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Biochemistry

Calcium control of actin-activated myosin adenosine triphosphatase from *Dictyostelium discoideum*

(cell movement/cell shape/nonmuscle contraction/affinity chromatography)

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ABSTRACT **A protein fraction from the cellular slime mold *Dictyostelium discoideum* confers Ca^{2+}-sensitivity on the activation of purified myosin adenosinetriphosphatase (ATP phosphohydrolase, EC 3.6.1.3) from *Dictyostelium* by purified *Dictyostelium* actin. That is, the fraction inhibits the actomyosin adenosine triphosphatase activity in the absence of Ca^{2+} but not in the presence of Ca^{2+}. This Ca^{2+}-sensitizing factor affects only the actin-activated myosin adenosine triphosphatase and not the enzyme activity of myosin alone. The Ca^{2+}-sensitivity is conserved when muscle actin replaces *Dictyostelium* actin, but is lost when muscle myosin replaces *Dictyostelium* myosin. The factor appears to be a protein since it is nondialyzable, is heat labile, and can be precipitated with ammonium sulfate. The factor can be purified 70-fold on an actin-affinity column.**

Cell motility and its control are fundamental to living organisms, and therefore have been the subject of intensive biochemical research in recent years (for reviews, see refs. 1–3). In vertebrate striated muscle, which is the best understood contractile system, contraction is regulated by changes in Ca^{2+} concentration which affect the tropomyosin–troponin–actin complex. At very low concentrations of Ca^{2+}, the tropomyosin–troponin complex inhibits the interaction of actin and myosin, and thus prevents the contraction of the muscle. At higher concentrations of Ca^{2+}, the inhibition is relieved because Ca^{2+} binds to troponin and triggers a structural change in the tropomyosin–troponin–actin complex. In invertebrates, Ca^{2+} is the primary regulator of muscle contraction, but in certain organisms such as mollusks, Ca^{2+} controls contraction by binding to the myosin (4, 5).

Many cells other than muscle contain actin and myosin, and these proteins are probably involved in maintenance of cell shape and in a variety of forms of cell motility (3, 6, 7). There is evidence suggesting that Ca^{2+} controls the interaction of actin and myosin in nonmuscle cells. For example, Ca^{2+}-sensitive actomyosins have been isolated from blood platelets (8), equine leukocytes (9), and chick embryo brain (10). In the case of blood platelets (11), it has been possible to show that the control is exerted through an actin-linked system. These cells exist only in highly evolved vertebrates, and control of their contractile machinery may be expected to be similar to that of muscle contraction.

Actin and myosin are also found in primitive organisms such as slime molds. Indirect evidence suggests that the Ca^{2+} control systems found in higher organisms are related to those which occur in these primitive cells. Changes in Ca^{2+} concentration affect the aggregation of *Dictyostelium* amoebae (12), the streaming of *Physarum* cytoplasm (13), and giant amoebae cytoplasm (14). Furthermore, several investigators have reported results with crude extracts which indicate the existence of Ca^{2+}-sensitivity in the *Physarum* actomyosin complex (15–17). Tanaka and Hatano (15) found that a *Physarum* extract made superprecipitation of muscle actomyosin sensitive to Ca^{2+}. Nachmias and Asch (16) reported that the ATPase (adenosine triphosphatase; ATP phosphohydrolase, EC 3.6.1.3) activity in a crude *Physarum* actomyosin preparation was Ca^{2+}-sensitive. Kato and Tonomura (17) showed that activation of a crude *Physarum* myosin preparation by impure *Physarum* actin was greater in the presence of Ca^{2+} than in the presence of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid]. The same authors were able to isolate a crude fraction that was like tropomyosin-troponin (18).

Work in our laboratory has focused on biochemical and structural studies of actomyosin-like proteins from nonmuscle cells. Previous reports described the purification and characterization of myosin (19) and actin (20, 21) from the cellular slime mold, *Dictyostelium discoideum*. Here we report the isolation of a protein fraction from *Dictyostelium* which makes the interaction of purified *Dictyostelium* actin with purified *Dictyostelium* myosin sensitive to Ca^{2+}. Streptomycin sulfate treatment is crucial in unmasking the calcium-sensitizing activity present in crude extracts.

