Myosin Light Chain Kinase and Myosin Light Chain Phosphatase from *Dictyostelium*: Effects of Reversible Phosphorylation on Myosin Structure and Function

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Abstract. We have partially purified myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) from *Dictyostelium discoideum*. MLCK was purified 4,700-fold with a yield of ~1 mg from 350 g of cells. The enzyme is very acidic as suggested by its tight binding to DEAE. *Dictyostelium* MLCK has an apparent native molecular mass on HPLC G3000SW of ~30,000 D. Mg^{2+} is required for enzyme activity. Ca^{2+} inhibits activity and this inhibition is not relieved by calmodulin. cAMP or cGMP have no effect on enzyme activity. *Dictyostelium* MLCK is very specific for the 18,000-D light chain of *Dictyostelium* myosin and does not phosphorylate the light chain of several other myosins tested. Myosin purified from log-phase amebas of *Dictyostelium* has ~0.3 mol P_i/mol 18,000-D light chain as assayed by glycerol-urea gel electrophoresis. *Dictyostelium* MLCK can phosphorylate this myosin to a stoichiometry approaching 1 mol P_i/mol 18,000-D light chain. MLCP, which was partially purified, selectively removes phosphate from the 18,000-D light chain but not from the heavy chain of *Dictyostelium* myosin. Phosphatase-treated *Dictyostelium* myosin has $\leq$0.01 mol P_i/mol 18,000-D light chain. Phosphatase-treated myosin could be rephosphorylated to $\geq$0.96 mol P_i/mol 18,000-D light chain by incubation with MLCK and ATP. We found myosin thick filament assembly to be independent of the extent of 18,000-D light-chain phosphorylation when measured as a function of ionic strength. However, actin-activated Mg^{2+}-ATPase activity of *Dictyostelium* myosin was found to be directly related to the extent of phosphorylation of the 18,000-D light chain. MLCK-treated myosin moved in an in vitro motility assay (Sheetz, M. P., and J. A. Spudich, 1983, *Nature (Lond.)*, 305:31–35) at ~1.4 μm/s whereas phosphatase-treated myosin moved only slowly or not at all. The effects of phosphatase treatment on the movement were fully reversed by subsequent treatment with MLCK.

In nonmuscle cells modulation of the degree and location in the cell of actin and myosin filament assembly as well as actin–myosin interaction may be critical for events such as cytokinesis, endocytosis, and chemotaxis (77). This is in contrast to the situation in muscle cells where more stable arrays of actin and myosin filaments occur. One attractive system for the investigation of the molecular basis of filament assembly and actin–myosin interaction in nonmuscle cells is the slime mold *Dictyostelium discoideum*. It is possible to grow biochemical quantities of these cells, and there is an exciting potential for correlating changes in the biochemical properties of actin and myosin with well-documented motile and nonmotile stages in the life cycle of the organism (7), as well as with chemotaxis (71) and shape changes (68) of the amebas in response to chemoattractants such as cAMP. For example, Malchow et al. (35) and Berlot et al. (6) have shown changes in myosin phosphorylation as a result of cAMP stimulation. Whereas Malchow et al. (35) suggested that at the onset of the chemotactic response to cAMP, myosin heavy chains exist in a phosphorylated state and are then rapidly dephosphorylated, the direct in vivo phosphorylation experiments of Berlot et al. (6) showed that stimulation of amebas with cAMP results in rapid phosphorylation of the myosin heavy chain as well as the myosin light chain.

For *Dictyostelium* as well as for other nonmuscle cells and for vertebrate smooth muscle, the interaction of contractile proteins is regulated at least in part by a myosin-linked regulatory system that involves phosphorylation of the myosin molecule. Actin-linked regulatory systems may also be present in smooth muscle (36, 41, 65) and in *Dictyostelium* (42), but these are as yet poorly characterized. Myosin-linked regulation of actin–myosin interaction is reviewed by Adelstein and Eisenberg (2) and by Kendrick-Jones and Scholey (27). Regulation of enzymes by phosphorylation is

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reviewed by Cohen (11) and by Krebs and Beavo (32). In the present report we compare *Dictyostelium* myosin with the following myosins in particular. (*a*) Vertebrate smooth muscle myosin is composed of two heavy chains of 200,000 D and two each of two light chains of 20,000 and 15,000 D. Phosphorylation of the 20,000-D light chain enhances the actin-activated Mg^{2+} ATPase activity of the molecule (9, 63, 64). (*b*) *Acanthamoeba* myosin I is a single-headed myosin consisting of a heavy chain of ~150,000 D and possibly one each of two different light chains (38, 53, 54). Heavy-chain phosphorylation is required for actin-activated Mg^{2+} ATPase activity (40). (*c*) *Acanthamoeba* myosin II consists of two heavy chains of 185,000 D and two pairs of light chains of 17,500 and 17,000 D (39, 55). Phosphorylation of the heavy chains of *Acanthamoeba* myosin II inhibits actin-activated Mg^{2+} ATPase activity (12).

Investigations in our laboratory have emphasized regulation of the properties of purified *Dictyostelium* myosin by phosphorylation. *Dictyostelium* myosin is composed of two heavy chains of 210,000 D and two each of two light chains of 18,000 and 16,000 D (10). Myosin purified from amebas grown in [^{32}P]phosphate has ~0.3 mol P_i/mol 210,000-D heavy chain and ~0.1 mol P_i/mol 18,000-D light chain (33). There are at least two heavy-chain phosphorylation sites, one a serine residue and the other a threonine residue (Berlot, Devreotes, and Spudich, manuscript submitted for publication). Both sites are in the carboxy-terminal half of the myosin tail (46, 48). With a partially purified *Dictyostelium* heavy chain kinase and bacterial alkaline phosphatase, it was possible to manipulate the extent of the heavy-chain phosphorylation and demonstrate that phosphorylation inhibits thick filament assembly and actin-activated ATPase activity (33). Maruta et al. (37) have also observed that heavy-chain phosphorylation inhibits the actin-activated Mg^{2+} ATPase activity of *Dictyostelium* myosin.

Here we focus on light-chain phosphorylation of *Dictyostelium* myosin. We report the purification from log-phase amebas of a specific *Dictyostelium* myosin light chain kinase (MLCK)[1] and a specific myosin light chain phosphatase (MLCP). Properties of *Dictyostelium* myosin examined as a function of light-chain phosphorylation include actin-activated Mg^{2+} ATPase activity, filament assembly, and myosin movement in an in vitro assay. An essential feature of our experiments is reversibility of phosphorylation. We have been able to dephosphorylate and then completely rephosphorylate the 18,000-D light chain of intact *Dictyostelium* myosin. With reconstitution of phosphorylation we have observed concomitant reconstitution of properties identical to those of myosin previously treated with kinase alone. This argues that the effect that we see with phosphatase treatment is due to removal of phosphate only, and not an artifact resulting from, for example, protease contamination of the phosphatase preparation.

Materials and Methods

Materials

Reagent-grade chemicals were obtained from the following sources: Amersham Corp., Arlington Heights, IL ([γ-^{32}P]ATP catalog no. PB10168 at 10 mCi/ml in H_2O); J. T. Baker, Phillipsburg, NJ (KI); Bio-Rad Laboratories, Richmond, CA (Bio-Gel A-0.5m agarose beads, Bio-Gel HT hydroxylapatite [HAP], Affi-Gel Blue 100–200 mesh and 75–150 μm, Bio-Gel A-15m agarose beads 200–400 mesh); Calbiochem-Behring Corp., La Jolla, CA (ATP, Aquacide III); Mallinckrodt, Inc., Los Angeles, CA (sodium pyrophosphate); Schwarz/Mann, Inc., Orangeburg, NY (ultrapure ammonium sulfate, ultrapure urea, sucrose); Sigma Chemical Co., St. Louis, MO (dithiothreitol [DTT], diisopropylfluorophosphate [DIFP], *N*-α-*p*-tosyl-L-lysine chloromethyl ketone [TLCK], L-1-tosylamide-2-phenylethyl chloromethyl ketone [TPCK], phenylmethylsulfonyl fluoride [PMSF] NaN_3, triethanolamine [Teola], Pipes, Tris, *N*-Tris[hydroxymethyl]-2-aminoethyl sulfonic acid [TES], BSA, *p*-nitro-phenyl phosphate); Whatman Chemical Separation Inc., Clifton, NJ (DEAE cellulose: DE-52); Varian Associates, Palo Alto, CA. (Toyo Soda column G3000SW, 60 cm in length).

Methods

Growth of Cells. Stock cultures of *Dictyostelium discoideum*, strain Ax-3, were maintained as described (66). When ~100 g of wet cells were desired, as for a myosin preparation or a MLCP preparation, cells were grown in 6-liter flasks on a rotary shaker platform. Each flask contained 2 liters of HL-5 medium (prepared as described [66]). Flasks were inoculated to an OD_{660} of 0.04 and harvested in late log-phase growth at an OD_{660} of 0.80. About 12 g of cells were obtained from each flask.

When >100 g of *Dictyostelium* amebas was desired, as for an MLCK preparation, cells were grown in HL-5 medium in 5-gal carboys similar to those used in the laboratory of Edward D. Korn, National Institutes of Health, Bethesda, MD (31, 73), to grow *Acanthamoeba*. To achieve a *Dictyostelium* doubling time in carboys of 9–10 h, which is equivalent to that obtained in shaker flasks, we modified the Weihing and Korn procedure (73) as follows. First, we used a high air-flow rate of 80 ml/s maintained with a line regulator (no. 3478, Matheson Gas Products, Inc., Secaucus, NJ) with a range of 1–200 psi. Therefore all rubber tubing to glass tubing connections were wired together tightly. We did not use an air filter on the air outflow line inasmuch as this impeded air flow. Secondly, we used a minimal amount of antifoam and allowed the culture to foam somewhat. Excess foam exited through the air outflow line and was collected in a waste container. The HL-5 medium for *Dictyostelium* contains glucose, which was autoclaved separately for 15 min only and then added to the carboy at the time of inoculation. Carboys containing 13 liters of HL-5 medium were inoculated to an OD_{660} of 0.06–0.10 and harvested 35–40 h later at an OD_{660} of 0.80. About 80 g of cells was obtained from each carboy. Of the two dozen cultures grown in carboys, none was contaminated with bacteria or yeast.

Preparation of Dictyostelium Myosin. Myosin was purified from amebas of *Dictyostelium* as described (10, 42), with modifications. 100 g of washed packed cells was resuspended in 2 vol/g (~200 ml) of 10 mM Tris, pH 7.5, 2 mM EDTA, 1 mM DTT, and 40 mM sodium pyrophosphate. The resuspension was then combined with an equal volume (about 300 ml) of 10 mM Tris, pH 7.5, 2 mM EDTA, 1 mM DTT, 40 mM sodium pyrophosphate, 60% sucrose, 1 mM TLCK, 1 mM TPCK, and 1 mM PMSF. Cells were then lysed by sonication at 0°C with constant stirring. A sonicator-cell disruptor (Heat Systems-Ultrasonics, Inc. Farmingdale, NY) operated at speed 7 and equipped with a medium-sized tip was used. 100-ml batches of cells were sonicated for a total of 40 s with 10-s intervals of sonication followed by 10-s intervals of no sonication. After sonication, KCl was added from a 3 M stock to a concentration of 0.1 M. The cell lysate was clarified by centrifugation at 27,000 *g* for 30 min, followed by ultracentrifugation at 100,000 *g* for 2 h. Actomyosin was precipitated by dialysis of the supernatant against 10 mM Pipes, pH 6.8, 0.5 mM DTT, 50 mM KCl, 1 mM EDTA, 0.5 mM PMSF, and 0.02% azide. ~400–500 mg of precipitate was collected by centrifugation at 27,000 *g* for 30 min.

