Dictyostelium Myosin: Characterization of Chymotryptic Fragments and Localization of the Heavy-chain Phosphorylation Site

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ABSTRACT Chymotrypsin cleaves *Dictyostelium* myosin in half, splitting the heavy chain (210,000 daltons) into two fragments of 105,000 daltons each. One of the two major fragments is soluble at low ionic strength and has a native molecular weight of ~130,000. As judged by SDS polyacrylamide gel electrophoresis, this soluble fragment consists of the two intact myosin light chains of 18,000 and 16,000 daltons and a 105,000-dalton polypeptide derived from the myosin heavy chain. The soluble fragment retains actin-activated ATPase activity and the ability to bind to actin in an ATP-dissociable fashion. The maximal velocity of the actin-activated ATPase activity of the soluble fragment is 80% of that of uncleaved myosin, although its apparent K_m for actin is 12-fold greater than that of myosin. In addition to the major soluble 105,000-dalton fragment discussed above, chymotryptic cleavage of the *Dictyostelium* myosin also generates fragments that are insoluble at low ionic strength. The major insoluble fragment is 105,000 daltons on an SDS polyacrylamide gel and forms thick filaments that are devoid of myosin heads. A less prevalent insoluble fragment has a molecular weight of 83,000 and is probably a subfragment of the insoluble 105,000-dalton fragment. The heavy chain of myosin is phosphorylated in vivo and the phosphorylation site has been localized to the insoluble fragments, which derive from the tail portion of the myosin molecule.

Myosin isolated from *Dictyostelium discoideum* is composed of heavy chains of 210,000 daltons and two classes of light chains of 18,000 and 16,000 daltons, as judged by SDS polyacrylamide gel electrophoresis (PAGE). *Dictyostelium* myosin has many of the properties of skeletal muscle myosin: assembly into filaments in solutions of low ionic strength, ATP-dissociable binding to actin, and an actin-activated ATPase activity (4).

Proteolytic cleavage of myosin isolated from skeletal and smooth muscle has been useful in determining which regions of the molecule are responsible for specific properties of myosin (9). These studies indicate that skeletal muscle myosin contains a coiled-coil α-helical rod portion that is required for formation of bipolar thick filaments. The rod is connected by a hinge region to a globular head that contains actin and nucleotide binding sites (9).

To determine which regions of the *Dictyostelium* myosin molecule are associated with specific functions, we cleaved *Dictyostelium* myosin proteolytically with chymotrypsin. Two fragments with different solubilities[1] were obtained, similar to the results obtained with skeletal muscle myosin. Recently, it has been demonstrated that the 210,000-dalton heavy chain of *Dictyostelium* myosin is phosphorylated in vivo to ~0.2 mol phosphate/mol heavy chain (7). Using chymotryptic cleavage, we have shown that the heavy chain phosphorylation site is in the rod portion of the myosin molecule.

MATERIALS AND METHODS

Cell Culture

Dictyostelium strain Ax3 was grown at 23°C in HL5 medium (4) in flasks on a rotary shaker platform. Cells were harvested in late log phase. For in vivo labeling studies, cells were grown in [^{32}P]orthophosphate (15–20 μCi/ml) in the defined medium of Franke and Kessin (5) with total phosphate reduced to 0.4 mM.

Proteins Used in This Study

RNA-free myosin was isolated and purified from *Dictyostelium discoideum*

[1] Throughout this paper we refer to the "soluble fragment" as that which does not sediment upon centrifugation at 100,000 *g* in 50 min in buffer F containing 0.05 M KCl. The "insoluble fragments" do sediment under these conditions.

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amoebae as described elsewhere (11). The purified myosin was stored on ice in 0.5 M KCl, 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT) and 1 mM EDTA (storage buffer). Actin was purified from *Dictyostelium discoideum* by the method of Uyemura et al. (17). TLCK(1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride)-treated chymotrypsin was purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of the Chymotryptic Fragments

