**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 $\alpha$-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 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 15-20 $\mu$Ci/ml orthophosphate 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* 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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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 MgCl₂, 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.

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 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 sample 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 \mu mol P_i/min/\mu mol fragment, which is 80\% of that of a single globular head of myosin. The apparent $K_m$ for actin was $8.2 \times 10^{-6}$ 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 $\alpha$-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.

**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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