For gel filtration chromatography, the actomyosin precipitate was solubilized in KI and ATP as follows. Pellets were resuspended to ~30 ml with 10 mM Teola, pH 7.5, 50 mM KCl. Next an equal volume of 10 mM Teola, pH 7.5, 1 mM EDTA, 1 mM DTT, 10 mM ATP, 10 mM $MgCl_2$, and 1.2 M KI was added and the sample was homogenized and then clarified at 100,000 *g* for 30 min. The actomyosin was concentrated by ammonium sulfate precipitation. The supernatant was brought to 50 mM in Teola, pH 7.5. Solid ammonium sulfate was added to 55% saturation. The precipitate was collected by centrifugation at 27,000 *g* for 30 min. Pellets were brought to 11 ml with 10 mM Teola, pH 7.5, and 50 mM KCl. Then 11 ml of 10 mM Teola pH 7.5, 1 mM EDTA, 1 mM DTT, 10 mM ATP, 10 mM $MgCl_2$, and 1.2 M KI were added, and the sample was homogenized and then clarified at 100,000 *g* for 60 min. The resulting 9.5-ml column sample, containing ~150 mg of protein, was applied to a 2.5 × 90-cm agarose A15m, 200–400

1. *Abbreviations used in this paper*: HAP, hydroxylapatite; HSS, high-speed supernatant; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; Teola, triethanolamine; TES, *N*-Tris (hydroxymethyl) methyl-2-aminoethyl sulfonic acid.

mesh column equilibrated with 10 mM TES, pH 7.5, 20 mM sodium pyrophosphate, 5% sucrose, 1 mM DTT, 50 mM KCl, and 0.02% azide and run at 60 cm of pressure. The column had been preloaded with 50–60 ml of 10 mM Teola, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.6 M KI, 5 mM ATP, and 5 mM $MgCl_2$. The myosin peak was located by assaying for ATPase activity. The fractions on the trailing edge of the peak were avoided in later pooling because they usually contained some degraded myosin.

The myosin peak containing ~10 mg of protein in 50 ml was separated from contaminating RNA on a column (9 × 2.8 cm) of DEAE-cellulose equilibrated with 100 ml of 10 mM TES, pH 7.5, 20 mM sodium pyrophosphate, 5% sucrose, 1 mM DTT, and 0.02% sodium azide, and run at 30 cm of pressure. When larger volumes of buffer were used in equilibration, RNA did not bind well to the column, presumably because of bound pyrophosphate. Myosin was eluted from the column with 110 ml of 0.15 M KCl in column buffer.

The purified myosin was concentrated by polymerization as follows. The DEAE pool containing ~7 mg of protein in 22 ml was dialyzed against 10 mM Pipes, pH 6.8, 0.5 mM DTT, 50 mM KCl, 1.0 mM EDTA, 0.5 mM PMSF, and 0.02% sodium azide. $MgCl_2$ was added to 10 mM and the solution was kept at 0°C for 60 min. Filaments were collected by centrifugation at 100,000 *g* for 60 min. The myosin pellets were homogenized gently in 1–1.5 ml of 0.5 M KCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide, and clarified by centrifugation at 27,000 *g* for 15 min. The clarified solution, which contained about 7–8 mg of myosin, was stored at 0°C. Aliquots were combined with an equal volume of glycerol and frozen at −4°C.

Myosin isolated by this method generally had 0.3 mol P_i/mol 18,000-D light chain, as assayed by glycerol-urea gel electrophoresis (see below). One preparation had 0.7 mol P_i/mol 18,000-D light chain.

Preparation of Dictyostelium Myosin Labeled In Vivo with ^{32}P. Myosin was purified from amebas grown in [^{32}P]phosphate according to Kuczmarski and Spudich (33).

Preparation of Rabbit Skeletal Muscle Myosin. Myosin was prepared from rabbit skeletal muscle by the method of Kielley and Harrington (30), stored as an ammonium sulfate pellet, and dialyzed into appropriate buffer before use.

Assay for MLCK Activity. MLCK activity was assayed by incubating purified myosin and MLCK in 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 0.75 mM [γ-^{32}P]ATP, which had 500 cpm/pmol ATP. Samples were mixed at 22°C in the following order: (*a*) stock 5× or 10× Tris-Mg^{2+} solution, (*b*) H_2O, (*c*) MLCK sample, (*d*) myosin, (*e*) 5× [γ-^{32}P]ATP stock. Total assay volume was 20 μl. Generally 8–14 μg of myosin was used per assay. The extent of 18,000-dalton light chain labeling under these conditions was directly related to MLCK concentration and the time of incubation up to ~20 min. Samples were incubated for 10 min at 22°C and then the reaction was stopped by addition of 20 μl of SDS polyacrylamide gel sample buffer and incubated at 100°C for 4 min. For a fast qualitative measure of phosphorylation, 6 μl of each of the samples was run on a microslab gel, stained, destained, and dried on the same day. An autoradiogram was exposed overnight. Such autoradiograms can detect unambiguously as little as 0.025 pmol ^{32}P/pmol 18,000-D light chain. When such a band is cut out of the gel and counted in a scintillation counter, it is only 30 cpm above a background of 30 cpm. For a quantitative measure of phosphorylation, the entire volume of each of the samples was run on a large slab gel. The 18,000-dalton light chain was cut from the gel that had been stained, destained, and dried, digested at 90°C in 30% peroxide for 10 h, and counted in a scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Alternatively, samples were cut from the gel and counted directly. Such samples had 70% of the counts of those digested in peroxide.

[γ-^{32}P]ATP was purchased at 1 mCi/ml in 50% ethanol or at 10 mCi/ml in H_2O and used within 1 wk. We discovered that, for the material purchased at 1 mCi/ml in 50% ethanol, no labeling of *Dictyostelium* myosin light chain occurred when the stock was over 2 wk old. The reason for this result remains unknown, but a thin-layer chromatograph of the 2-wk-old [γ-^{32}P]ATP showed that it was not contaminated with labeled ADP, AMP, or adenosine. The kinase activity found in the 40–60% $(NH_4)_2SO_4$ cut of *Dictyostelium* high-speed supernatant (HSS) (see the 0–60% cut in Fig. 1) was not affected by the age of the [γ-^{32}P]ATP stock.

For assay of 18,000-dalton light-chain phosphorylation by glycerol-urea gel electrophoresis, incubations were carried out using 1 mM ATP without [γ-^{32}P]ATP label, and were stopped by freezing in a dry ice-acetone bath and then stored at 4°C before further processing.

Assay for MLCP Activity. The substrate, *Dictyostelium* myosin labeled with ^{32}P on the 18,000-D light chain ([^{32}P]myosin), was prepared by incubating myosin with *Dictyostelium* MLCK and [γ-^{32}P]ATP, as described below.

Semiquantitative assay of myosin phosphatase activity was performed as follows. The assay was initiated by addition of [^{32}P]myosin to phosphatase in 25 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 1 mM $CaCl_2$. Generally ~5 μg of [^{32}P]myosin was used per assay (total volume, 20 μl). Samples were incubated for 15 min at 22°C and then stopped by the addition of 20 μl of SDS PAGE sample buffer and incubation at 100°C for 4 min. 6 μl of each of the samples was run on a microslab gel, stained, destained, and dried on the same day. An autoradiogram was exposed overnight or for a shorter time period as appropriate. Autoradiograms were scanned with a scanning densitometer (model RFT; Transidyne General Corp., Ann Arbor, MI), peak heights were measured, and relative percent MLCP activity was calculated.

Quantitative assay of myosin phosphatase activity was performed as described above with the following modifications. ~10 μg [^{32}P]myosin were used per assay. The assay incubation was stopped by the addition of 30 μl of ice-cold 25% TCA and 2 mM potassium phosphate, incubated at 0°C for 10 min, and centrifuged for 2 min in an Eppendorf microfuge (Brinkmann Instrument Co., Westbury, NY). 40 μl of the supernatant, which contained liberated [^{32}P]orthophosphate, was counted in a Beckman scintillation counter.

In practice, the semiquantitative assay was generally used for column fractions because it requires less [^{32}P]myosin substrate per assay. Its disadvantage is that it takes longer to process as compared with the quantitative assay. The quantitative method was used to assay each step of the preparation to measure the degree of purification. By conserving [^{32}P]myosin in this way, one preparation of ~2.5 mg could be used for a complete myosin phosphatase purification.

Assay for Alkaline Phosphatase Activity. Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as substrate. Assay conditions were 50 mM Tris, pH 8, and 10 mM $MgCl_2$ at 22°C for 30 min. Components were combined in the following order: (*a*) 10× Tris-$MgCl_2$ mixture, (*b*) H_2O, (*c*) phosphatase sample, and (*d*) *p*-nitrophenyl phosphate. 200 μg of *p*-nitrophenyl phosphate was used in an assay volume of 100 μl. The reaction was stopped by adding 1 ml of 20 mM NaOH and the OD_{410} was read.

Treatment of Dictyostelium Myosin with Kinase. 0.5–2.0 mg of *Dictyostelium* myosin was dialyzed against 20 mM Tris, pH 7.5, 5% sucrose, 50 mM KCl, 1 mM DTT, and 0.02% azide. The myosin was incubated for 1 h at 22°C with an appropriate amount of *Dictyostelium* MLCK (~0.1 mg of HAP peak pool/mg of myosin) under the conditions described above for the MLCK assay. The reaction was stopped by adding KCl to 0.5 M. The myosin was separated from kinase and [γ-^{32}P]ATP by gel filtration on a 0.7 × 25-cm (10 ml) column of A-0.5m, 200–400 mesh, equilibrated with 20 mM potassium phosphate, pH 7.5, 2 mM sodium pyrophosphate, 0.4 M KCl, 1 mM EDTA, 1 mM DTT, 0.02% sodium azide, and 5 mM ATP, and run at 20 cm of pressure. The myosin peak was located by OD_{280} and then concentrated by polymerization and resuspended in an appropriate volume of storage buffer as described above for the purification of *Dictyostelium* myosin. The amount of myosin degradation, if any, was assessed by SDS gel electrophoresis. The extent of 18,000-D light-chain phosphorylation was measured with glycerol-urea gel electrophoresis.

Treatment of Dictyostelium Myosin with Phosphatase. 0.5–2.0 mg of *Dictyostelium* myosin was dialyzed against 20 mM Tris, pH 7.5, 5% sucrose, 50 mM KCl, 1 mM DTT, 0.02% azide. The myosin was incubated with *Dictyostelium* myosin phosphatase (~0.1 mg of phosphatase preparation/mg myosin) in 25 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 1 mM $CaCl_2$ for 2 h at 22°C. The reaction was stopped by adding KCl to 0.5 M. The myosin was separated from phosphatase by chromatography on a 0.7 × 25-cm column (10 ml) of A-0.5m, 200–400 mesh, equilibrated with 10 mM Tris, pH 7.5, 0.5 M KCl, 1 mM EDTA, 1 mM DTT, and 0.02% azide. The myosin peak was located by OD_{280} and then concentrated by polymerization and sedimentation. It was then resuspended in storage buffer as described above for the purification of *Dictyostelium* myosin. The degree of myosin degradation, if any, was assessed by SDS Gel electrophoresis, and the extent of 18,000-D light-chain dephosphorylation was measured by glycerol-urea gel electrophoresis.