10 mg of myosin in 2 ml of storage buffer was dialyzed overnight at 4°C against 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT (buffer F) containing 0.05 M KCl, to allow myosin thick filaments to form. Chymotrypsin was dissolved in 0.01 M Tris-HCl, pH 8.0, and its concentration was determined spectrophotometrically, using $A^{1\%}_{1cm} = 20.4$ at 280 nm. Chymotryptic cleavage was carried out at 22°C for 4 min, using a 1:200 (wt/wt) ratio of chymotrypsin to dialyzed myosin. Nonaggregated myosin was cleaved in the same manner, except that the dialysis buffer contained 0.5 M KCl. Cleavage was terminated by addition of phenylmethanesulfonyl fluoride (100 mM in 95% ethanol) to a final concentration of 0.5 mM. The reaction mixture was centrifuged (100,000 *g* for 50 min), and the supernate applied to a 50 ml DE-52 column equilibrated with buffer F. The protein was eluted with a linear KCl gradient (0–0.15 M). The fractions containing the purified soluble fragment were pooled and concentrated by vacuum dialysis against buffer F containing 0.05 M KCl. The yield of purified soluble fragment from 10 mg of myosin was ~0.8 mg.

The method of Szent-Györgyi et al. (15) was used to purify insoluble fragments. The material that sedimented after chymotryptic cleavage was dissolved in 1.0 ml of buffer F containing 0.5 M NaCl. 3 vol of cold ethanol were added and after 60 min at 4°C the mixture was centrifuged at 159,000 *g* for 30 min. The pellet was redissolved in 1 vol of buffer F containing 0.5 M NaCl and dialyzed against 1,000 vol of the same buffer. The dialyzed sample was centrifuged in a Beckman airfuge (Beckman Instrument Co., Spinco Div., Palo Alto, Calif.) for 15 min at 120,000 *g* to remove denatured protein, and the resulting supernate was dialyzed against buffer F containing 0.05 M KCl.

Electron Microscopy

Actin filaments were decorated with the soluble fragment, using the procedures described previously (6, 14). Solutions containing purified myosin or insoluble fragments in buffer F containing 0.05 M KCl were applied to carbon-coated parlodian grids for 30 s followed by negative staining with 1% aqueous uranyl acetate. Grids were examined with a Philips 201 electron microscope.

Molecular Weight Determination of the Native Soluble Fragment

The molecular weight of the purified, soluble fragment in buffer F containing 0.05 M KCl was determined by short-column equilibrium ultracentrifugation in a Beckman model E ultracentrifuge, according to the method of Chervanka (3), where M_w^{app} was determined from the slope of the plot of $\ln(C_r)$ vs. r^2. The partial specific volume ($\bar{V}$) of the fragment was taken as 0.728 ml/g, the value commonly used as an approximation for fragments of skeletal muscle myosin (12). To attain equilibrium, the sample was centrifuged for 20 h at 4,800 rpm at 20°C.

ATPase Assay

ATPase activity was measured using [γ-^{32}P]ATP, as described previously (4). Actin-activated ATPase activity was measured in 0.015 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.0 mM ATP, 25 mM PIPES buffer, pH 7.0, with 0.06 mg/ml myosin or soluble fragment and 0–1.4 mg/ml *Dictyostelium* actin. The actin-activated ATPase activity was calculated by subtracting the activity found for myosin or soluble fragment alone.

PAGE and Radioautography

SDS PAGE was carried out in the discontinuous buffer system of Laemmli (8). Slab gels were scanned with a Transidyne RFT scanning densitometer (Transidyne General Corp., Ann Arbor, Mich.) at 550 nm. The relative amount of protein present in a single band was quantitated by cutting out and weighing the corresponding peak. The molecular weight of each of the protein bands seen on the gels was determined by comparison of the relative mobility of the protein with that of the following standards run on the same gel: phosphorylase *b* (94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), and soybean trypsin inhibitor (20,000 daltons).

Gels were dried on a Hoefer slab gel drying apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) before being radioautographed using Kodak X-Omat x-ray film with a Dupont Cronex intensifying screen.

Other Procedures

Protein concentrations were determined by the procedure of Bradford (2).

RESULTS

Preparation of Chymotryptic Fragments of Myosin

The susceptibility of *Dictyostelium* myosin to chymotryptic cleavage depended on the state of myosin assembly. Chymotryptic cleavage of myosin in the form of individual molecules (in buffer F containing 0.5 M KCl) produced major fragments with molecular weights of approximately 185,000, 105,000, and 83,000. However, chymotryptic cleavage of myosin in the form of thick filaments (in buffer F containing 0.05 M KCl) resulted in a major band on SDS gels of 105,000 daltons (Fig. 1). Analysis of the time-course of chymotryptic proteolysis of filamentous myosin indicated that cleavage occurred preferentially at a site producing 105,000-dalton fragments and that these fragments were relatively resistant to further degradation (Fig. 1). After digestion in 0.05 M KCl, intact myosin and the insoluble fragments were separated from the soluble fragment by centrifugation. The soluble fragment was further purified by DEAE chromatography (Fig. 2).