MATERIALS AND METHODS

Isolation of *Dictyostelium* Myosin. Myosin was extracted and purified from amoebae of *Dictyostelium discoideum* as described elsewhere (19), with two modifications. Dr. Margaret Clarke found that replacing the 200–400 mesh agarose column with a 100–200 mesh agarose column resulted in faster separation of the actin and myosin with the same resolution and that the activity of this myosin was considerably higher than that reported previously. The maximal actin-activated myosin ATPase when either *Dictyostelium* actin or muscle actin was used was 0.20 μmol of P_i/min per mg of myosin, a stimulation of 43-fold over the activity of the *Dictyostelium* myosin alone.

The second modification was an additional purification step in which DEAE-cellulose was used to remove a small amount of contaminating RNA. Myosin from the agarose column step was pooled, brought to 40 mM sodium pyrophosphate by the addition of 0.2 M sodium pyrophosphate (pH 7.5), and rapidly dialyzed (2 hr) against 40 mM sodium pyrophosphate (pH 7.5) to remove KCl. The dialyzed pool was then applied to a 2.4 × 8.5 cm DEAE-cellulose column (DE-52, Whatman Biochemicals Ltd.) equilibrated with 40 mM sodium pyrophosphate (pH 7.5). Nonadsorbing material was removed with two column volumes of equilibration buffer. Myosin was then eluted with 0.15 M KCl, 40 mM sodium pyrophosphate (pH 7.5). A similar procedure using DEAE-Sephadex has been described for muscle myosin (22), but we found that DEAE-cellulose resulted in a much higher recovery of *Dictyostelium* myosin (70–75%). The myosin fractions were pooled and dialyzed against 10 mM imidazole·HCl (pH 6.5), 0.1 M KCl, 0.1 mM dithiothreitol.

Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

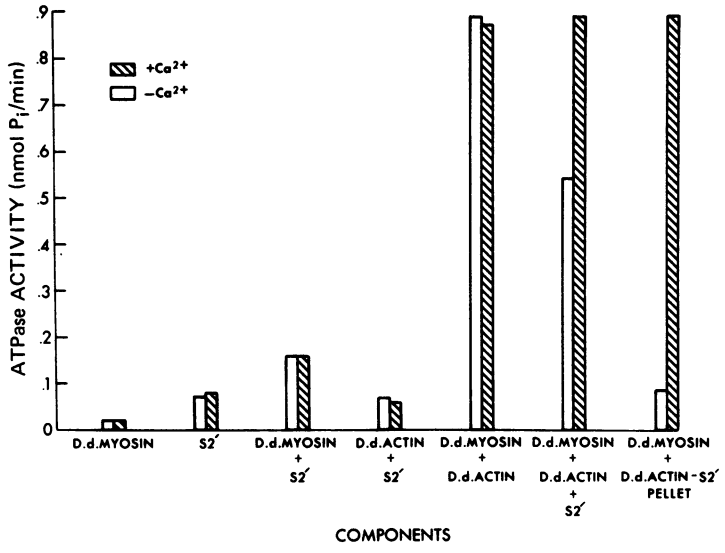

FIG. 1. Effect of S2′ on the actin-activated ATPase of *Dictyostelium* myosin. The assay conditions and preparation of the actin-S2′ pellet are described in *Materials and Methods*. D.d. = *Dictyostelium discoideum*. The values are averages of duplicate assays which differed by less than 10%. The actin-S2′ pellet was resuspended and used in a standard ATPase assay with myosin in the presence and absence of Ca^{2+}. Controls were performed with actin sedimented without S2′ (ATPase activity = 0.85 nmol P_i/min with Ca^{2+} and 0.90 without Ca^{2+}) and with S2′ sedimented without actin (ATPase activity < 0.008 nmol P_i/min with and without Ca^{2+}).

After dialysis, $MgCl_2$ was added to a final concentration of 10 mM, the solution was left at 0° for 2 hr, and the aggregated myosin was collected by centrifugation for 30 min at 100,000 × *g*. The pellet was dissolved in 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 0.1 mM dithiothreitol, 0.6 M KCl. The A_{280}/A_{260} ratio of the myosin was 1.6. Removal of the contaminating RNA did not significantly alter any of the previously reported (19) biochemical or structural properties of the *Dictyostelium* myosin. However, unpublished experiments in this laboratory have shown that higher levels of RNA inhibit the *Dictyostelium* myosin ATPase and actin-activated myosin ATPase.