In Vitro Motility Assay. The in vitro movement of *Dictyostelium* myosin was measured by the assay of Sheetz and Spudich (62), as described in detail by Sheetz, et al. (61). Bead samples were prepared using myosin at 25 and 100 μg/μl.

Myosin ATPase Assay. Myosin ATPase activity was measured using [γ-^{32}P]ATP as described by Clarke and Spudich (10). The Ca^{2+} ATPase activity of myosin was assayed in 10 mM Tris, pH 8.0, 10 mM $CaCl_2$, 0.6 M KCl, and 1 mM ATP. Actin-activated myosin Mg^{2+} ATPase activity was measured in 50 mM Tris, pH 8, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 15 mM KCl, and 1 mM ATP with myosin concentration 0.06 mg/ml and actin concentration 0–0.4 mg/ml. The actin-activated ATPase activity was calculated by subtracting the value for myosin alone.

Myosin Assembly Assay. Thick filament formation was measured as described by Kuczmarski and Spudich (33).

HPLC Methods. We used a Waters HPLC system (Waters Associates, Milford, MA) equipped with a Toyo Soda 60-cm G3000SW gel filtration column (range of mol wt 1,000–300,000). The column was equilibrated with prefiltered buffer until a stable baseline was achieved. Samples were dialyzed against column buffer and clarified in a microfuge (Beckman Instruments, Inc., Fullerton, CA) before application. Column runs were done at 22°C, but the column sample was stored at 0°C before application and fractions were placed at 0°C immediately after collection.

Biochemical Methods. Protein analysis was performed by the method of Bradford (8) with BSA as a standard.

PAGE in SDS was carried out on slab gels with a Tris/glycine buffer (5, 34) and the gels were stained with Coomassie Brilliant Blue R (20) and dried on a slab drying apparatus (Hoefer Scientific Instruments, San Francisco, CA).

Very dilute samples of protein (as for example, the HPLC fractions in Fig. 4) were precipitated with an equal volume of 10% TCA on ice for 30 min. The precipitate was collected by centrifugation in an Eppendorf microfuge for 5 min. The pellets were then washed with 5% TCA and again centrifuged in the microfuge for 5 min. For SDS PAGE, sample buffer containing 200 mM Tris, pH 8.8, was used. The amount of protein present in such samples was measured by densitometry of the gel. The bands were cut out and weighed, and compared with the weight of a scan of a known amount of protein.

Glycerol-urea gel electrophoresis was performed by the method of Perrie and Perry (49), with modifications used in the laboratory of Robert S. Adelstein (National Institutes of Health–James R. Sellers, personal communication). Samples that had been frozen in a dry ice-acetone bath and then stored at −4°C before processing were freeze-dried in a Savant Instruments Speed-Vac (Hicksville, NY) at 22°C. Freshly prepared sample buffer containing urea was added directly to the freeze-dried samples. Gels were scanned with a scanning densitometer (Transidyne General Corp.), peaks were cut out and weighed and percent phosphorylation of 18,000-D light chain was calculated.

Routine scanning densitometry of SDS polyacrylamide slab gels was performed using a scanning densitometer (Transidyne General Corp.). High-resolution densitometry was performed using a flatbed densitometer (Perkin-Elmer Corp., Norwalk, CT) coupled to a VAX computer. The average density was calculated by an appropriate program and plotted.

Autoradiograms of previously dried slab gels were made by exposing them to X-Omat AR x-ray film (Eastman Kodak Co., Rochester, NY) with a DuPont Cronex intensifying screen (DuPont Co., Wilmington, DE). Scanning densitometry of autoradiograms was performed using a scanning densitometer (Transidyne General Corp.).

Results

Purification of MLCK from Dictyostelium

Steps in the Purification of MLCK: Preparation of HSS. ~350 g of amebas of *Dictyostelium discoideum* was grown in four carboys. Cells were harvested by centrifugation in 1-liter bottles in a centrifuge (International Equipment Co., Needham Heights, MA) at 18,000 *g* for 7 min. The cells were immediately placed on ice. They were washed in 10 mM Tris, pH 7.5, and again collected by centrifugation. The cells were next combined with 2 vol/g (~750 ml) 10 mM Teola, pH 7.5, 0.4 mM DTT, 30% sucrose, 40 mM sodium pyrophosphate, 1 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 1 mM PMSF, 0.5 mM TPCK, and 1 mM TLCK. In some preparations 1 mM DIFP was also included. Cells were lysed by sonication as described in Materials and Methods for the purification of myosin. The lysate was centrifuged at 50,000 *g* for 30 min. The supernatant was collected and diluted vol/vol with 10 mM Teola, pH 7.5, 0.4 mM DTT, 25 mM sodium pyrophosphate, 1 mM EDTA, 5 mM EGTA, and 0.02% sodium azide. In some preparations 1 mM DIFP was also included. Next this material was clarified by centrifugation at 100,000 *g* for 1 h. The HSS was collected.

Ammonium Sulfate Fractionation. After the addition of Teola, pH 7.5, to 50 mM, the HSS was fractionated with ammonium sulfate. For MLCK assays, fractions were dialyzed against 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide. *Dictyostelium* MLCK activity fractionated in 70–100% $(NH_4)_2SO_4$ (Fig. 1). The 80–100% $(NH_4)_2SO_4$ cut, which constituted about a 30-fold purification of MLCK activity as compared with the HSS (Table I), contained ~1% of the total protein and ~40% of the total MLCK activity. In contrast, the 40–60% $(NH_4)_2SO_4$ cut (see the 0–60% cut in Fig. 1) contains kinase activity that phosphorylates at least 100 different proteins.

The amount of enzyme present in the 80–100% $(NH_4)_2SO_4$ cut is independent of the phase of growth of *Dic-*

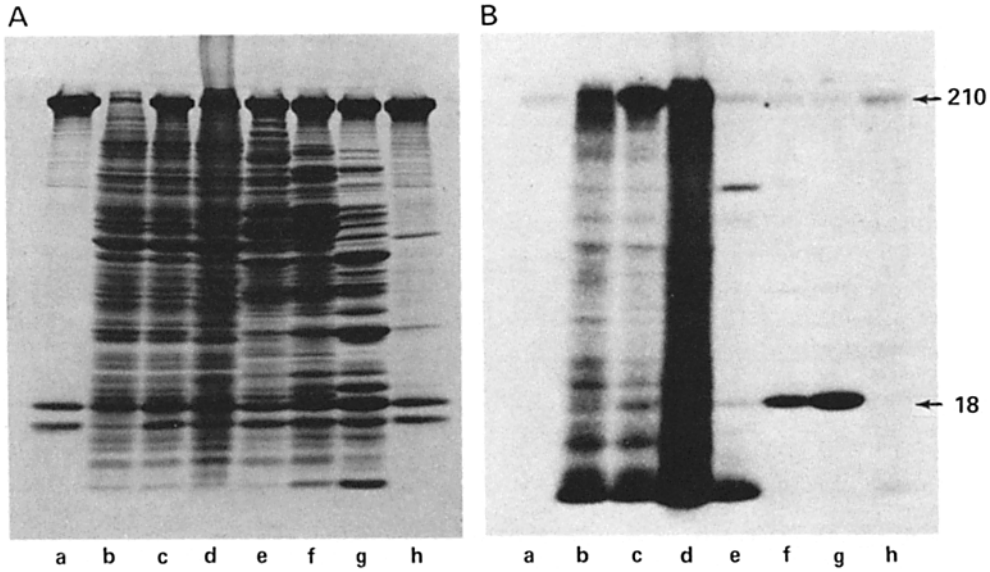

Figure 1. $(NH_4)_2SO_4$ fractionation of *Dictyostelium* MLCK. (*A*) SDS gel electrophoresis on 12% polyacrylamide of $(NH_4)_2SO_4$ cuts of *Dictyostelium* HSS incubated together with [γ-^{32}P]ATP and *Dictyostelium* myosin and (*B*) corresponding autoradiogram. Myosin (14 μg) was incubated with 30 μg of each cut as described in Materials and Methods. (*a*) Myosin only, (*b*) HSS only, (*c*) HSS and myosin, (*d*) 0–60% + myosin, (*e*) 60–70% + myosin, (*f*) 70–80% + myosin, (*g*) 80–100% + myosin, (*h*) 100% $(NH_4)_2SO_4$ supernatant + myosin. The positions of *Dictyostelium* myosin heavy chain (210,000 D) and light chain (18,000 D) are indicated.

Table I. Purification of Dictyostelium MLCK

Step	Volume	Protein concentration	Total protein	Total activity	Specific activity	Total-fold purification
	ml	*mg/ml*	*mg*	*pmol/min*	*pmol/min/mg*	
HSS	1,450	13.8	20,000	6,000	0.3	–
$(NH_4)_2SO_4$	30.5	8.3	250	2,500	10	33
DE-52	21.5	0.32	6.9	2,150	310	1,030
HAP	3.0	0.31	0.9	1,280	1,420	4,700

The starting material for this preparation was 350 g of *Dictyostelium discoideum* amebas. Samples were dialyzed into 10 mM Tris, pII 7.5, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 5% sucrose, and 0.01% sodium azide (DE-52 buffer) before quantitative assay of MLCK activity as described in Materials and Methods.

tyostelium amoebae in HL-5 liquid medium. We found no difference in the pattern of fractionation of MLCK or in the amount of enzyme present in the 80–100% cut for cells grown to OD 0.5 (log-phase growth) as compared with OD 1.0 (stationary phase) or OD 0.5 starved for 6 h in phosphate buffer (MKK_2 buffer prepared as described by Dinauer et al. [19]) to induce the early stages of differentiation of *Dictyostelium* amebas to form slugs.

For large MLCK preparations, the 80–100% $(NH_4)_2SO_4$ cut was routinely collected. The 70–80% cut was not collected because it contains many more proteins than the 80–100% cut, as judged by SDS PAGE (Fig. 1). The 80–100% cut was collected as follows. After addition of Teola, pH 7.5, to 50 mM, the HSS was made 70% in $(NH_4)_2SO_4$. Solid ammonium sulfate was added in increments with constant slow stirring at 0°C. The sample was then centrifuged at 27,000 *g* for 30 min. The 70% supernatant was then made 80% in $(NH_4)_2SO_4$. Again the sample was centrifuged and the supernatant was then made 100% in $(NH_4)_2SO_4$. The sample was centrifuged and the 80–100% $(NH_4)_2SO_4$ pellet was homogenized gently in a small volume of 10 mM Tris, pH 7.5, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 5% sucrose, 0.02% sodium azide, 1 mM PMSF, 0.25 mM TPCK, and 0.5 mM TLCK, and dialyzed against the same solution. In some preparations 1 mM DIFP was also included.

