Purification and Characterization of a Myosin I Heavy Chain Kinase from *Acanthamoeba castellanii*

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In previous work from this laboratory, a partially purified protein kinase from the soil amoeba *Acanthamoeba castellanii* was shown to phosphorylate the heavy chain of the two single-headed *Acanthamoeba* myosin isoenzymes, myosin IA and IB, resulting in a 10- to 20-fold increase in their actin-activated Mg$^{2+}$-ATPase activities (Maruta, H., and Korn, E. D. (1977) *J. Biol. Chem.* 252, 8329–8332). A myosin I heavy chain kinase has now been purified to near homogeneity from *Acanthamoeba* by chromatography on DE-52 cellulose, phosphocellulose, and Procion red dye, followed by chromatography on histone-Sepharose. Myosin I heavy chain kinase contains a single polypeptide of 107,000 Da by electrophoretic analysis. Molecular sieve chromatography yields a Stokes radius of 4.1 nm, consistent with a molecular weight of 107,000 for a native protein with a fractional ratio of approximately 1.3:1. The kinase catalyzes the incorporation of 0.9 to 1.0 mol of phosphate into the heavy chain of both myosins IA and IB. Phosphoserine has been shown to be the phosphorylated amino acid in myosin IB. The kinase has highest specific activity toward myosin IA and IB, about 3–4 amol of phosphate incorporated/min/mg (30°C) at concentrations of myosin I that are well below saturating levels. The kinase also phosphorylates histone 2A, isolated smooth muscle light chains, and, to a very small extent, casein, but has no activity toward phosvitin or myosin II, a third *Acanthamoeba* myosin isoenzyme with a very different structure from myosin IA and IB. Myosin I heavy chain kinase requires Mg$^{2+}$ but is not dependent on Ca$^{2+}$, Ca$^{2+}$/calmodulin, or cAMP for activity. The kinase undergoes an apparent autophosphorylation.

Three different myosin ATPases have been purified from *Acanthamoeba castellanii*, myosin II (1, 2), myosin IA (3, 4), and myosin IB (4). Myosin II consists of two heavy chains of 185,000 Da and two pairs of light chains of 17,500 and 17,000 Da arranged in a double-headed molecule (1, 2). By virtue of its size and subunit composition, its distinct head and tail domains (2), and its propensity for forming filaments at low ionic strength and upon addition of Mg$^{2+}$ (5, 6), myosin II is structurally similar to other characterized muscle and non-muscle myosins. By contrast, myosin IA is an unconventional single-headed enzyme with a native molecular weight of about 150,000 Da, comprised of one 130,000-Da heavy chain and a light chain of 17,000 Da (3, 4). Myosin IB is also a single-headed enzyme with a native molecular weight of about 150,000, but contains one 125,000-Da heavy chain and a light chain of 25,000 Da (4). Both myosin I isoenzymes are obtained with variable amounts (always less than 0.5 mol/mol by Coomassie blue stain) of a peptide of 14,000 Da (4). Myosin IA and IB are both globular molecules (3, 4) with no detectable tail region and no known ability to self-associate. Peptide mapping (7) and immunological analysis (8) support the conclusions that myosins IA, IB, and II are separate gene products and that the myosin I isoenzymes as isolated are identical with the native molecules in the cell.

For all three *Acanthamoeba* myosins, the magnitude of their actin-activated Mg$^{2+}$-ATPase activity is governed by the level of heavy chain phosphorylation (4, 10–12). Myosin II possesses three heavy chain phosphorylation sites, all of which have been localized to a 9000-Da chromotryptic peptide isolated from near the COOH terminus of the molecule (13, 14). The actin-activated Mg$^{2+}$-ATPase activity of myosin II is inversely correlated with the phosphorylation state of the enzyme, i.e. the fully dephosphorylated enzyme has the highest actomyosin II Mg$^{2+}$-ATPase activity (10, 13). In parallel with the effect of phosphorylation on ATPase activity, dephosphorylated myosin II associates more readily into filaments than the phosphorylated molecule (6). A myosin II heavy chain kinase which phosphorylates all three sites has been partially purified (13) and a protein phosphatase active toward myosin II has been highly purified (15).