Preparation of *Dictyostelium* Control Proteins. A crude actomyosin-free fraction from *Dictyostelium* amoebae, called S2, was obtained as described (19). Briefly, *Dictyostelium discoideum* amoebae were homogenized in a buffer containing 30% sucrose. The cell debris was removed by low-speed centrifugation, and the supernatant (S1) was dialyzed for 15 hr to remove the sucrose. Actomyosin was removed by low-speed centrifugation, and the supernatant (S2) was made 1 mM in dithiothreitol.

A 10% (wt/vol) solution of streptomycin sulfate (Pfizer Laboratories) at pH 7.1 was added to S2 to a final concentration of 0.5%. The solution was left at 0° for 30 min before removing the precipitate by centrifugation at 27,000 × *g* for 20 min. The supernatant was then dialyzed for 15 hr against 10 mM Tris·HCl (pH 8.0), 0.5 mM dithiothreitol. The dialysate was centrifuged at 100,000 × *g* for 30 min and the supernatant containing the Ca^{2+}-sensitizing factor was labeled S2′.

In some experiments, an actin-S2′ pellet was used and was prepared as follows: S2′ (3 mg/ml) in EGTA buffer [10 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 0.5 mM EGTA] was centrifuged for 2 hr at 100,000 × *g*. The supernatant solution (0.8 ml) and 0.5 mg of actin were mixed and brought to a final volume of 8 ml with EGTA buffer. This mixture was spun at 100,000 × *g* for 2 hr, resulting in an actin-S2′ pellet.

Isolation of *Dictyostelium* Actin. *Dictyostelium* actin was prepared by the method of Spudich (20).

Isolation of Muscle Proteins. Actin was purified from an acetone powder of rabbit striated muscle using the procedure of Spudich and Watt (23). Myosin was obtained from rabbit striated muscle as described by Tonomura *et al.* (24). Tropomyosin and troponin from rabbit striated muscle were gifts from Dr. Robert Crooks.

Assay of ATPase Activity. ATPase activity was measured using [γ-^{32}P]ATP, essentially as described previously (19). Standard reaction mixtures for measuring actin-activated myosin ATPase activity contained 0.5 mM [γ-^{32}P]ATP, 25 mM Tris·HCl (pH 8.0), 2.5 mM $MgCl_2$, 12 mM KCl, 5.4 μg of myosin, 6.7 μg of actin, 50–55 μg of S2′, and either 0.5 mM EGTA or 0.2 mM $CaCl_2$, in 0.1 ml total volume, unless otherwise noted. The actin-activated myosin ATPase activity was calculated by subtracting the activity found for myosin alone or, where S2′ was also used, for the mixture of S2′ and myosin.

Protein Determination. Protein concentration was determined by the method of Lowry *et al.* (25), as modified by Hartree (26), after acid precipitation. Crystallized bovine albumin (Pentex, Miles Laboratories) was used as a standard.

Affinity Chromatography. DNase-agarose was prepared by the procedure of Lazarides and Lindberg (27) or purchased from Worthington Biochemical. Muscle G-actin (1–2 ml of 3 mg/ml) was passed through a 1 ml column of DNase-agarose equilibrated with G buffer [5 mM Tris·HCl (pH 7.5), 2 mM ATP, 0.5 mM dithiothreitol, 0.2 mM $MgCl_2$]. Excess actin was washed from the column with G buffer. The column was then equilibrated with 10 mM Tris (pH 8.0), 2.5 mM $MgCl_2$, 0.2 mM $CaCl_2$ 1 mM dithiothreitol. S2′ was loaded onto the column in the same buffer, and unbound protein was removed by further washes with this buffer. The calcium-sensitizing activity was eluted with column buffer containing 1 M KCl. Each ml of DNase-agarose bound approximately 1 mg of actin. Each ml of this complex could adsorb the Ca^{2+}-sensitizing activity from 7 to 10 ml of S2′ (2.5 mg/ml).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. The discontinuous buffer system of Laemmli (28) was used according to the procedure of Ames (29).