DEAE Chromatography. The 80–100% $(NH_4)_2SO_4$ cut, which had been dialyzed as described above, was clarified by centrifugation at 100,000 *g* for 1 h and applied to a DEAE cellulose column as illustrated in Fig. 2 (*left*). A 30-fold purification and 90% recovery are achieved with this step (Table I) for two reasons. First, with the inclusion of 5 mM EGTA in the column buffer, most protein does not bind to the column, whereas MLCK does. Secondly, the enzyme binds tightly to DEAE, which suggests that it is very acidic. It elutes behind the main protein peak (Fig. 2, *left*) at ~0.13 M KCl. A total fold purification of about 1,000 is achieved (Table I). The peak was pooled and dialyzed against 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, 5% sucrose, and 0.02% sodium azide.

HAP Chromatography. The pooled and dialyzed DEAE MLCK peak was applied to a HAP column (Fig. 2, *right*). *Dictyostelium* MLCK activity eluted on the leading edge of

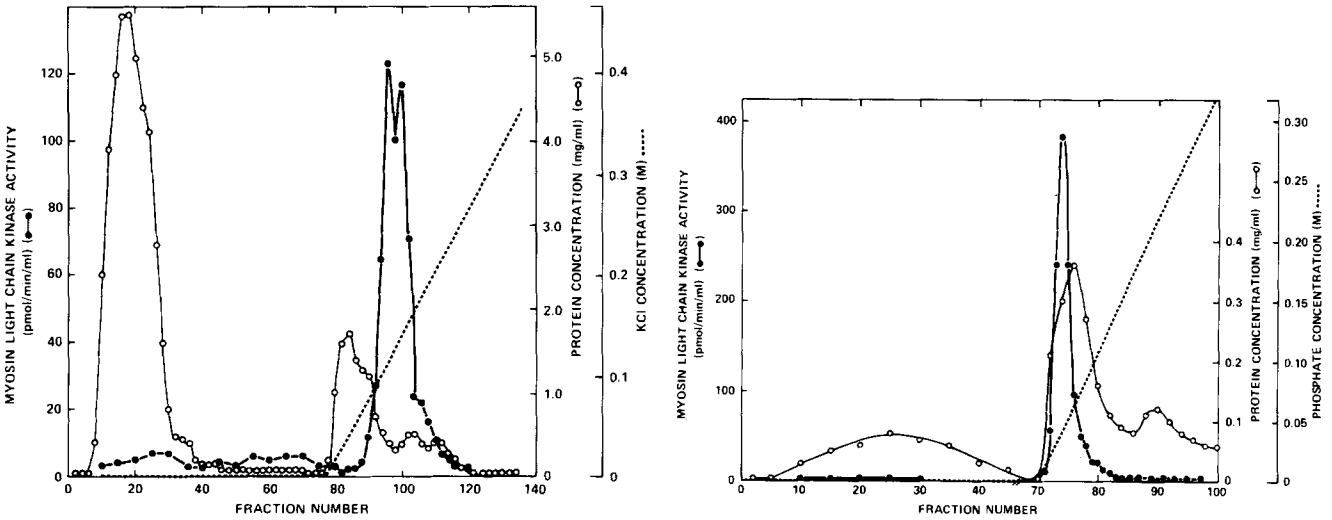

Figure 2. (*Left*) DEAE chromatography of *Dictyostelium* MLCK. 250 mg of 80–100% cut of *Dictyostelium* HSS in 30 ml was applied to a 2 × 8-cm column (25 ml) of DEAE-cellulose (DE-52) preequilibrated with 10 mM Tris, pH 7.5, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 5% sucrose, and 0.02% sodium azide. The column was run at 20 cm of pressure and eluted with a 150-ml linear 0–0.5 M KCl gradient in column buffer. Fractions were dialyzed against column buffer without KCl and 8-μl aliquots were assayed for MLCK activity as described in Materials and Methods. (*Right*) HAP chromatography of *Dictyostelium* MLCK. 7 mg of pooled DEAE peak in 21 ml was applied to a 4 × 0.8-cm column (2.5 ml) of HAP preequilibrated with 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, 5% sucrose, and 0.02% sodium azide. The column was run at 20 cm of pressure and eluted with a 50-ml linear 0.01–0.5 M potassium phosphate gradient in column buffer. Fractions were dialyzed against 10 mM Tris, pH 7.5, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 5% sucrose, and 0.02 sodium azide, and 8-μl aliquots were assayed for MLCK activity as described in Materials and Methods.

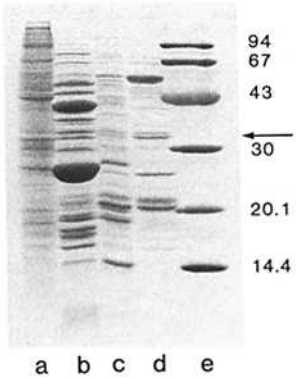

Figure 3. SDS gel electrophoresis on 15% polyacrylamide of the purification of *Dictyostelium* MLCK. 15 μg of protein was applied to each lane. (*a*) HSS, (*b*) 80–100% $(NH_4)_2SO_4$ cut, (*c*) DEAE pool, (*d*) HAP peak fraction. Molecular mass standards are shown on the right. (*Arrow*) 33,000-D doublet.

the main protein peak at ~0.04 M potassium phosphate. A 4.5-step fold purification with ~60% recovery of activity was achieved (Table I). An attempt to improve on this by making the phosphate gradient shallower was unsuccessful. The total fold purification of MLCK after HAP chromatography was ~4,700-fold. An SDS gel of the HAP peak fraction of MLCK activity is compared in Fig. 3 with the HSS, 80–100% $(NH_4)_2SO_4$ cut, and DEAE pool. The DEAE pool consists of at least 20 major bands, whereas the HAP peak fraction consists of about six major bands. There is a single band of ~50,000 daltons, a doublet at ~33,000 D, a single band at ~26,000 D and a doublet at ~20,000 D. The peak of MLCK activity comigrated only with the 33,000-D doublet.

An additional point of interest is that the lower band of the 33,000-D doublet, but not the upper band, is the only other protein besides the myosin light chain that is phosphorylated during the MLCK reaction. This phosphorylation also occurs when the MLCK preparation is incubated with ATP in the absence of myosin.

HAP fractions were dialyzed (see legend to Fig. 2) and stored either at 0°C or in aliquots in liquid N_2.

Comments on the Purification Procedure. As described above, ~1 mg of *Dictyostelium* MLCK can be isolated from 350 g of wet cells by 80–100% ammonium sulfate precipitation and chromatography on DEAE and HAP. The overall purification is ~4,700-fold (Table I). This material is relatively stable. In one experiment, HAP peak at 0.3 mg/ml stored at 0°C lost 35% of its activity over a 5-d period. This material can also be stored in liquid N_2 in which case less activity is lost. About 350 g of wet cells is necessary for a good MLCK preparation because it is important that protein concentration in the HAP peak be ≥0.1 mg/ml. More dilute concentrations of enzyme lost activity rapidly and activity was not stable to storage in liquid N_2.

Because of the known sensitivity to proteolysis of MLCK purified from other sources, we took several precautions to avoid proteolysis during our purification. Amebas were lysed under conditions that minimize proteolysis (70). EGTA and EDTA were included in all buffers, except the HAP and

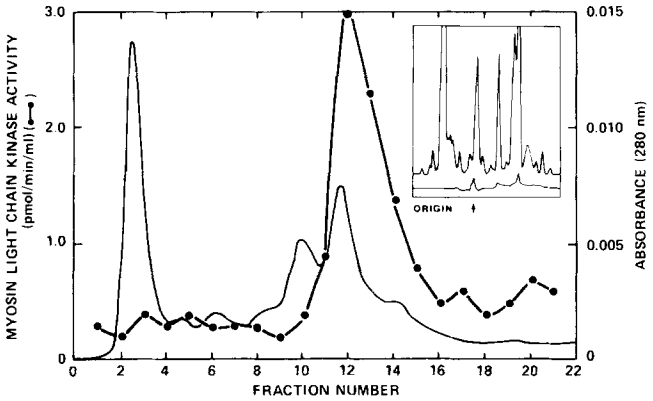

Figure 4. HPLC on G3000SW of *Dictyostelium* MLCK. An aliquot (62 μg in 200 μl) of HAP peak fraction 74 was dialyzed against 10 mM Tris, pH 7.5, 2 mM EDTA, 1 mM DTT, and 0.1 M KCl and then applied to a 60-cm HPLC gel filtration column, Toyo Soda G3000SW, mol wt range 1,000–300,000. The column was preequilibrated with the same buffer and run at 0.5 ml/min. 0.5-ml fractions were placed on ice as soon as they were collected. 10-μl aliquots were assayed immediately for MLCK activity, according to Materials and Methods. The remainder of each fraction was used for SDS polyacrylamide gel electrophoresis (see *inset* and Fig. 5). (*Inset*) MLCK activity peak fraction 13 consists of 30% 33,000-mol wt doublet. Aliquots (0.5 ml) of HPLC G3000SW fractions were precipitated with TCA and analyzed by 15% SDS PAGE. Upper gel scan: 15 μg of HAP peak fraction. The same gel appears in Fig. 3, lane *d*. Lower gel scan: 4 μg of MLCK activity peak fraction 13. (*Arrow*) 33,000-mol wt doublet. Our most pure fraction of MLCK activity illustrated here consists of ~30% 33,000 mol wt doublet. Two other proteins are present in this fraction, both of mol wt <33,000, but neither of these comigrates with MLCK activity. (Note that the densitometer used for these gel scans does not resolve the two components of the 33,000-mol wt doublet).

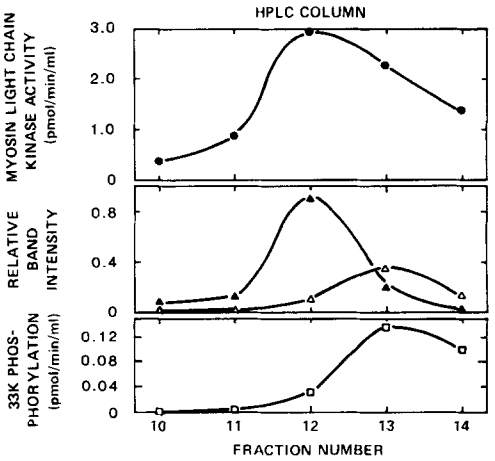

Figure 5. Comigration of *Dictyostelium* MLCK activity and 33,000-D doublet with chromatography on HPLC G3000SW. Aliquots (0.5 ml) from the experiment illustrated in Fig. 4 were precipitated with TCA, analyzed by 15% SDS PAGE, and scanned with a very high resolution densitometer which was capable of resolving the two components of the 33,000-D doublet (see Materials and Methods). The upper panel shows MLCK activity (*solid circles*). The middle panel shows the amount of upper band (*solid triangles*) as compared with lower band (*open triangles*) of the 33,000-D doublet in each fraction. The lower panel (*open squares*) illustrates phosphorylation of the lower band of the 33,000-D doublet which occurred during the MLCK assay. The extent of phosphorylation was determined as described in Materials and Methods.

HPLC column buffers which had EDTA alone. The proteolysis inhibitors PMSF, TPLK, and TLCK were used routinely. DIFP was also used in some preparations. In one preparation, the cells were split into two batches and DIFP was used in one batch and omitted from the second batch. The two batches were processed separately through the DEAE chromatography step. No significant difference in the yield or properties of the MLCK activity was found.