For myosins IA and IB, heavy chain phosphorylation regulates the actin-activated Mg$^{2+}$-ATPase activity in a manner opposite to that observed for myosin II, i.e. fully phosphorylated myosin I has the highest actomyosin I Mg$^{2+}$-ATPase activity (4, 12, 16). Maruta and Korn (16) showed that a partially purified cofactor, found by Pollard and Korn (17) to be required for actin activation of myosin I Mg$^{2+}$-ATPase, is a specific myosin I heavy chain kinase and that the magnitude of actomyosin I Mg$^{2+}$-ATPase activity is directly proportional to the extent of myosin I heavy chain phosphorylation (12). The myosin I kinase obtained by Maruta and Korn was quite impure, containing at least 12 prominent bands on electrophoresis gels (16) and being contaminated with proteases (12). The purpose of the present work was to purify myosin I heavy chain kinase to homogeneity in order to allow its characterization and also to facilitate physical and kinetic studies of the mechanism by which phosphorylation of the heavy chain of myosin I affects its enzymatic activity. In this paper we describe the purification to near homogeneity of a myosin I heavy chain kinase and the initial characterization of this kinase.

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1 Albanesi, J. P., Hammer, J. A., III, and Korn, E. D., to be submitted for publication.
**EXPERIMENTAL PROCEDURES**

**Materials**—DE-52 cellulose and P11 phosphocellulose were obtained from Whatman, Bio-Gel A-0.5m (200-400 mesh), Bio-Gel P-100 (50–100 mesh), and the dye reagent used for the Bradford assay were purchased from Bio-Rad. Procion red dye chromatography beads, obtained from Amicon Corp., were washed with 8 M urea (2 column volumes) and equilibration buffer (8 column volumes) before use. Histone-Sepharose was prepared using histone 2A (Sigma H-9250) and CNBr-activated Sepharose 4B (Pharmacia) by the procedure described in the Affinity Chromatography Manual from Pharmacia. Coupling was performed at 4°C for 24 h using 7.5 mg of histone 2A per ml of swollen packed gel (>98% of the protein was coupled). The remaining active groups were blocked by a 2-h incubation at 21°C with 1 M ethanolamine (pH 8.0). The following proteins were obtained asgifts: *Acanthamoeba myosin II*, purified by the method of Collins and Korn (11) and partially purified *Acanthamoeba myosin II* heavy chain kinase (13) (Dr. Graham Cote, *National Institutes of Health*); rabbit skeletal muscle F-actin, purified according to Eisenberg and Kielley (19) (Dr. Lois Greene, *National Institutes of Health*); histone-Sepharose was prepared using histone 2A (Sigma H-89205) and CNBr-activated Sepharose 4B (Pharmacia) by the procedure described in the Affinity Chromatography Manual from Pharmacia. Dialyzed overnight at 4°C for 24 h using 7.5 mg of histone 2A per ml of swollen packed gel (>98% of the protein was coupled). The remaining active groups were blocked by a 2-h incubation at 21°C with 1 M ethanolamine (pH 8.0). The following proteins were obtained as gifts: *Acanthamoeba myosin II*, purified by the method of Collins and Korn (11) and partially purified *Acanthamoeba myosin II* heavy chain kinase (13) (Dr. Graham Cote, *National Institutes of Health*); rabbit skeletal muscle F-actin, purified according to Eisenberg and Kielley (19) (Dr. Lois Greene, *National Institutes of Health*); bovine brain calmodulin (Dr. Claude Klein, *National Institutes of Health*); Casein (C-8765), phosphoenol, the inhibitor of cAMP-dependent protein kinase (P-8140), ATP, cAMP, phosphoserine, phosphothreonine, TES, imidazole (grade III), leupeptin, pepstatin, PMSF, EGTA, and EDTA were obtained from Sigma. [γ-32P]ATP was purchased from New England Nuclear. All other chemicals were reagent grade.

**Purification of Myosin I Isoenzymes**—Myosin IA and IB were purified as described in the accompanying paper (18). Myosin IB was routinely >90% pure; the purity of myosin IA used in these experiments varied between 60–90% and was used less frequently. In all preparations, the purified myosin isoenzymes were shown to be devoid of kinase activity (see below). A small amount of myosin I heavy chain kinase still associated with myosin IA and IB after DE-52 chromatography was removed from the myosins by chromatography on ADP-agarose as described previously (4). Myosin IA and IB were stored in 20 mM imidazole (pH 7.5), 100 mM KCl, 25–40% glycerol, 1 mM dithiothreitol, and 0.02% sodium azide at 4°C. Myosins IA and IB were stable in terms of enzymatic activity and SDS-PAGE profiles for between 1 and 2 weeks; routinely, the myosins were used within less than 1 week. Myosin I isoenzymes as isolated contain negligible phosphate.