The chymotryptic fragments that sedimented at low ionic strength after cleavage of the myosin were purified by precipitation with ethanol. The precipitated protein was redissolved in buffer F containing 0.5 M NaCl, and the denatured protein was removed by centrifugation. The purified sedimentable material contained 105,000- and 83,000-dalton fragments, as judged by SDS PAGE (Fig. 2).

Characterization of the Soluble Fragment of Myosin

The 105,000-dalton heavy chain of the soluble fragment is noncovalently associated with 18,000- and 16,000-dalton light chains that comigrate with the light chains of *Dictyostelium* myosin on SDS polyacrylamide gels (Fig. 3). In the 4-min

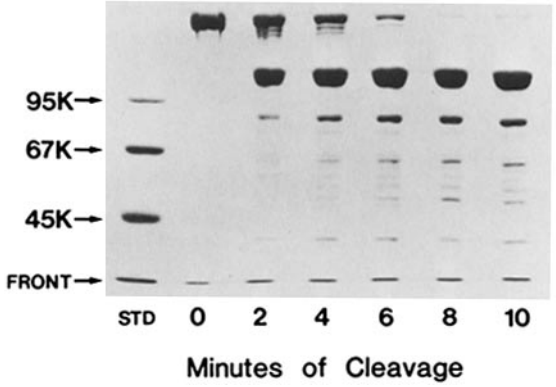

FIGURE 1 SDS gel (10%) showing the time-course of the chymotryptic cleavage of filamentous myosin. A 6.0 mg/ml solution of myosin in storage buffer was dialyzed overnight against buffer F containing 0.05 M KCl to allow filaments to form. Filamentous myosin was incubated with chymotrypsin as described in Materials and Methods for the times indicated. Each lane contains 10 μg of protein.

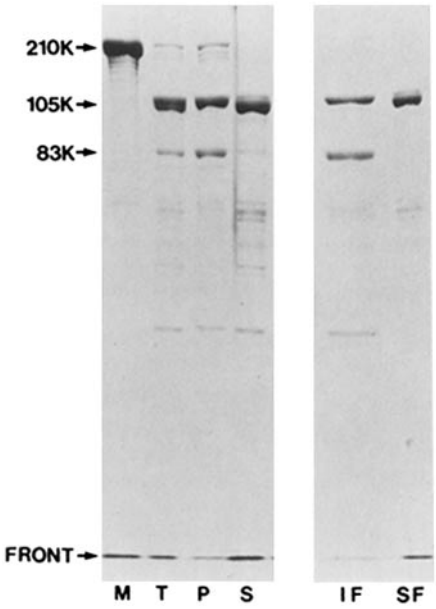

FIGURE 2 SDS gel (10%) showing steps in the purification of chymotryptic fragments of myosin. (*M*) Myosin after overnight dialysis against buffer F containing 0.05 M KCl. (*T*) Total cleavage products after incubation of the dialyzed myosin with chymotrypsin, at a 200:1 (wt/wt) ratio of myosin to chymotrypsin for 4 min, as described in Materials and Methods. The doublet appearing at 105,000 daltons consists of two proteins of similar molecular weight that separate upon centrifugation at low ionic strength. (*P*) Pellet obtained after centrifugation of the total cleavage products. The sedimentable material includes uncleaved myosin and 105,000- and 83,000-dalton insoluble fragments. (*S*) Supernate remaining after centrifugation of the total cleavage products. The major band is the 105,000-dalton heavy chain of the soluble myosin fragment. Each of these first four lanes contains 5 μg of protein. (*IF*) Insoluble fragments (3 μg) purified by ethanol precipitation. (*SF*) Soluble fragment (3 μg) purified by DEAE chromatography.

sample, the molar ratio of the 105,000-dalton heavy chain: 18,000-dalton light chain:16,000-dalton light chain of the soluble fragment was determined by quantitative densitometry to be 0.9:1.0:1.0. In the 8-min sample, there was a decrease in the amount of 18,000-dalton light chain with a concomitant increase in a peptide of ~17,000 daltons (Fig. 3, bottom). Therefore, the soluble fragment was prepared by digestion of filamentous myosin for 4–6 min to avoid degradation of the 18,000-dalton light chain. The molecular weight of the purified native soluble fragment was determined by ultracentrifugation to be ~130,000 daltons. This indicates that the soluble fragment is a monomer in its native state, similar to muscle myosin subfragment 1 (S1).