Properties of Dictyostelium MLCK. To gain further information about the molecular weight of the myosin light chain kinase, we analyzed the HAP pool by HPLC (Fig. 4). The *Dictyostelium* MLCK eluted as a single peak of activity with an apparent native molecular weight of ~30,000 as compared with standards of mol wt 240,000 (β-phycoerythrin); 67,000 (BSA); 43,000 (ovalbumin); and 17,000 (myoglobin) run on the same column under identical conditions. As we found for the HAP column, the peak of MLCK activity on HPLC comigrates with a 33,000 doublet (Fig. 5). Interestingly, as we found for the HAP pool, the lower band of the 33,000-D doublet, but not the upper band, is phosphorylated during the MLCK reaction (Fig. 5, *lower panel*). The increase in the specific activity of the two HPLC fractions with maximal activity (fractions 12 and 13) as compared with the column load was marginal (data not shown), probably because of loss of enzyme activity as a result of the substantial dilution that occurred (from 0.3 mg/ml in the HAP pool to ~0.01 mg/ml in fractions 12 and 13). In our most purified material, HPLC fraction 13, the 33,000-D doublet is ~30% of the total protein present (Fig. 4, *inset*).

The effects of various ions and cyclic nucleotides on MLCK activity were examined. Assays for MLCK activity were generally carried out in 10 mM Tris, pH 7.5, 5 mM Mg^{2+}, 1 mM ATP. Addition of 1–2 mM cAMP or cGMP had no effect on MLCK activity. KCl inhibited MLCK activity significantly. 50–100 mM KCl reduced activity to 50% as compared with samples without KCl. Mg^{2+} was required for MLCK activity. Significant activity occurred when 2–10 mM Mg^{2+} was included with the buffer. Ca^{2+} could not substitute for the Mg^{2+} requirement. Samples having 1–10 mM Ca^{2+} in buffer that lacked Mg^{2+} had no measurable MLCK activity. When added to the usual assay buffer that included Mg^{2+}, 1 mM Ca^{2+} inhibited MLCK activity. 1–2 mM Ca^{2+} reduced MLCK activity to ≤50% as compared with samples without Ca^{2+}. This is illustrated in Fig. 6 (lanes *d* and *e*).

Dictyostelium MLCK activity in 1 mM Ca^{2+} was not affected by the addition of calmodulin purified from either bovine brain (Fig. 6, lanes *e* and *f*) or *Dictyostelium* (data not shown). In contrast, gizzard smooth muscle light chain kinase was activated by calmodulin and 1 mM Ca^{2+} using either smooth muscle myosin (lane *n*) or rabbit skeletal muscle myosin (lane *m*) as substrate, as expected (3).

Dictyostelium MLCK is specific for *Dictyostelium* myosin among other myosins tested. As illustrated in Fig. 6, *Dictyostelium* MLCK phosphorylated *Dictyostelium* myosin (lane *d*), but not rabbit skeletal muscle myosin (lane *h*), smooth muscle myosin isolated from turkey gizzard (lane *j*), or *Acanthamoeba* myosin II (data not shown). Histone and casein were not phosphorylated by the enzyme.

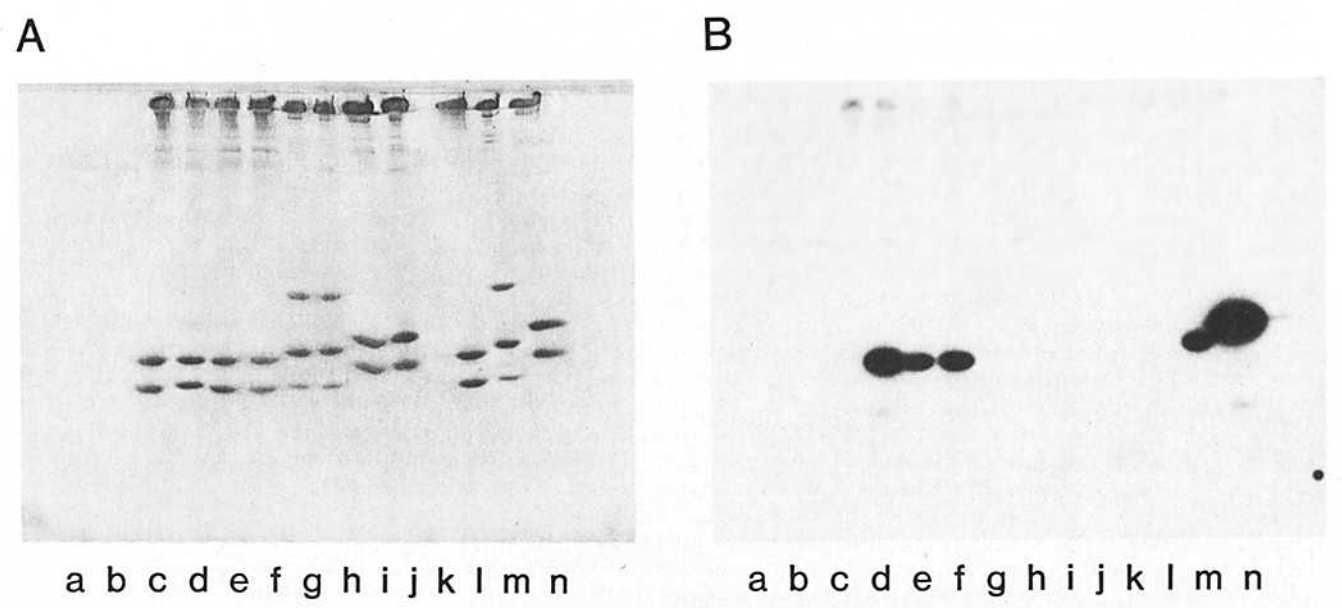

Figure 6. Dictyostelium MLCK will phosphorylate *Dictyostelium* myosin but not skeletal or smooth muscle myosins, and the activity of the enzyme is not affected by the addition of calmodulin. SDS gel electrophoresis on (*A*) 12% polyacrylamide and (*B*) corresponding autoradiogram. Protein in 20-μl assay: *Dictyostelium* myosin, turkey gizzard smooth muscle myosin or rabbit skeletal muscle myosin, 10 μg; *Dictyostelium* HAP peak MLCK, 0.35 μg; turkey gizzard smooth muscle MCLK, 0.15 μg; bovine brain calmodulin, 0.05 μg. Buffer: for *Dictyostelium* MLCK, 20 mM Tris, pH 7.5, 5 mM Mg^{2+}, 0.75 mM ATP; for smooth muscle MLCK, 20 mM Tris, pH 7.5, 4 mM $MgCl_2$, 1 mM DTT, 0.75 mM ATP. 1 mM Ca^{2+} was also included where indicated below. [γ-^{32}P]ATP was 500 cpm/pmol ATP. Incubation was 10 min at 22°C. Samples were processed as in Materials and Methods. (*a*) *Dictyostelium* MLCK alone, (*b*) *Dictyostelium* MLCK + calmodulin, (*c*) *Dictyostelium* myosin alone, (*d*) *Dictyostelium* myosin + *Dictyostelium* MLCK, (*e*) *Dictyostelium* myosin + *Dictyostelium* MCLK + $CaCl_2$, (*f*) *Dictyostelium* myosin + *Dictyostelium* MLCK + $CaCl_2$ + calmodulin, (*g*) skeletal muscle myosin alone, (*h*) skeletal muscle myosin + *Dictyostelium* MLCK, (*i*) smooth muscle myosin alone, (*j*) smooth muscle myosin + *Dictyostelium* MLCK, (*k*) smooth muscle MLCK + Ca^{2+} + calmodulin, (*l*) *Dictyostelium* myosin + smooth muscle MLCK + Ca^{2+} + calmodulin, (*m*) skeletal muscle myosin + smooth muscle MLCK + Ca^{2+} + calmodulin, (*n*) smooth muscle myosin + smooth muscle MLCK + Ca^{2+} + calmodulin.