Other Methods. Unless otherwise noted, all aspects of cell fractionation and protein manipulation were carried out at 0–4°. All pH values were measured at 25°.

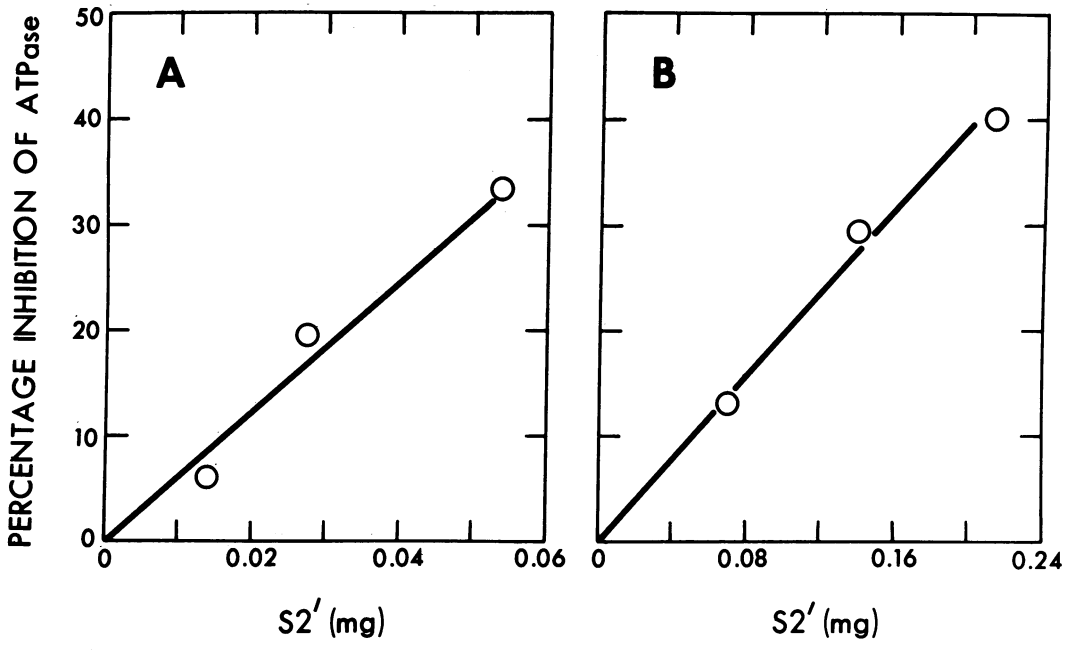

FIG. 2. Ca^{2+}-sensitive inhibition of actin-activated myosin ATPase as a function of S2′ concentration. Percentage inhibition was determined by comparing the rate of ATP hydrolysis in the presence or absence of Ca^{2+}, as defined in Footnote *. (A) *Dictyostelium* actin and *Dictyostelium* myosin. (B) muscle actin (6.0 μg) and *Dictyostelium* myosin. More S2′ was needed to inhibit the actomyosin ATPase in experiment B than in experiment A because a different and less effective S2′ preparation was used in experiment A. The effectiveness of a given S2′ preparation as an inhibitor of actin-activated *Dictyostelium* myosin ATPase was the same whether *Dictyostelium* actin or muscle actin was used (see Fig. 3).

RESULTS

Control of the actin-myosin interaction

As previously reported (19), the enzymatic interaction of purified *Dictyostelium* myosin with purified *Dictyostelium* actin does not require Ca^{2+}, nor is the enzymatic activity of the crude actomyosin fraction (P2) affected by Ca^{2+}. We found that under standard assay conditions, addition of 43 μg of S2 (see *Materials and Methods*) inhibited the actin-activated *Dictyostelium* myosin ATPase by 70%, but this inhibition was not relieved by Ca^{2+}. However, treatment of the S2 fraction with streptomycin sulfate, which removes nucleic acids, revealed a Ca^{2+}-sensitizing activity in the resulting supernatant fraction, called S2′.