**Purification of Myosin I Heavy Chain Kinase**—Approximately 1 kg of *Acanthamoeba castellanii* was grown, harvested, and purified as described by Pollard and Korn (5). The cell pellet was disrupted (% strokes in a tight-fitting glass Dounce homogenizer) in 2 volumes of 30 mM imidazole (pH 7.0), 75 mM KCl, 12 mM sodium pyrophosphate, 5 mM dithiothreitol, 0.1% leupeptin, 1% pepstatin, and 0.6 mM PMSF and the homogenate centrifuged at 100,000 × g for 3 h (Beckman type 50 rotor). All procedures were performed at 4°C. The supernatant (about 2 liters) was titrated to pH 8.0 with 1 M Tris, dialyzed for 12 h against 28 liters of buffer containing 10 mM Tris (pH 8.0), 7.5 mM sodium pyrophosphate, 1 mM dithiothreitol, and 0.6 mM PMSF, and centrifuged at 40,000 × g for 20 min. The supernatant was loaded onto a DE-52 column (5 × 80 cm) equilibrated with 20 mM Tris (pH 8.0), 10 mM KCl, and 1 mM dithiothreitol. The material collected during loading plus 1 liters of column wash was used for purification of myosin I heavy chain kinase while the myosin I isoenzymes were eluted from the column as described in the accompanying paper (18).

**Phosphocellulose Chromatography**—Solid ammonium sulfate (to 2 M) was added to the material which did not adsorb to DE-52, and the precipitate was collected and resuspended in 150 ml of 20 mM TES (pH 7.0), 50 mM KCl, 5% glycerol, 1 mM dithiothreitol, 0.1% leupeptin, 1% pepstatin, and 0.6 mM PMSF, and dialyzed overnight against 2 liters of the same buffer. This material was applied to a phosphocellulose P-11 column (5 × 20 cm) equilibrated with 20 mM TES (pH 7.0), 25 mM KCl, and 1 mM dithiothreitol. The column was washed with the equilibration buffer, eluted with a linear KCl gradient (12 liters, 25 to 600 mM KCl), and fractions were assayed for protein and myosin I kinase activity (see below and Fig. 1).

**Procion Red Dyed Chromatography**—The phosphocellulose peak eluting at 0.12 M KCl was dialyzed overnight against 2 liters of 29 mM imidazole (pH 7.2), 100 mM KCl, and 1 mM dithiothreitol, loaded onto a Procion red dye column (1.5 × 20 cm) equilibrated with the same buffer, washed, and eluted with a linear KCl gradient (156 ml, 100–1300 mM KCl) (Fig. 2).

**Histone-Sepharose Chromatography**—The Procion red dye peak eluting at 0.9 M KCl was dialyzed overnight against 3 liters of 20 mM imidazole (pH 7.0), 75 mM KCl, 5% glycerol, and 1 mM dithiothreitol and applied to a histone-Sepharose column (1 × 15 cm) equilibrated with the same buffer (without glycerol). The column was washed with 3 column volumes of equilibration buffer and eluted with 100 ml of a linear 9 to 40 mM MgCl2-ATP gradient (no KCl present) (Fig. 3). The myosin I kinase peak was dialyzed twice against 500 ml of 20 mM imidazole (pH 7.5), 75 mM KCl, 50% glycerol, 1 mM dithiothreitol, and 0.02% sodium azide and stored at −20°C.

**Assay of Actin-activated Mg2+-ATPase of Myosin I**—Myosin I heavy chain kinase was assayed indirectly during its purification by its ability to increase the actin-activated Mg2+-ATPase of myosin I. The Asse assay mixture contained 15 mM imidazole (pH 7.5), 2 mM MgCl2, 1 mM EGTA, and 2 mM [γ-32P]ATP (0.5 μCi/μmol) and the following additions: 5–10 μg of myosin IA or IB, 50 μg of skeletal muscle F-actin, and 1–10 μl of the column fraction to be assayed in a final volume 0.5 ml. ATPase activity was measured by the release of 32P from the [γ-32P]ATP as described by Pollard and Korn (3) following 5–10 min of incubation at 30°C. Both purified myosin I kinase and the impure kinase fractions and negligible Mg2+-ATPase activity. The volume of the column fractions assayed was adjusted so that the most active fraction always gave less than the maximum possible activation of actomyosin ATPase activity. While not strictly quantitative, this assay was rapid and convenient and provided sufficient indication of the relative distribution of kinase activity to allow purification. Myosin I was never available in sufficient quantity to allow assay of the kinase under conditions of excess substrate.