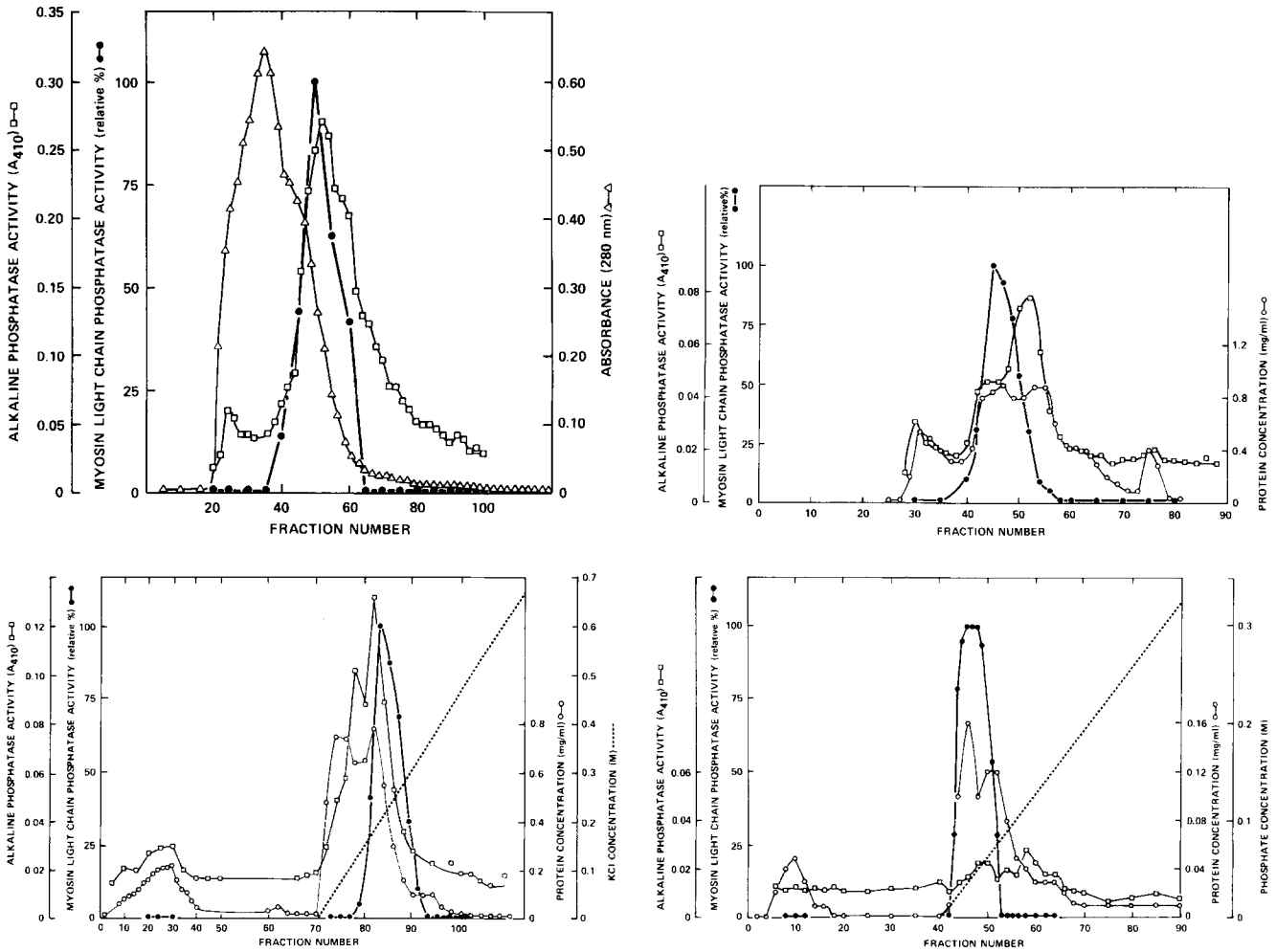

Figure 7. (*Top left*) Affi-Gel Blue chromatography of *Dictyostelium* myosin phosphatase. The 30–60% $(NH_4)_2SO_4$ cut (1.9 g of protein; 55 ml) of the *Dictyostelium* HSS was applied to a 2.2 × 18-cm column of Affi-Gel Blue 100–200 mesh, 75–150 μm run at 14 cm of pressure. The column was preequilibrated with 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide and developed with the same buffer. 6-μl aliquots of each fraction were assayed for myosin phosphatase activity and 10-μl aliquots were assayed for alkaline phosphatase activity, as described in Materials and Methods. (*Top right*) Gel filtration on A-0.5m of *Dictyostelium* myosin phosphatase. The Affi-Gel Blue pool (~500 mg of protein concentrated into 6.5 ml as described in Results) was applied to a 70 × 2.5-cm column of agarose A-0.5m, 100–200 mesh, which was run at 30 cm of pressure. The column was preequilibrated with 20 mM Tris, pH 7.5, 0.5 M KCl, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide and developed with the same buffer. 10-μl aliquots of each fraction were assayed for alkaline phosphatase activity and 6-μl aliquots of each fraction were assayed for myosin phosphatase activity, as described in Materials and Methods, after dialysis of fractions against 20 mM Tris, pH 7.5, 25 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide. (*Bottom left*) DEAE chromatography of *Dictyostelium* myosin phosphatase. The A-0.5m pool (17 mg of protein; 10 ml) was applied to a 4.0 × 1.2-cm (4.5 ml) column of DEAE Sephadex (DE-52) run at 15 cm of pressure. The column was preequilibrated with 20 mM Tris, pH 7.5, 25 mM KCl, 1 mM EGTA, 1 mM DTT, and 0.02% sodium azide and developed with an 80-ml linear 0.025–0.75 M KCl gradient in column buffer. 10-μl aliquots of each fraction were assayed for myosin phosphatase activity and 40-μl aliquots were assayed for alkaline phosphatase activity, as described in Materials and Methods. (*Bottom right*) HAP chromatography of *Dictyostelium* myosin phosphatase. The pooled DEAE peak (2 mg of protein; 6.5 ml) was applied to a 4.5 × 1.2-cm (5 ml) column of HAP run at 15 cm of pressure. The column was preequilibrated with 10 mM potassium phosphate, pH 7.5, 1 mM DTT, and 0.02% sodium azide and eluted with a 120-ml linear 0.01–0.4 M potassium phosphate gradient in column buffer. 50-μl aliquots of each fraction were assayed for alkaline phosphatase activity as described in Materials and Methods. 6-μl aliquots of each fraction were assayed for myosin phosphatase activity after dialysis of fractions against 20 mM Tris, pH 7.5, 25 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide.

Stoichiometry of phosphorylation of *Dictyostelium* myosin with *Dictyostelium* MLCK is ≥0.96 mol P_i/mol 18,000-D light chain (described below).

The residue on *Dictyostelium* myosin is 18,000-D light chain, which is phosphorylated in vivo and in vitro by *Dictyostelium* MLCK, is serine (E. R. Kuczmarski, Northwestern University School of Medicine, personal communication; Berlot, Devreotes, and Spudich, manuscript submitted for publication).

Purification of MLCP from Dictyostelium

Steps in the Purification: Preparation of HSS. ~70 g of amebas of *Dictyostelium* was harvested by centrifugation, washed, and again collected as described for the purification

Table II. Purification of Dictyostelium MLCP

Step	Volume	Total protein	Total activity	Specific activity	Total-fold purification
	ml	*mg*	*pmol/min*	*pmol/min/mg*	
HSS	410	4,900	64,000	13	–
30–60% cut	55	1,900	28,000	15	1.1
Affi-Gel	110	520	16,000	31	2.4
A-0.5m	50	90	4,200	47	3.6
DE-52	10	17	1,800	106	8.2
HAP	6.5	2.0	870	430	33

The starting material for this preparation was 70 gm of *Dictyostelium discoideum* amebas. Samples were dialyzed into 20 mM Tris, pH 7.5, 25 mM KCl, 1 mM EGTA, 1 mM DTT, 0.02% sodium azide (DE-52 buffer) and phosphatase activity was assayed quantitatively as described in Materials and Methods.

of MLCK. The cells were next combined with 2 vol/g of 10 mM Teola, pH 7.5, 40 mM sodium pyrophosphate, 30% sucrose, 0.4 mM DTT, 2 mM EDTA, 1 mM PMSF, 0.5 mM TPCK, and 1 mM TLCK. Cells were lysed by sonication as described in Materials and Methods for the purification of myosin. The lysate was centrifuged at 27,000 *g* for 30 min. The supernatant was collected and diluted vol/vol with 10 mM Teola, pH 7.5, 25 mM sodium pyrophosphate, 0.4 mM DTT, and 2 mM EDTA. The diluted lysate was clarified by centrifugation at 100,000 *g* for 1 h. The HSS was retained.

Ammonium Sulfate Fractionation. After addition of Teola, pH 7.5, to 50 mM, the HSS was made 30% in $(NH_4)_2SO_4$ with constant stirring at 0°C. The sample was centrifuged at 27,000 *g* for 30 min and the 30% supernatant was retained. The supernatant was made 60% in $(NH_4)_2SO_4$ and centrifuged, and the 30–60% $(NH_4)_2SO_4$ pellets were retained. The pellets were homogenized gently into a minimal volume (total volume of pellets plus buffer 50–60 ml) of 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide and dialyzed against the same buffer. This step separates MLCP from *Dictyostelium* MLCK activity, which fractionates in 70–100% $(NH_4)_2SO_4$ (as described above).

Affi-Gel Blue Chromatography. The dialyzed 30–60% $(NH_4)_2SO_4$ cut was clarified by centrifugation at 100,000 *g* for 1 h and the supernatant applied to an Affi-Gel Blue column as illustrated in Fig. 7 (*top left*). *Dictyostelium* myosin phosphatase activity eluted on the trailing edge of the run through, along with alkaline phosphatase activity. A 2.4-fold purification and 60% recovery of activity were achieved with this step (Table II). This step separates light chain phosphatase from heavy chain kinase activity, which binds to Affi-Gel Blue (E.R. Kuczmarski, Northwestern University School of Medicine, personal communication).

A-0.5m Chromatography. The myosin phosphatase activity peak from the Affi-Gel Blue column was pooled and brought to 50 mM in Teola, pH 7.5. Solid $(NH_4)_2SO_4$ was added to 65% in increments with continuous gentle stirring at 0°C and the precipitate was sedimented at 27,000 *g* for 30 min. The pellet was homogenized gently with 20 mM Tris, pH 7.5, 0.5 M KCl, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide to a total volume of 6.5 ml. This sample was clarified by centrifugation at 27,000 *g* for 30 min and loaded on an agarose A-0.5m column as illustrated in Fig. 7 (*top right*). Myosin phosphatase activity eluted as a single peak with a dissociation constant (K_D) of 0.36. Alkaline phosphatase activity eluted as a much broader peak in the same region and slightly behind the peak of myosin phosphatase activity. A 1.5-fold purification and 26% recovery of activity were achieved with this step (Table II). The peak was pooled and dialyzed against 20 mM Tris, pH 7.5, 25 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.02% azide.

DEAE Chromatography. The dialyzed A-0.5m myosin phosphatase activity peak was chromatographed on DEAE cellulose (DE-52) as shown in Fig. 7 (*bottom left*). Myosin phosphatase activity binds to DEAE and elutes at 0.22 M KCl, on the trailing edge of the protein peak. Alkaline phosphatase activity elutes slightly ahead of the myosin phosphatase activity. A 2.2-fold purification and 43% recovery of activity were achieved with this step (Table II). Fractions having myosin phosphatase activity were pooled and dialyzed against 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide.

HAP Chromatography. The final step in the purification of *Dictyostelium* myosin phosphatase was chromatography on HAP. The dialyzed activity peak from DEAE was chromatographed as illustrated in Fig. 7 (*bottom right*). Myosin phosphatase activity bound to HAP and eluted with the main peak of protein at about 0.05 M potassium phosphate. The myosin phosphatase peak had very little alkaline phosphatase activity. A 4.2-fold purification and 48% recovery of activity were achieved with this step (Table II).

The *Dictyostelium* myosin phosphatase HAP peak was concentrated by dialysis against dry Aquacide III and then dialyzed into 20 mM Tris, pH 7.5, 25 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.02% sodium azide. 75% of the protein and 47% of the phosphatase activity were recovered after this procedure.

In summary, ~1.5 mg of partially purified *Dictyostelium* myosin phosphatase can be isolated from 70 g of wet cells by 30–60% ammonium sulfate precipitation and chromatography on Affi-Gel Blue, A-0.5m, DEAE, and HAP. The overall purification is 33-fold (Table II). This material is stable for at least 1 wk when stored at 0°C. The quantity of material obtained is adequate for dephosphorylating several mg of *Dictyostelium* myosin.

Kinetic Properties of Dictyostelium Myosin Phosphatase. *Dictyostelium* myosin phosphatase dephosphorylates *Dictyostelium* myosin such that ≤0.01 mol P_i/mol 18,000-D light chain remains. In one experiment, purified myosin was incubated without or with 0.1 μg of phosphatase/μg of myosin for 2 h at 22°C under standard buffer conditions. By scanning densitometry of glycerol-urea gels of the myosins, the phosphorylated sample had 0.7 mol P_i/mol 18,000-D light chain, an amount higher than previously found (33), whereas the dephosphorylated sample had no detectable phos-

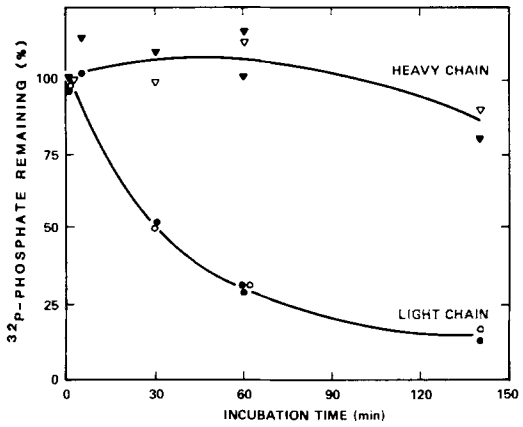

Figure 8. Dictyostelium myosin phosphatase removes ^{32}P from the light chain but not from the heavy chain of *Dictyostelium* myosin purified from amebas grown in [^{32}P]phosphate. 15 μg of myosin labeled in vivo as described in Materials and Methods was incubated together with either 3.5 μg of DEAE purified phosphatase (*open symbols*) or 2.0 μg of HAP purified phosphatase (*solid symbols*) in 25 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 1 mM $CaCl_2$ at 22°C for the times designated. To quantify phosphate remaining on either heavy chain or light chain, samples were run on SDS polyacrylamide gels and an autoradiogram was developed. The autoradiogram was scanned, the peaks were cut out and weighed, and the relative percent phosphorylation was calculated.

phate. In a parallel experiment, using myosin previously labeled in vitro with *Dictyostelium* MLCK and [γ-^{32}P]ATP, the phosphatase-treated sample had ≤0.01 mol P_i/mol 18,000-D light chain.