Fig. 1 shows that the actin-activated *Dictyostelium* myosin ATPase activity is the same in the presence or in the absence of Ca^{2+}. Addition of S2′ renders the actomyosin ATPase sensitive to Ca^{2+}. That is, S2′ inhibits the actomyosin ATPase, and this inhibition is relieved by addition of Ca^{2+}. This difference in ATPase activities in the presence or absence of Ca^{2+} is not found upon assaying a mixture of actin and S2′, or myosin and S2′, indicating that the effect is specific for actin-activated myosin ATPase. These results eliminate the possibility that the S2′ effect is due to two separate activities, a Ca^{2+}-insensitive inhibitor of the actomyosin ATPase and a Ca^{2+}-sensitive ATPase unrelated to actomyosin.

The percent inhibition* of the actomyosin ATPase varies linearly with the amount of S2′ (Fig. 2). At S2′ levels higher than those shown in Fig. 2, the Ca^{2+}-sensitivity curve begins to level off; that is, Ca^{2+} becomes less effective in relieving the inhibition. This result probably reflects the impurity of S2′; for example, S2′ may contain nonspecific Ca^{2+}-insensitive factors which result in a lower actomyosin ATPase activity. A greater percent inhibition can be obtained by further fractionation of S2′. Some components present in S2′ bind to actin under the assay conditions. We therefore mixed actin with S2′ and sedimented the actin and its associated proteins by high-speed centrifugation. When the resulting pellet (actin-S2′ pellet) was resuspended and assayed with *Dictyostelium* myosin, much greater inhibition resulted, which was still completely relieved by Ca^{2+} (Fig. 1, last column).

To test whether the effect of S2′ is specific for the *Dictyostelium* contractile system, we examined its effect on muscle actin activation of *Dictyostelium* myosin and on *Dictyostelium* actin activation of muscle myosin. Fig. 3 shows that the Ca^{2+}-sensitivity is preserved when muscle actin replaces *Dictyostelium* actin, but is lost when muscle myosin replaces *Dictyostelium* myosin. S2′ appears to increase the actin-activation of muscle myosin ATPase slightly with or without Ca^{2+}.

Preincubation of actin and myosin with S2′ in EGTA does not reduce the subsequent rate of ATP hydrolysis produced upon addition of Ca^{2+}†. Thus, the lower rate of ATP hydrolysis observed when the actomyosin-S2′ mixture is assayed in EGTA is not due to an irreversible alteration of the actomyosin which occurs under the EGTA assay conditions. Instead, Ca^{2+} acts like a switch in turning on the actin-activated myosin ATPase.

Properties of the Ca^{2+}-sensitizing factor

The Ca^{2+}-sensitizing factor has properties characteristic of a

* Throughout this report, "percent inhibition" is determined by comparing the rate (R) of ATP hydrolysis when S2′ plus actomyosin was used in the presence or absence of Ca^{2+}, i.e., $[1-(R_{+S2',-Ca^{2+}}/R_{+S2',+Ca^{2+}})] \times 100$, and represents the degree of inhibition that can be relieved by Ca^{2+}.

† Under standard assay conditions with added S2′ (see *Materials and Methods*) *Dictyostelium* actomyosin hydrolyzed 16 nmol of ATP in 15 min in the presence of Ca^{2+} regardless of whether the actomyosin was preincubated for 15 min at 25° in 0.5 mM EGTA before adding 1 mM Ca^{2+} and ATP. The actomyosin hydrolyzed only 8 nmol of ATP in 15 min in the presence of EGTA.