**Measurement of Myosin I Heavy Chain Kinase Activity**—In all experiments with purified myosin I kinase, kinase activity was determined directly from a time course of the initial rate of substrate phosphorylation. Kinase assays were performed at 30°C in reaction mixtures containing 20 mM imidazole (pH 7.5), 50 mM KCl, 4 mM MgCl2, 1 mM [γ-32P]ATP, and 0.4 mM dithiothreitol in a total volume of 200 μl. The concentration of myosin I kinase, the concentration added as substrate, the specific activity of [γ-32P]ATP and variations in the buffer conditions are given in the figure legends.

Assays were initiated by addition of substrate and then kinase to

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**FIG. 1. Purification of myosin I heavy chain kinase on phosphocellulose.** The DE-52 flow-through fraction was chromatographed on phosphocellulose P-11, and aliquots were assayed for kinase activity (2.5 μl) and protein concentration as described under "Experimental Procedures."
Experimental Procedures.

The phosphocellulose peak eluted at 0.12 M KC1 was applied to the column and aliquots of each fraction were assayed for protein concentration and for myosin I kinase activity (1 µl) as described under “Experimental Procedures.”

A reaction mixture pre-equilibrated to 30 °C. Aliquots of 40 µl were removed at intervals of 20 or 45 s, spotted onto 2.3-cm filter paper discs (Whatman, grade 3MM) which were then dipped into 10% trichloroacetic acid containing 5% sodium pyrophosphate to terminate the reaction, and immediately washed on a suction manifold with 15 ml of 10% trichloroacetic acid. At the end of the incubation, all the filter paper discs were washed four times for 20 min with gentle agitation in 200 ml of 10% trichloroacetic acid plus 5% sodium pyrophosphate, washed once for 5 min with absolute ethanol, once for 5 min with absolute ether, air dried, and counted in 15 ml of Aquasol (New England Nuclear) in a Beckman model 250 scintillation counter.

The amount of myosin I heavy chain kinase added was such that less than 15% of the total substrate was phosphorylated. Under these conditions, the incorporation of 32P into substrate was linear with time and proportional to the amount of kinase added. Kinase activity (micromoles of phosphate incorporated/min) was calculated by linear regression analysis of the phosphorylation time course. Control reactions containing only substrate or only kinase showed negligible phosphorylation. Autophosphorylation of myosin I kinase did not contribute significantly to the measured values. Finally, autoradiography of SDS-polyacrylamide gels of myosin I phosphorylated under these conditions showed that all of the 32P was incorporated into the intact myosin I heavy chain.

Stoichiometry of Phosphate Incorporation into Myosin I—The maximal extent of phosphorylation of myosins IA and IB by myosin I heavy chain kinase was determined by the filter paper assay essentially as described above but with higher concentrations of kinase. In calculating the phosphate content of myosin I, corrections were made for 32P incorporated into proteins other than myosin I. This correction was based on densitometric scans of autoradiograms of SDS-polyacrylamide gels of maximally phosphorylated myosin I, where the radioactivity in bands other than the intact myosin I heavy chain was less than 15% of the total protein-bound 32P. The amount of myosin I per assay was estimated from the protein concentration corrected for the percentage of Coomassie blue stain in SDS-polyacrylamide gels not present in the intact myosin I heavy chain (usually not more than 10%).

Estimation of the Stokes Radius of Myosin I Heavy Chain Kinase—Purified myosin I kinase (40 µg) and three standards of known Stokes radius, ovalbumin (Rc = 2.85 nm), bovine serum albumin (Rc = 3.5 nm), and aldolase (Rc = 4.5 nm), were fractionated by gel filtration on a column (1 x 48 cm) of Bio-Gel A-5.5 (200–400 mesh) equilibrated with 20 mM imidazole (pH 7.4), 100 mM KCl, 20% glycerol, 1.5 mM dithiothreitol, and 0.02% sodium azide. Myosin I kinase activity was detected by its ability to activate the actin-activated Mg2+-ATPase activity of myosin I. For estimation of the Stokes radius of myosin I kinase, the data were plotted by the method of Ackers (20). The three standards gave a straight line with a correlation coefficient of 0.994.