The soluble fragment "decorates" *Dictyostelium* actin filaments to form the "arrowhead" pattern characteristic of muscle actin decorated with muscle myosin S1 (data not shown). Binding to actin was also demonstrated by cosedimentation of the soluble fragment with actin filaments in the absence of ATP. Actin (0.4 mg/ml) was mixed with soluble fragment (0.2 mg/ml) in buffer F containing 0.05 M KCl, incubated at 22°C for 2 h, and centrifuged in a Beckman airfuge at 120,000 *g* for 15 min. Greater than 90% of the soluble fragment sedimented. The material that pelleted was redissolved in buffer F containing 0.05 M KCl and 10 mM ATP and centrifuged again. Less than 10% of the soluble fragment sedimented. Thus, addition of ATP caused dissociation of the complex. As a control, a solution containing the soluble fragment without actin was centrifuged in buffer F containing 0.05 M KCl without ATP; the supernate of this control contained >90% of the soluble fragment.

Another important property retained by the soluble fragment is the actin-activated ATPase activity (Fig. 4). The maximal velocity of the actin-activated ATPase activity of the purified

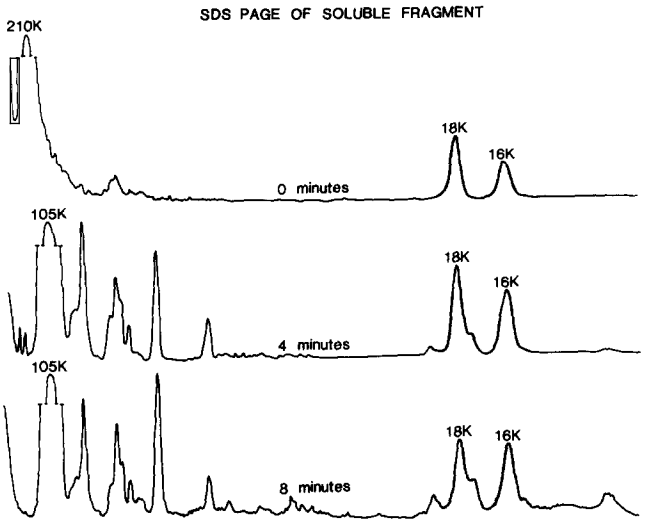

FIGURE 3 Scans of SDS gels (13%) comparing the light chains of myosin with those of the soluble chymotryptic fragment. Myosin was incubated with chymotrypsin as described in Materials and Methods for the times indicated. The 4- and 8-min reaction mixtures were centrifuged at 100,000 *g* for 15 min, and gel samples were prepared from the supernates. The lanes were scanned with a densitometer at 550 nm. Each lane contains 10 μg of protein.

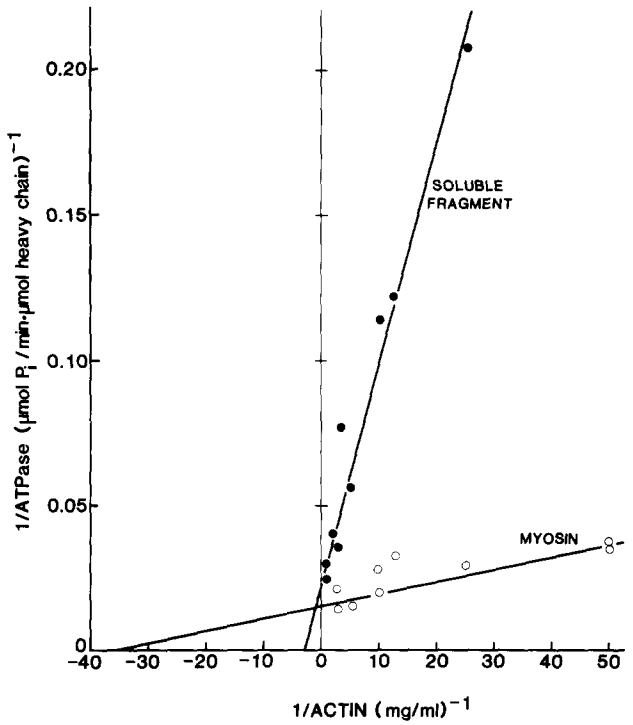

FIGURE 4 Lineweaver-Burke plot of the actin-activated ATPase activity of myosin (○) and of the soluble fragment (●). The actin-activated ATPase activity was measured, using actin concentrations ranging from 0.02 to 1.4 mg/ml. The data were normalized with respect to micromoles of heavy chain of myosin and of soluble fragment used in the assay.