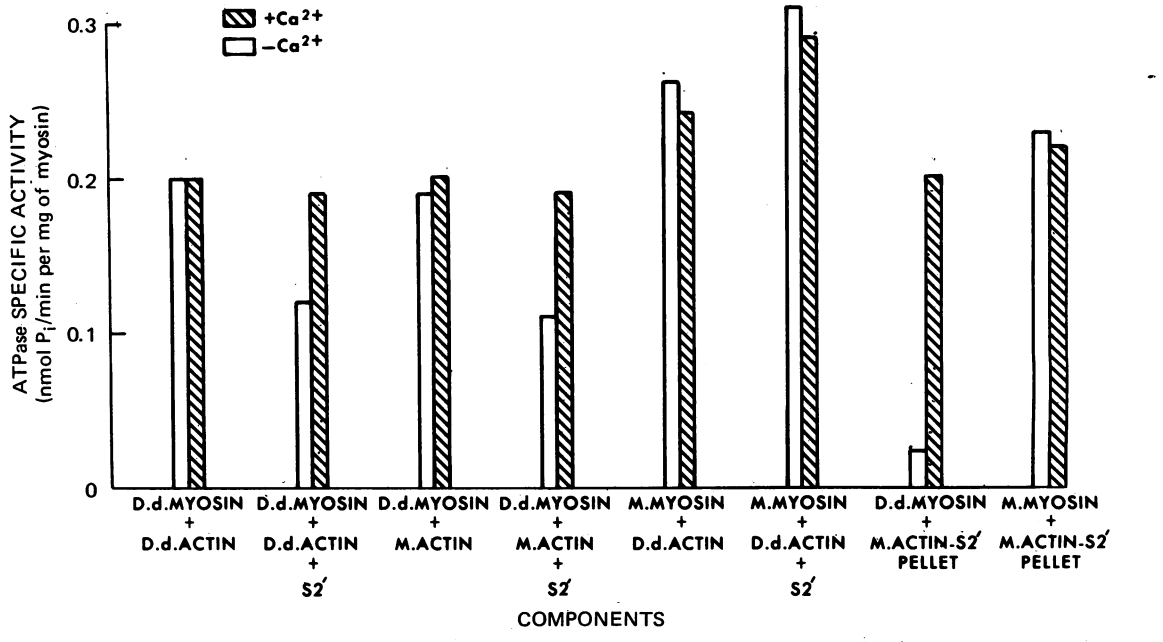

FIG. 3. Effect of S2′ on the actin-activated myosin ATPase of heterologous actomyosin. Details on preparation of the actin-S2′ pellet are in *Materials and Methods*. D.d. = *Dictyostelium discoideum*, M. = muscle. The values are averages of duplicate assays which differed by less than 10%.

protein. First, Ca^{2+}-sensitivity of the actin-activation of *Dictyostelium* myosin ATPase is lost if S2′ is incubated at 100° for 10 min (the percent inhibition was 81% before boiling and 0% after boiling). Second, the factor appears to be nondialyzable since its preparation involves two extensive dialysis steps. Third, addition of ammonium sulfate to 40% saturation precipitates approximately 70% of the Ca^{2+}-sensitizing activity.

Actin-affinity chromatography

The extremely high affinity of actin for DNase I (27) suggested a simple method for making an actin-affinity column. In column buffer, the resin retains the Ca^{2+}-sensitizing activity. The activity is then eluted with 1 M KCl. The specific activity of the affinity column fraction is 70 times that of the crude S2′ (Fig. 4). Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (Fig. 4) shows that the partially purified fraction has two major components with approximate molecular weights of 15,000 and 30,000. There are also components with molecular weights similar to those of muscle tropomyosin and muscle troponin T, but these are present in the preparation in only small amounts. No components with molecular weights similar to muscle troponin I or troponin C have been detected.

DISCUSSION

An unambiguous demonstration of Ca^{2+}-controlled ATPase of actomyosin requires separation of actin, myosin, and the Ca^{2+}-sensitizing factor. Ca^{2+}-sensitivity in a crude actomyosin preparation could be due to hydrolysis of ATP by contaminating enzymes which require Ca^{2+}. Separation of the actomyosin preparation into two fractions, crude actin and crude myosin, allows one to demonstrate that the Ca^{2+}-sensitivity does not reside in either the actin or the myosin fraction alone, but requires their recombination. However, there is no guarantee that the Ca^{2+}-sensitive ATPase activity observed in the recombined preparation is due to actomyosin rather than to some other recombined factors which result in Ca^{2+}-dependent hydrolysis of ATP.