The phosphatase preparation does not contain contaminating proteases, as judged by the following data. First, no proteolysis of myosin occurred with phosphatase treatment as assessed by SDS PAGE (data not illustrated). Secondly, the phosphatase-treated myosin can be rephosphorylated by incubation with *Dictyostelium* MLCK and ATP, and the rephosphorylated myosin has properties similar to those of myosin treated with kinase only (see below).

Dictyostelium myosin phosphatase appears to be a relatively specific enzyme in that it will remove serine phosphate from the light chain but not from the heavy chain of *Dictyostelium* myosin. In the experiment illustrated in Fig. 8, in vivo labeled myosin having ^{32}P on both the 210,000-D heavy chain and the 18,000-D light chain was incubated for various amounts of time with *Dictyostelium* myosin phosphatase. After incubation for 1 h, none of the heavy chain phosphate was removed whereas ~70% of the light chain phosphate was removed. After incubation for 2 h, ~5% of the heavy chain phosphate was removed and 85% of the light chain phosphate was removed. Later preparations of phosphatase removed all detectable light chain phosphate (see above).

Effects of 18,000-D Light-Chain Phosphorylation and Dephosphorylation on the Properties of Dictyostelium Myosin

We prepared *Dictyostelium* myosin, MLCK and MLCP, and used the same protein preparations in all of the following experiments, which examine effects of light-chain phosphorylation and dephosphorylation on properties of myosin.

Myosin-coated Bead Movement In Vitro. 0.5–2 mg of *Dictyostelium* myosin was treated with either *Dictyostelium* MLCK or *Dictyostelium* myosin phosphatase as described in Materials and Methods. The extent of phosphorylation of the 18,000-D light chain of *Dictyostelium* myosin was related to the rate at which the myosin moved in an *in vitro* assay (61), as illustrated in Fig. 9. The untreated myosin, which had 0.33 mol phosphate/mol 18,000-D light chain, moved at rates between 0.8 and 1.2 μm/s (*upper panel*). After treatment with kinase, the myosin had 0.96 mol phosphate/mole 18,000-D light chain and moved more rapidly at rates between 1.1 and 1.6 μm/s (*second panel*). In both of these cases, the majority of the beads that settled onto the *Nitella* substratum moved (60–80% of the beads moved in all cases except for dephosphorylated myosin). In contrast, myosin that was treated with phosphatase and then bound to beads had ≤0.01 mol phosphate/mol 18,000-D light chain and its rate of movement was zero for most of the beads (>99%) that settled onto the *Nitella* substratum. The few beads that moved (<1%) did so at a rate of 0.4–0.8 μm/s (*third panel*). Finally, it was possible to rephosphorylate the phosphatase-treated myosin. Such myosin was found to move at rates equivalent to those for myosin that had been treated with kinase only. In one type of experiment, phosphatase-treated myosin was treated with MLCK and ATP while bound to beads. The movement of such kinase-treated myosin is illustrated in the lower panel. Alternatively, phosphatase-treated myosin was purified by gel filtration chromatography and then treated with MLCK and ATP before incubation with beads (lower panel). Both of these samples moved at rates of 0.9–1.7 μm/s.

Actin-activated Mg^{2+} ATPase Activity. Extent of phos-

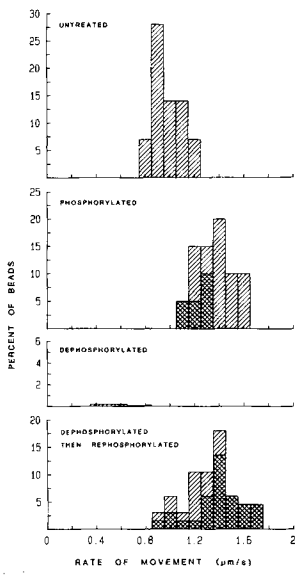

Figure 9. Movement of *Dictyostelium* myosin in vitro is related to the extent of phosphorylation of the 18,000-D light chain. *Dictyostelium* myosin was treated with *Dictyostelium* MLCK or *Dictyostelium* myosin phosphatase and samples were assayed for movement as described in Materials and Methods. The percentage of the beads, or bead aggregates (61), that moved at each velocity shown is plotted, where n = total number of beads or bead aggregates that settled onto the *Nitella* substratum. Those beads that did not move are not plotted. (*Top panel*) Untreated myosin bound to beads (n = 15, 67% moved). (*Second panel, hatched*) Myosin phosphorylated with kinase and then bound directly to beads; (*cross-hatched*) myosin phosphorylated with kinase, purified by gel filtration, and then bound to beads; the total height for each bar shown represents the sum of the two data sets (n = 20, 75% moved). (*Third panel*) Myosin dephosphorylated with phosphatase and then bound directly to beads (n = 730, 0.9% moved). (*Lower panel, cross-hatched*) Myosin dephosphorylated with phosphatase, bound to beads, and subsequently rephosphorylated with kinase while bound to beads; (*hatched*) myosin dephosphorylated with phosphatase, purified by gel filtration, rephosphorylated with kinase, and subsequently bound to beads; the total height for each bar shown represents the sum of the two data sets (n = 61, 72% moved).

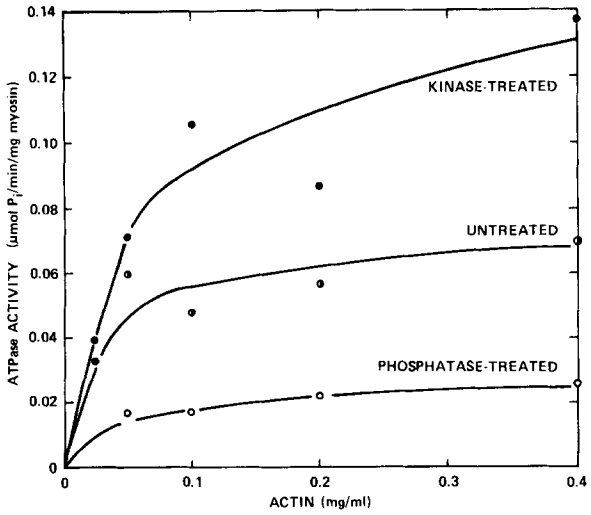

Figure 10. Actin-activated ATPase activity of *Dictyostelium* myosin is related to the extent of phosphorylation of the 18,000-D light chain. *Dictyostelium* myosin was treated with *Dictyostelium* MLCK or *Dictyostelium* phosphatase and actin-activated ATPase activity measured using rabbit skeletal muscle actin as described in Materials and Methods.

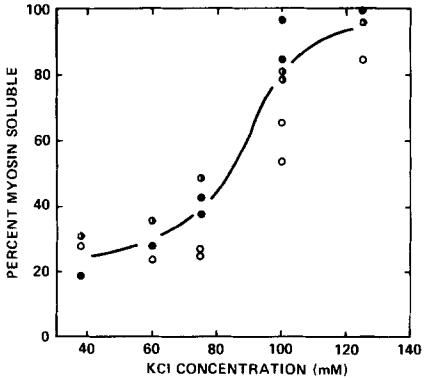

Figure 11. Dictyostelium myosin thick filament formation as a function of ionic strength is independent of the extent of phosphorylation of the 18,000-D light chain. *Dictyostelium* myosin was treated with *Dictyostelium* MLCK or *Dictyostelium* phosphatase and thick filament assembly assayed as described in Materials and Methods. Buffer conditions: 10 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM DTT. Untreated myosin (*half-solid circles*), MLCK-treated myosin (*solid circles*), myosin phosphatase-treated myosin (*open circles*).

phorylation of the 18,000-D light chain of *Dictyostelium* myosin was found to be directly related to the actin-activated Mg^{2+} ATPase activity of the molecule. As shown in Fig. 10, samples of phosphatase-treated, untreated, and kinase-treated myosin were combined with various concentrations of actin and the actin-activated Mg^{2+} ATPase activity was measured. The actin-activated Mg^{2+} ATPase activity of the kinase-treated sample is four to five times that of the phosphatase-treated sample. The actin-activated Mg^{2+} ATPase activity of the untreated sample, which had 0.33 mol phosphate/mol 18,000-D light chain, was found to be intermediate between the values for the kinase-treated and phosphatase-treated samples.

Assembly. Under the conditions that we used, we did not observe a significant effect of phosphorylation of the 18,000-D light chain of *Dictyostelium* myosin on assembly of the molecule into thick filaments. Samples of *Dictyostelium* myosin that were untreated or treated with *Dictyostelium* MLCK or myosin phosphatase were assayed for filament assembly in 10 mM Tris, pH 7.4, 0.1 mM EDTA, and 0.1 mM DTT, with varying concentrations of KCl as illustrated in Fig. 11. The sample of phosphatase-treated myosin appeared to have a slightly higher degree of polymerization as compared with the other two samples, but the differences among the three samples were small.

Discussion

Dictyostelium MLCK

We do not know whether the enzyme that we have purified is the only MLCK in *Dictyostelium*. We were persuaded to study this particular enzyme because of its striking specificity for *Dictyostelium* 18,000-D light chain apparent very early in the purification with ammonium sulfate fractionation (Fig. 1).

Dictyostelium MLCK differs from MCLK isolated to date from vertebrate smooth muscle (3, 69), vertebrate skeletal muscle (44, 75), vertebrate cardiac muscle (74), and vertebrate nonmuscle sources such as brain (26), platelet (25), and BHK-21 (76). For example, smooth muscle MLCK, which has been studied most extensively, is a Ca^{2+}/calmodulin-dependent enzyme of 130,000 mol wt (3, 69). In contrast, for *Dictyostelium* MLCK we show here that a doublet of 33,000 mol wt appears to be important for activity, and this activity is not Ca^{2+}/calmodulin dependent. An issue of importance is whether this *Dictyostelium* enzyme has suffered proteolysis during purification. In early attempts to purify MLCK from skeletal muscle (52) and from platelets (18), for example, proteolyzed Ca^{2+}/calmodulin-independent enzymes were isolated. In later work MLCK-from skeletal muscle (44, 75) and platelets (15, 25) were shown to be larger Ca^{2+}/calmodulin-dependent enzymes. Early attempts to purify MLCK from chicken gizzard (16, 17) and from bovine brain (15) yielded Ca^{2+}/calmodulin-dependent enzymes of 105,000 D. The most recent work shows that MLCK from chicken gizzard (69) and bovine brain (26) are of 130,000 D. Further, Walsh et al. (72) have produced a Ca^{2+}/calmodulin-independent enzyme of 80,000 D by limited proteolysis of Ca^{2+}/calmodulin-dependent turkey gizzard smooth muscle MLCK. Because *Dictyostelium* is a large phylogenetic distance from the vertebrate MLCKs that have been studied, it would not be surprising if it had distinctive properties. Factors supporting the argument that the *Dictyostelium* MLCK that we have isolated is a distinctive enzyme and not a breakdown product of a larger protein are as follows. First, we took a number of precautions to avoid proteolysis as described in Results. Secondly, our enzyme initially fractionates in 80–100% $(NH_4)_2SO_4$, which is different from other MLCK, such as turkey gizzard smooth muscle MLCK (3), which fractionates in 40–60% ammonium sulfate.