Phosphoamino Acid Analysis—Following maximal phosphorylation of myosin IB (100 µg) by myosin I heavy chain kinase in the presence of [γ-32P]ATP, myosin was separated from ATP by gel filtration column (1 x 40 cm) equilibrated with 0.1 M ammonium bicarbonate (pH 7.5). The radioactive myosin peak in the void volume was pooled, lyophilized, and subjected to partial acid hydrolysis in 6 N HCl at 105 °C for 30, 60, 90, and 180 min. The hydrolysates were repeatedly lyophilized to remove HCl, mixed with authentic phosphoserine and phosphothreonine standards, and analyzed by electrophoresis at pH 1.9 on cellulose thin layer sheets exactly as described by Cote et al. (13).

Miscellaneous Methods—Protein concentrations were determined using the colorimetric assay of Bradford (21), with bovine serum albumin as a standard. The values determined for purified myosin I using the Bradford assay were approximately 20–25% lower than values determined using the protein assay of Lowry et al. (22) with bovine serum albumin as a standard. KC1 concentrations in column eluates were estimated by conductivity measurements. Discontinuous SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (23), followed by Coomassie blue staining according to Fairbanks et al. (24). Gels were destained in 10% acetic acid. For autoradiography of 32P-labeled proteins and phosphoamino acids, dried gels and thin layer sheets were exposed to x-ray film (Kodak X-Omat AR-2) beneath an intensifying screen (Dupont, Cronex Lightning Plus). Densitometric scans of autoradiograms and Coomassie blue-stained gels were performed at 600 nm using a Helena Laboratories Quick-Scan gel scanner. Care was taken to scan samples within the predetermined linear response range of the instrument.

RESULTS

Purification of Myosin I Heavy Chain Kinase—Myosin I heavy chain kinase was detected during its purification by its ability to activate the myosin I Mg2+-ATPase in the presence of actin. This assay provided a means of detecting specifically protein kinases that affect a physiologically significant property of myosin I, i.e. activation of its ATPase. Chromatography on DE-52 was the first step in the separation of myosin I kinase from myosin IA and IB, which elute together at 0.1 M KC1 (4). As shown previously by Maruta and Korn (16), the myosin peak contains some kinase activity. We found, however, that about 85% of the myosin activity recovered from the column was in the material which did not adsorb to the DE-52 resin. Therefore, the DE-52 flow-through fraction was used for subsequent kinase purification.

Phosphocellulose chromatography of the DE-52 fraction yielded two peaks of activity, a large peak (PC1) at 0.12 M KC1 and a smaller peak (PC2) at 0.36 M KC1 (Fig. 1). PC1 usually contained 60–70% of the total activity eluted, was well
separated from the bulk of the eluted protein, and was used for further myosin I kinase purification. While the activity in PC2 was not routinely purified, in one experiment both PC1 and PC2 were further purified by chromatography on histone-Sepharose. These partially purified kinases were found to incorporate maximally 0.8 mol of phosphate per mol of myosin I B in vitro and when added together (data not shown), suggesting that the two fractions phosphorylated the same site in myosin IB.

The phosphocellulose peak (PC1) was further purified by Procion red dye chromatography, which yielded a major peak of activity at 0.9 M KCl, well separated from the bulk of the eluted protein (Fig. 2). In addition to activating the actin-activated Mg\(^{2+}\)-ATPase activity of myosin I, this fraction was found to phosphorylate histone 2A with a high specific activity (see Table 1). Consequently, histone-Sepharose was used as a purification step.

All the myosin I kinase activity bound to the histone-Sepharose column and was eluted as a single peak with a gradient of Mg\(^{2+}\)-ATP (Fig. 3). The specific activity of myosin I kinase was essentially constant in fractions across the peak, indicating an essentially homogenous protein preparation (data not shown). SDS-PAGE of the pooled kinase peak revealed more than 94% of the Coomassie blue stain in a 107,000-Da band (average of four separate determinations) (Fig. 4, lane 5).