soluble fragment was 46 μmol P_i/min/μmol fragment, which is 80% of that of a single globular head of myosin. The apparent K_m for actin was 8.2×10^{-6} M, which is 12-fold greater than that of myosin.

Properties of the Insoluble Fragments

The chymotryptic fragments purified from the sedimentable cleavage products (Fig. 2) form filamentous structures at low ionic strength. The filaments formed are similar in size to those of myosin, but they do not have the globular projections that extend from the shaft of the myosin filament (Fig. 5). The thick filaments seem to be composed of thinner filaments of 25–30 Å diameter, which corresponds well with that of the α-helical coiled-coil regions of muscle myosin (6).

The Insoluble Fragments Contain the Heavy-chain Phosphorylation Site

The 210,000- and 18,000-dalton subunits of *Dictyostelium* myosin are phosphorylated in vivo (7). For determination of which chymotryptic fragment of the heavy chain contains the in vivo phosphorylation site, myosin purified from [^{32}P]-labeled cells ([^{32}P]myosin) was digested with chymotrypsin. The soluble and insoluble fragments were separated by centrifugation and analyzed by SDS PAGE followed by radioautography (Fig. 6). The insoluble 105,000-dalton fragment had the ^{32}P label, but the soluble 105,000-dalton fragment did not. Thus, the in vivo heavy chain phosphorylation site is on the tail portion of the myosin molecule.

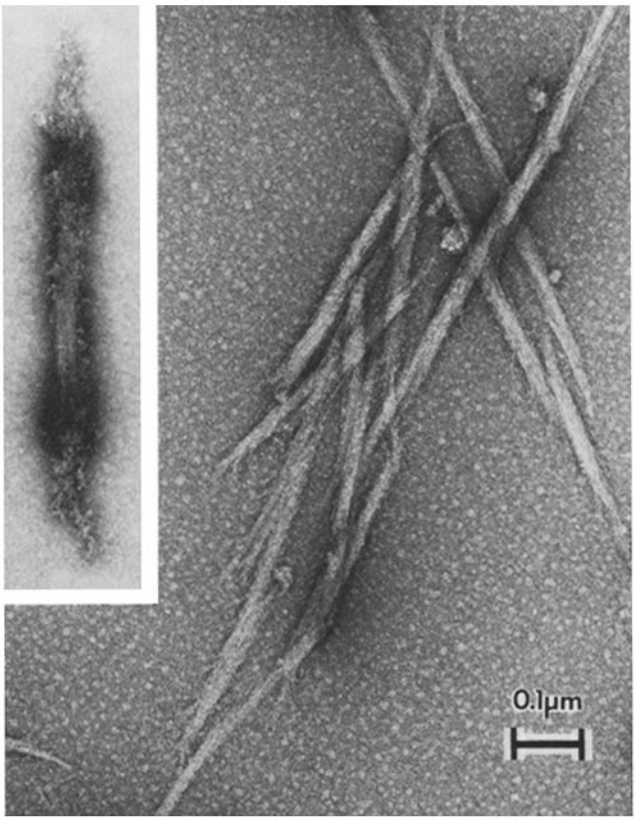

FIGURE 5 Electron micrographs of negatively stained filaments formed by myosin (*inset*) and the purified insoluble fragments. Note that the myosin heads that extend away from the core of the myosin thick filament are not seen in the thick filaments formed by the insoluble fragments.

