not those formed between CaM and myosin light chain kinase or calcineurin.

One of the most specific calmodulin–enzyme interactions is the protozoan calmodulin-specific activation of *Tetrahymena* guanylate cyclase (Kakiuchi et al., 1981b; Kudo et al., 1981; Watanabe & Nozawa, 1982). It will be of interest to determine whether the same unique features of *Tetrahymena* calmodulin that enable the production of protozoan-specific antibody also dictate the specificity of guanylate cyclase activation.

The inability of the antibody to recognize the carboxyl-terminal half of the *Tetrahymena* calmodulin molecule (fragment 76–147) when it is complexed to PDE is indicated by the failure of antibody to (1) precipitate PDE activity from mixtures of PDE and fragment 76–147 and to (2) relieve the inhibition by fragment 76–147 of the activation of PDE by native calmodulin. Thus, the immunological characteristics of native *Tetrahymena* calmodulin and fragment 76–147, while very similar in radioimmunoassays, appear to be quite different when they are complexed to PDE, due to either the absence in the fragment of that portion of calmodulin responsible for precipitation or the differential alterations in its conformation in the complex. However, further experimentation will be required to fully characterize the interactions between the antibody, calmodulin, and PDE.

In conclusion, a precipitating calcium-dependent antibody against *Tetrahymena* calmodulin has been produced and characterized. The ability of this antibody to bind *Tetrahymena* calmodulin in PDE–CaM complexes makes it a useful tool for investigating the interaction of *Tetrahymena* calmodulin with calmodulin-activated enzymes.

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Supplementary Material Available

A table giving amino acid compositions of *Tetrahymena* calmodulin and *Tetrahymena* calmodulin limited tryptic peptides (1 page). Ordering information is given on any current masthead page.

Registry No. Ca, 7440-70-2; 3′,5′-cyclic nucleotide phosphodiesterase, 9040-59-9.

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Submicrosecond and Microsecond Rotational Motions of Myosin Head in Solution and in Myosin Synthetic Filaments As Revealed by Time-Resolved Optical Anisotropy Decay Measurements†

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ABSTRACT: Rotational Brownian motions of the head portion (subfragment 1) of rabbit skeletal myosin were studied by the measurement of flash-induced absorption anisotropy decay and phosphorescence anisotropy decay of the triplet probe 5-eosinylmaleimide bound to the myosin head. Fluorescence anisotropy decay of the fluorescent probe *N*-(1-pyrenyl)maleimide was also examined in some cases. Most of the triplet signals were observed in the presence of 60% (w/w) sucrose, which simply reduced the rate of motion via viscosity damping, to obtain good time resolution. Anisotropy decay of eosin on isolated head fragment was single exponential over two decades; the data indicated that the largest diameter of the head was 14–17 nm if the head was modeled as a prolate ellipsoid of revolution and 12–13 nm if oblate. Anisotropy decay in myosin synthetic filaments consisted of a fast and a slow component and a small constant part; myosin monomers and heavy meromyosin gave similar but somewhat faster decays with a smaller residual anisotropy. For each sample, the decay curves between −10 and 30 °C overlapped with each other after reducing the time scale to that at 20 °C in the absence of sucrose, showing that no gross conformational changes occurred between these temperatures. The fast decay was in the submicrosecond range on the reduced time scale and could be explained by a wobbling motion of the head around the head–rod junction within a cone of semiangle 35° for filament and 41° for solubilized proteins. The slow decay had a relaxation time of a few microseconds and indicated that a part of the rod portion next to the head also wobbled extensively. Analysis in which the rod end was assumed to wobble uniformly in a cone suggested that the effective length of the wobbling part was about 14 nm, and the cone angle was estimated to be about 48° in filament and 57–60° for solubilized proteins.

The molecular mechanism of muscle contraction is not yet fully understood. In striated muscles of vertebrates, sliding motion of the thick and thin filaments past each other leads to contraction (Huxley & Niedergerke, 1954; Huxley & Hanson, 1954), although shortening of the filaments themselves might also take place under certain conditions [see review by Pollack (1983)]. During contraction, the cross bridges projecting from the thick (myosin) filament approach the thin (actin) filaments; the force pulling the two sets of filaments against each other is apparently mediated by the cross bridges (Huxley, 1969). Where and how the force is generated, however, are still challenging problems.

A prevailing model has been one in which a cross bridge first attaches to an actin filament with a certain angle of tilt and subsequently changes the tilt angle, generating tension in an elastic arm which connects the bridge to the backbone of the myosin filament; after tension is exerted, the cross bridge is detached from the actin filament for the next cycle of reaction (Huxley, 1969; Huxley & Simmons, 1971). Recent studies, however, do not necessarily support this model, at least in its simplest form: Attempts at finding a variation in the angle of attachment of cross bridges using fluorescence (Yanagida, 1981) or electron paramagnetic resonance (Cooke et al., 1982) have revealed a rather uniform tilt, contrary to expectation from the model. Three-dimensional analyses of electron micrographs (Wakabayashi & Toyoshima, 1981) have shown a tilt angle different from a previously suggested one (Reedy et al., 1965) upon which the model was based. Clearly more information, particularly about dynamic aspects, is needed before a final answer is reached.

Two aspects should be distinguished when one considers the molecular dynamics of muscle contraction. One is the "conformational change(s)" of participating molecules: a different equilibrium structure may be induced when a protein

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