Molecular sieve chromatography of myosin I kinase on Bio-Gel A-0.5m yielded a single symmetrical peak of kinase activity which eluted with the 107,000-Da band (Fig. 4, lane 6). A Stokes radius of 4.1 nm was estimated, consistent with a native molecular weight of 107,000 for a protein with a frictional ratio of approximately 1.3:1. Furthermore, as is typical of many protein kinases (25), the 107,000-Da polypeptide underwent an apparent autophosphorylation when incubated with Mg\(^{2+}\) and \([\gamma-\text{P}]\text{ATP}\) (Fig. 4, lanes 7 and 8). These results, along with the high specific activity of myosin I kinase (see below), all indicate that the histone-Sepharose peak is a highly purified protein kinase of \(M_c = 107,000\) which exists in solution as a roughly globular monomer and which phosphorylates both myosin I and histone 2A.

In a typical preparation, 0.2 to 0.5 mg of myosin I heavy chain kinase was obtained from 1000 g of cells (50 g of total protein and 25 g of protein in the 100,000 \(\times g\) extract). It was difficult to obtain accurate quantitative measurements of kinase specific activity and yield at each step of the purification procedure because the purified myosin I substrate, which was always from a previous preparation, was not stable over the course of the kinase isolation (see under “Discussion”). The SDS-polyacrylamide gel in Fig. 4 indicates the approximate degree of purification of the 107,000-Da band at each chromatographic step (Fig. 4, lanes 2-5).

Myosin I heavy chain kinase was stable in terms of enzymatic activity and SDS-PAGE profile for at least 3 months when stored in 50% glycerol at \(-20^\circ\text{C}\) and lost less than 20% of its activity after 6 months of storage. In some fully active preparations, however, instead of the 107,000-Da band, SDS-PAGE showed two equimolar bands of approximately 50,000 Da and 60,000 Da (Fig. 4, lane 9). This material eluted at the same position on gel filtration (data not shown) as the 107,000-Da kinase. It is likely, therefore, that in these preparations the 107,000-Da kinase had been proteolytically cleaved to two peptides that stayed together under non-denaturing conditions.

Purified myosin I kinase was not contaminated by detectable levels of either a protease or a protein phosphatase. When myosins IA and IB were maximally phosphorylated by purified kinase and the proteins separated from ATP on Sephadex G-25 and incubated for 2 h at \(30^\circ\text{C}\), there was no detectable loss of myosin I heavy chain phosphate. Furthermore, incubation of the purified kinases with myosin I kinase (1:50 ratio of kinase to myosin, w/w) for 30 min at \(30^\circ\text{C}\) followed by SDS-PAGE revealed no proteolysis of the myosin I heavy chain. This latter observation is important because Maruta and Korn (12) previously showed that the actin-activated Mg\(^{2+}\)-ATPase of myosin I can be activated by proteolysis of the heavy chain, as well as by phosphorylation.

Optimal Conditions for Assay of Myosin I Heavy Chain Kinase—Because of the difficulty in obtaining pure myosin I isoenzymes in substrate concentrations, histone 2A, which is a good substrate (see below), was used in many of the experiments to determine optimal assay conditions. The kinase was relatively insensitive to alterations in pH, exhibiting a broad pH optimum centered about pH 7.5 (Fig. 5A). The activity of myosin I kinase toward myosin IB was inhibited by KCl concentrations greater than 75 mM and was 50% inhibited at 140 mM KCl, relative to the rate at 46 mM KCl (Fig. 5B). The sensitivity to increasing ionic strength was affected by the concentration of myosin I used in the assay. When the myosin I concentration was reduced from 2.4 to 1 \(\mu\text{M}\), myosin I kinase activity was significantly inhibited above 55 mM KCl and was 50% inhibited at about 90 mM KCl (data not shown). Therefore, for all experiments involving phosphorylation of myosin I, the final KCl concentration of the reaction was kept between 40–50 mM. The myosin I isoenzymes have been shown to exist as soluble monomers at KCl concentrations as low as 20 mM (18). Phosphorylation of histone 2A (67 \(\mu\text{M}\)) was not as sensitive to ionic strength, being significantly inhibited only above 310 mM KCl (data not shown).

The activity of myosin I kinase toward histone 2A was dependent on Mg\(^{2+}\) (Fig. 5C), being completely inactive in its absence, and appeared to require low concentrations of free Mg\(^{2+}\) for optimal activity, as shown by the increase in activity when total Mg\(^{2+}\) was in excess of ATP. The kinase did not, however, demonstrate a pronounced optimum concentration
for free Mg$^{2+}$, as has been observed for cyclic nucleotide-dependent kinases (26). Similarly, the phosphorylation rate with myosin IB as substrate was essentially constant from 1 to 9 mM free Mg$^{2+}$ (Fig. 5C). The rate of phosphorylation of myosin IB (2 µM) varied by less than 10% between incubations containing 0.1 mM EGTA (4.36 µmol/min-mg), 20 µM free Ca$^{2+}$ (4.68 µmol/min-mg), 20 µM free Ca$^{2+}$ plus 2 µM calmodulin (4.28 µmol/min-mg), 50 µM cAMP (4.11 µmol/min-mg), and 20 µg/ml of cAMP-dependent protein kinase inhibitor (4.70 µmol/min-mg).