In the experiments reported here, we have used highly purified actin, highly purified myosin, and a Ca^{2+}-sensitizing fraction called S2′. Ca^{2+} does not influence the ATPase activity of S2′ alone, or of S2′ plus actin, or of S2′ plus myosin. The Ca^{2+}-sensitive ATPase activity is achieved only when all three fractions are combined. Since the actin and myosin are both electrophoretically homogeneous, it is highly unlikely that

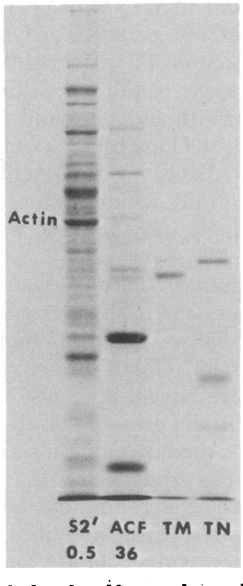

FIG. 4. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of S2′ applied to the actin-affinity column, the affinity column fraction (ACF) eluted with 1 M KCl, muscle tropomyosin, and muscle troponin. The amounts of protein applied to the gel are 19 μg, 6 μg, 1 μg, and 2 μg, respectively. Approximate molecular weights are 43,000 for actin, 35,000 for tropomyosin, 37,000 for troponin, 24,000 for troponin I, and 20,000 for troponin C. The gel in which the proteins were separated contained 10% acrylamide and the preliminary stacking gel contained 3% acrylamide. The numbers beneath the S2′ and ACF columns represent the percent calcium sensitivity per μg of protein in S2′ or ACF, when used in the standard actin-activated myosin ATPase assay.

sensitivity to Ca^{2+} is due to a contaminating ATPase which requires Ca^{2+}.

The Ca^{2+}-sensitizing factor binds to actin attached noncovalently to DNase-agarose. This column serves as an excellent purification step because it is easy to build, gentle, fast, and highly specific and yields a 70-fold purification. The two major proteins which bind to this column do not correspond to any of the components of muscle tropomyosin-troponin, although the column does bind two minor components with molecular weights similar to tropomyosin and troponin T. It seems likely that one or both of the major components is involved in the Ca^{2+}-control since the extent of their enrichment by this purification step is consistent with the very large increase in specific activity in this preparation.

Kato and Tonomura (18) have recently described a tropomyosin–troponin like Ca^{2+}-regulatory system from *Physarum polycephalum* (18). A major difference between their activity and the Ca^{2+}-sensitizing activity which we report here is that the *Physarum* control system will operate on muscle myosin while the *Dictyostelium* control system will not.

The loss of Ca^{2+}-sensitivity when muscle myosin replaces *Dictyostelium* myosin further demonstrates that S2′ specifically influences the actin interaction with myosin and indicates that the nature of the myosin is important to the regulation. This observation fits well with previous experiments using heterologous systems. For both *Acanthamoeba* myosin (30) and *Dictyostelium* myosin (19), the muscle tropomyosin–troponin complex inhibits the actin-activated ATPase, but unlike the situation with muscle myosin, Ca^{2+} does not significantly relieve the inhibition. Thus, relief of inhibition depends on the type of myosin used, even though in this case the Ca^{2+} is known to act on the actin-linked tropomyosin–troponin complex. Hence, the loss of Ca^{2+}-sensitivity when muscle myosin replaces *Dictyostelium* myosin does not permit one to conclude that the sensitizing factor acts by binding to the myosin. *Dictyostelium* myosin and rabbit muscle myosin could interact with actin in slightly different ways. Thus, it is possible that changes induced in the actin–tropomyosin–troponin complex upon binding Ca^{2+} are sufficient to allow binding of muscle myosin to the actin but not binding of *Dictyostelium* myosin.

Since the Ca^{2+}-sensitizing factor sediments in the presence of actin, we know that the factor has some affinity for actin. However, proteins presumably unrelated to contraction, such as aldolase (31), are known to associate with actin. Therefore this affinity does not necessarily indicate that the sensitizing factor acts through the actin.

An unambiguous determination of the site of action of the Ca^{2+}-sensitizing factor would require a sufficiently pure preparation to permit competition assays such as described by Spudich and Watt (23). Our present results demonstrate that *Dictyostelium* amoebae contain a protein fraction which specifically renders the interaction of *Dictyostelium* actin and *Dictyostelium* myosin sensitive to Ca^{2+}, and that the nature of the myosin is important to the regulation.

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