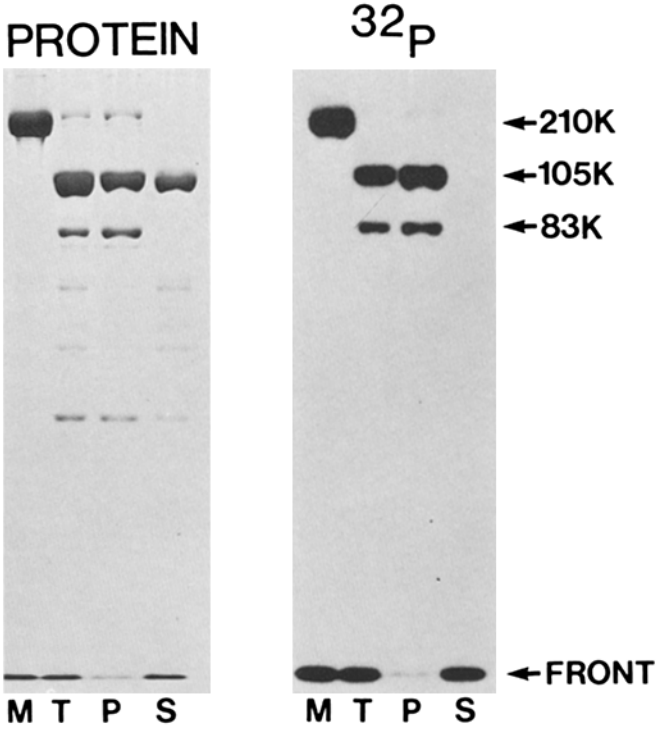

FIGURE 6 SDS gel (10%) and radioautogram localizing the in vivo site of myosin heavy-chain phosphorylation to the insoluble fragments. [^{32}P]myosin was cleaved with chymotrypsin as described in Materials and Methods. Each lane contains ~4 μg of protein. (*M*) [^{32}P]myosin after dialysis against buffer F containing 0.05 M KCl. (*T*) Total cleavage products after incubation of [^{32}P]myosin with chymotrypsin. (*P*) Pellet obtained after centrifugation of the total cleavage products. (*S*) Supernate remaining after centrifugation of the total cleavage products. The ^{32}P-labeled 18,000-dalton light-chain migrates at the front on a 10% gel. Note that it is associated with the soluble fragment (*S*) and not with the insoluble fragments (*P*).

DISCUSSION

When *Dictyostelium* myosin is filamentous, chymotryptic cleavage is specific for a site near the middle of the heavy chain, suggesting a region of decreased structural order with increased susceptibility to proteolysis. When the myosin is not aggregated, at least one additional site is susceptible to chymotryptic cleavage. In the case of skeletal muscle myosin, cleavage of one of the light chains is a prerequisite for heavy chain chymotryptic digestion at the S1 site (18). The 18,000- and 16,000-dalton light chains of *Dictyostelium* myosin, however, are not altered by limited chymotryptic cleavage of the heavy chain.

The insoluble fragments of *Dictyostelium* myosin retain one of the properties of myosin, the ability to form thick filaments in solutions of low ionic strength. The soluble fragment, which contains the 18,000- and 16,000-dalton light chains, retains the ATP-dissociable binding to actin and the actin-activated ATPase activity of myosin. The maximal velocity of the actin-activated ATPase activity of the soluble fragment is similar to that of intact myosin, indicating that the maximal rate of substrate turnover at the active site is unaltered by the cleavage. The 12-fold increase in the apparent K_m for actin of the soluble fragment over that of native myosin indicates that myosin has a greater affinity for actin.

The soluble fragment prepared from *Dictyostelium* myosin has important advantages over the fragments prepared from muscle myosin. Under appropriate conditions, chymotrypsin is

highly specific for a cleavage site near the middle of the heavy chain and the associated light chains are not degraded, which allows for the preparation of an intact, reasonably homogeneous fragment. It may be possible to crystallize this soluble fragment and obtain detailed information about the actin and nucleotide binding sites of myosin.

Previous studies on the effects of phosphorylation of myosin isolated from various sources have been concerned with changes in the ATPase activity induced by phosphorylation. Phosphorylation of the 18,000- to 20,000-dalton light chains of macrophage (16), platelet (1), and rat myoblast (13) myosins and of the heavy chain of *Acanthamoeba* myosin I (10) affects the actin-activated Mg-ATPase of those myosins. The insoluble fragment of *Dictyostelium* myosin does not contain the actin or nucleotide binding sites but rather is the region of the molecule required for self-assembly. Localization of the site of heavy chain phosphorylation to this region of the molecule, therefore, suggests that some property of myosin other than its ATPase activity may be modulated by phosphorylation. Indeed, one effect of heavy chain phosphorylation is to inhibit myosin thick filament formation (7).

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