The dependence of the rate of phosphorylation of histone 2A by myosin I heavy chain kinase on the concentration of ATP was determined over a concentration range of 5 µM to 2 mM ATP (Fig. 6). The results obeyed classical Michaelis-Menten kinetics and when plotted by Lineweaver-Burk analysis yielded a $K_a$ for ATP of 43 µM (Fig. 6, inset).

**Phosphorylation of Myosin I Isoenzymes by Myosin I Heavy Chain Kinase**—To determine the site of phosphorylation and the stoichiometry of phosphate incorporation, myosin IA and IB were phosphorylated to a maximum extent by purified myosin I kinase. Both myosin IA and IB showed a maximum of 0.9–1.0 mol of phosphate per mol of myosin (Fig. 7). Autoradiography of SDS-polyacrylamide gels of maximally phosphorylated myosin IB showed all the $^{32}$P incorporated into the 125,000-Da heavy chain; no detectable phosphorylation of the 25,000-Da light chain was observed (Fig. 4, lanes 10–13). Similarly, no $^{32}$P was incorporated into the 17,000-Da light chain of maximally phosphorylated myosin IA (Fig. 4, lanes 14 and 15). Phosphoamino acid analysis of maximally phosphorylated myosin IB revealed a single phosphorylated amino acid, phosphoserine (Fig. 7). These results indicate that highly purified myosin I kinase phosphorylates both myosin IA and IB at a single site, that this site is within the heavy chain, and that, at least in myosin IB, this site is a serine residue. As shown previously (4, 16) and in more detail in an accompanying paper (18), only the phosphorylated
myosin I isoenzymes show actin-activated Mg$^{2+}$-ATPase activity, with the fully phosphorylated enzymes exhibiting an approximately 20-fold higher actomyosin I Mg$^{2+}$-ATPase than the unphosphorylated enzymes. Phosphorylation of myosin I does not effect the (K$^+$,EDTA)-ATPase, Ca$^{2+}$-ATPase, or Mg$^{2+}$-ATPase (in the absence of actin) activities of either myosin IA or IB (4, 16).

To determine the dependence of the rate of phosphorylation of myosin IB on the concentration of myosin, myosin I kinase

![Phosphorylation of Myosin IB](Image)

**FIG. 5. The effects of pH, KCl, and Mg$^{2+}$ on myosin I heavy chain kinase.** A, the specific activity of myosin I kinase with histone 2A as substrate was determined in incubations containing 67 μM histone 2A, 1 mM myosin I kinase, 50 mM KCl, 4 mM MgCl$_2$, and 1 mM [γ-$^32$P]ATP (50 μCi/μmol). Buffers used were 50 mM MOPS (pH 7.5), 50 mM imidazole (pH 7.5), or 50 mM Tris (pH 7.5). B, the initial rate of phosphorylation of myosin IB was determined in incubations containing 2.4 μM myosin IB, 1.1 mM myosin I kinase, 10 mM imidazole (pH 7.5), 10% glycerol, 1.5 mM MgCl$_2$, 0.5 mM dithiothreitol, and KCl ranging from 40 to 190 mM. C, histone 2A phosphorylation (p---p); magnesium acetate was varied from 0 to 30 mM in incubations containing 67 μM histone 2A, 1 mM myosin I kinase, 20 mM imidazole (pH 7.5), and 1 mM [γ-$^32$P]ATP (50 μCi/μmol). The ionic strength was held constant by reducing the concentration of KCl (100 mM in 0 mM Mg$^{2+}$ acetate). The open square indicates the addition of 1 mM EDTA. Myosin IB phosphorylation (p---p); MgCl$_2$ was varied from 2 to 11 mM in incubations containing 1.14 μM myosin IB, 0.2 mM myosin I kinase, 20 mM imidazole (pH 7.5), 10% glycerol, 0.5 mM dithiothreitol, and 1 mM [γ-$^32$P]ATP (80 μCi/μmol). The ionic strength was held constant by reducing the KCl concentration (75 mM KCl in 2 mM MgCl$_2$).