Myosin kinases purified thus far from sources other than vertebrate are notable in their diversity of properties, although none of them is like the *Dictyostelium* MLCK. A distinctive light chain kinase has been purified from *Limulus* skeletal muscle. Limulus MLCK is a doublet of 39,000 and 37,000 D and is Ca^{2+}/calmodulin dependent (59). Phosphorylation of *Limulus* myosin results in an increase in the

actin-activated Mg^{2+}-ATPase activity of the myosin (58). Other myosin kinases purified from sources other than vertebrate are heavy chain kinases. Myosin I heavy chain kinase purified from amoebae of *Acanthamoeba castellanii* (23) has a mol wt of 107,000 and is Ca^{2+}/calmodulin independent. Two *Acanthamoeba* myosin II heavy chain kinases have also been partially purified (14, 31). From *Dictyostelium*, Maruta et al. (37) have partially purified an enzyme from growth-phase amebas that has an apparent mol wt of 70,000 and is Ca^{2+}/calmodulin independent. In contrast, a second distinct myosin heavy chain kinase purified from amebas previously starved to induce aggregation has an apparent mol wt of 70,000 and is inactivated by Ca^{2+}/calmodulin. The partially purified *Dictyostelium* myosin heavy chain kinase of Kuczmarski and Spudich (33; and manuscript in preparation) has an apparent mol wt of 60,000.

Dictyostelium MLCK appears to be very specific as compared with other MLCKs investigated so far. Myosins from vertebrate smooth muscle and skeletal muscle, and *Acanthamoeba* myosin II were not phosphorylated by *Dictyostelium* MLCK. Further, we found that turkey gizzard smooth muscle MLCK will not phosphorylate *Dictyostelium* myosin. In contrast, turkey gizzard smooth muscle MLCK will partially phosphorylate vertebrate skeletal muscle myosin light chain (3). *Acanthamoeba* myosin I heavy chain kinase will also phosphorylate the 20,000-D light chain of smooth muscle myosin at what appears to be the same site that is phosphorylated by smooth muscle MLCK (24).

For *Dictyostelium* myosin 18,000-D light chain, as for vertebrate smooth muscle myosin 20,000-D light chain (3), vertebrate skeletal muscle myosin 18,500-D dithionitrobenzoate light chain (50), and *Acanthamoeba* myosin I heavy chain, the residue that is phosphorylated is serine. For *Dictyostelium* myosin light chain this is the case both for the myosin purified from amebas grown in [^{32}P]phosphate and for myosin labeled in vitro with purified *Dictyostelium* MLCK and [γ-^{32}P]ATP (E.R. Kuczmarski, Northwestern University School of Medicine, personal communication). In contrast, both serine and threonine phosphorylation of the heavy chain of *Acanthamoeba* myosin II (14) and *Dictyostelium* myosin has been measured in vivo and in vitro. *Dictyostelium* myosin purified by conventional methods from cells labeled in vivo by growth in [^{32}P]orthophosphate is labeled on the heavy chain at serine only (33). Myosin rapidly isolated from amebas by immunoprecipitation is labeled on the heavy chain at both serine and threonine (Berlot, C. H., and J. A. Spudich, unpublished observations). One partially purified heavy chain kinase from *Dictyostelium* is specific for threonine whereas another phosphorylates both threonine and serine (37; Kuczmarski, E. R., and J. A. Spudich, unpublished observations).

It is not clear how *Dictyostelium* MLCK might be regulated in vivo. As described above we have not been able to show Ca^{2+} dependence, Ca^{2+}/calmodulin dependence, or any effect of cAMP or cGMP on activity. We have preliminary evidence that the lower band of the 33,000-D doublet is associated with the MLCK activity, and this polypeptide is either autophosphorylated or is phosphorylated by another kinase that contaminates our MLCK preparation. This phosphorylation could be part of a regulatory mechanism, but this remains to be determined. *Acanthamoeba* myosin I heavy chain kinase is apparently autophosphorylated (23), but the significance of this phosphorylation is also unknown. In the case of turkey gizzard smooth muscle MLCK (1, 13) and human platelet MLCK (26), phosphorylation by cAMP-dependent protein kinase can occur, and this is a regulatory mechanism for the enzymes. Phosphorylation decreases the affinity of the 130,000-D MLCK for Ca^{2+}/calmodulin, and thereby inhibits the activity of the enzyme.

Dictyostelium MLCP

Here we describe a myosin phosphatase that will preferentially remove phosphate from the light chain but not from the heavy chain of *Dictyostelium* myosin. With this enzyme we are able to manipulate the extent of myosin light chain phosphorylation while leaving the extent of heavy chain phosphorylation unaffected and constant. Although the phosphatase preparation is impure, it is active without concomitant degradation of the *Dictyostelium* myosin, indicating that protease contamination is not a problem. We found that myosin treated with phosphatase and then with *Dictyostelium* MLCK has properties identical to those of myosin treated with MLCK alone.

Because our myosin phosphatase from *Dictyostelium* is as yet partially purified, we do not know whether it is similar to the MLCPs that have been purified from rabbit skeletal muscle and from turkey gizzard and chicken gizzard smooth muscle. The skeletal muscle enzyme purified by Morgan et al. (43) has a mol wt of 70,000. Two phosphatases have been purified from turkey gizzard smooth muscle by Pato and Adelstein (47). Phosphatase I consists of three polypeptides of mol wt 60,000, 55,000, and 38,000 and will also dephosphorylate smooth muscle MLCK at about one-half the rate that it dephosphorylates isolated 20,000-D light chain. Phosphatase II has a mol wt of 43,000. The phosphatase purified from chicken gizzard smooth muscle by Onishi et al. (45) consists of components of mol wt 67,000, 54,000, and 34,000.

Effects of Reversible Light Chain Phosphorylation on the Properties of Dictyostelium Myosin

Comparison of the rates of movement of phosphorylated *Dictyostelium* myosin and myosin that had been dephosphorylated with *Dictyostelium* myosin phosphatase showed that light chain phosphorylation is important for myosin movement on actin. Moreover, we were able to rephosphorylate myosin that had previously been treated with phosphatase; such myosin moved at rates comparable to those of myosin treated with kinase alone. These results are consistent with those found for smooth muscle myosin and for *Acanthamoeba* myosin I. Dephosphorylated smooth muscle myosin moves very poorly, if at all; when phosphorylated on the 20,000-D light chain it moves at $\sim$0.4 μm/s (60). Phosphorylated *Acanthamoeba* myosin I moves at a slower rate (0.06 μm/s) and movement is again phosphorylation dependent (4).

We did not observe an effect of phosphorylation of the 18,000-D light chain of *Dictyostelium* myosin on thick filament assembly. The conditions used were identical to those used by Kuczmarski and Spudich (33) who found that phosphorylation of the heavy chain of *Dictyostelium* myosin in-

hibits thick filament assembly. There is an effect of light chain phosphorylation on myosin filament assembly in the cases of myosins from chicken gizzard (67) and from calf thymus or porcine platelets (57). In these cases, myosin thick filaments remain intact at physiological ionic strength upon addition of ATP only if their regulatory light chains are phosphorylated.

The question of whether *Dictyostelium* myosin heavy chain phosphorylation, which occurs on the tail of the molecule, exerts its effect on the actin-activated ATPase activity of the heads of the molecule directly by a conformational change or indirectly by an effect on filament assembly is an important one. The answer is not yet clear because in their study of *Dictyostelium* myosin heavy chain phosphorylation, Kuczmarski and Spudich (33) measured actin-activated Mg^{2+} ATPase activity under conditions in which the unphosphorylated myosin was polymerized but the phosphorylated myosin was only partly polymerized. An additional complication is that for myosin in general there are insufficient data to make firm conclusions about the effects of myosin filament formation on actin-activated ATPase activity. For example, Reisler (56) showed equivalence of kinetic properties of the actin-activated ATPase for short bipolar skeletal muscle myosin minifilaments as compared to the soluble myosin fragment HMM. In contrast, Kiehart and Pollard (28) found that a subset of monoclonal antibodies that bind to the tip of the tail of *Acanthamoeba* myosin II inhibit filament formation, ATPase activity, and actomyosin contraction in cytoplasmic extracts. In kinetic experiments preformed *Acanthamoeba* myosin II filaments were disassembled by antibody and actin-activated ATPase activity was lost concomitantly (29).

It is attractive to speculate that in *Dictyostelium* amebas changes in phosphorylation of myosin occur during motile events such as chemotaxis, leading to enhanced actin-activated Mg^{2+} ATPase activity and myosin mobility. Progress in correlating the state of *Dictyostelium* myosin phosphorylation with changes in cell shape associated with chemotaxis has recently been made by Berlot et al. (6). They were able to specifically immunoprecipitate myosin from chemotactically competent amebas that had previously been labeled with [^{32}P]orthophosphate and then stimulated with cAMP to induce cell shape changes. It was found that a transient increase in phosphorylation of both the heavy chain and the 18,000-D light chain of myosin occurs and that the time courses of phosphorylation correlate with that of cell shape change and chemotaxis.

In our experiments we have examined properties of myosin as a function of extent of light-chain phosphorylation, while keeping heavy-chain phosphorylation constant at ~0.3 mol P_i/mol heavy chain. It will be interesting to examine possible relationships between the heavy-chain phosphorylation and the light-chain phosphorylation in terms of effects on myosin function. For example, what would be the range of variation of motility and actin-activated Mg^{2+} ATPase activity of light-chain–dephosphorylated/heavy-chain–phosphorylated myosin as compared with light-chain–phosphorylated/heavy-chain–dephosphorylated myosin? Data so far show that light-chain phosphorylation enhances actin-activated Mg^{2+} ATPase five- to sixfold, and that heavy-chain dephosphorylation enhances it about two-fold. Perhaps the combined effects of light-chain phosphorylation and heavy-chain dephosphorylation are simply additive and one would expect therefore a 10–12-fold difference in actin-activated Mg^{2+} ATPase activity. Alternatively, some cooperativity may exist between light chain and heavy chain sites and a more complex effect on actin-activated ATPase activity may occur. Persechini and Hartshorne (51), for example, have presented evidence suggesting that light-chain phosphorylation of both heads of smooth muscle myosin is required for the actin-activated ATPase activity of either head, and that phosphorylation of the second head of the myosin molecule is negatively cooperative. *Dictyostelium* myosin may be more complicated because heavy-chain phosphorylation is a variable also.

Now that we have available the enzymes necessary to quantitatively phosphorylate and dephosphorylate both the light and heavy chains of *Dictyostelium* myosin, we would like to determine the properties of myosin that is selectively phosphorylated at one site or the other or on both sites. Thorough studies of actin-activated ATPase activity, assembly, and movement in vitro as a function of site specific phosphorylation should now be possible. We anticipate that such experiments will yield information about the regulation of myosin in general as well as of *Dictyostelium* myosin in particular.

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