![Stoichiometry of Phosphate Incorporation](Image)

**FIG. 6. Determination of the $K_m$ of myosin I heavy chain kinase for ATP.** The dependence of the rate of phosphorylation of histone 2A on the concentration of ATP was measured in incubations containing 134 μM histone 2A, 1.9 mM myosin I kinase, 20 mM imidazole (pH 7.5), 100 mM KCl, 4 mM MgCl$_2$, and [γ-$^32$P]ATP (80 μCi/μmol) ranging from 5 μM to 2 mM. The open and closed circles are replicate experiments on separate days. Inset, the Lineweaver-Burk representation of the data.

![Dependence of Phosphorylation on Myosin IB](Image)

**FIG. 8. Dependence of the rate of phosphorylation on the concentration of myosin IB.** The initial rate of phosphorylation of myosin IB was measured in incubations containing myosin IB (0.3 to 2.4 μM), myosin I heavy chain kinase (0.4 nM), 10 mM imidazole (pH 7.5), 40 mM KCl, 10% glycerol, 1.5 mM MgCl$_2$, 0.5 mM dithiothreitol, and 0.75 mM [γ-$^32$P]ATP (75 μCi/μmol). Assays were performed with myosin IB varied from 0.3 to 2.4 μM (Fig. 8). We were limited to this narrow range because the highest concentration of purified myosin IB we obtained was only 0.75 mg/ml. The initial rate of phosphorylation of myosin IB over this concentration range increased in almost direct
proportion to the increase in myosin concentration, although the slope of the line appears to begin to fall off above 1.5 μM myosin IB. The data at the very low myosin I concentrations (<1 μM) suggest something other than simple hyperbolic kinetics. Replicate measurements of phosphorylation rates at very low myosin I concentrations showed considerable variability, and it may be that the type of assay performed underestimates the phosphorylation rate when the protein concentration in the assay is very low. Nevertheless, the results in Fig. 8 indicate that at the concentrations of myosin I used in most experiments (1–2 μM), the measured rate is well below the Vmax of myosin I heavy chain kinase for myosin I.

Substrate Specificity of Myosin I Heavy Chain Kinase—The activity of purified myosin I kinase toward myosin IA and IB was compared with its activity toward the general phosphoaccepting proteins, histone 2A, casein, and phosphorivin, and toward Acanthamoeba myosin II (Table II). As stated above, we were limited in the concentration of myosin I we could use. Nevertheless, even at low myosin IB concentrations (1–2 μM), the specific activity was on the order of 2–4 μmol/min·mg. Myosin I kinase phosphorylated the myosin IA isoenzyme at essentially the same rate as myosin IB. Myosin I kinase also phosphorylated histone 2A at a high rate, but phosphorylated casein at a very low rate, and phosphorivin not at all. Absolutely no phosphorylation of Acanthamoeba myosin II by myosin I kinase was observed, even at very high myosin I kinase concentrations (40 nM). In separate experiments, no phosphorylation of myosin IA or IB by a partially purified Acanthamoeba myosin II heavy chain kinase could be detected, either by filter paper assay or by autoradiography (data not shown). Therefore, Acanthamoeba contains at least two myosin heavy chain kinases, one of which is specific for the myosin I isoenzymes and the other specific for myosin II. Interestingly, myosin I heavy chain kinase also phosphorylated isolated light chains from turkey gizzard smooth muscle myosin at a significant rate.

DISCUSSION

After the discovery that heavy chain phosphorylation regulates the actin-activated Mg2+-ATPase activity of Acanthamoeba myosin I (4, 12, 16), heavy chain phosphorylation was also found to regulate Acanthamoeba myosin I (10, 11, 13, 14), Dictyostelium myosin (28, 29) (in both cases phosphorylation inhibits the actin-activatable ATPase activity), and Physarum myosin (30) (where phosphorylation activates the actin-activatable ATPase activity). Heavy chain phosphorylation also occurs in liver macrophage (31), lymphocyte (32), and brain (33) myosins, although the stoichiometry and consequences of these phosphorylations are unknown. The purification and characterization of myosin heavy chain kinases and the mechanism(s) by which heavy chain phosphorylation regulates myosin activity is, therefore, of general importance.

This paper is the first report of purification to homogeneity of any myosin heavy chain kinase. That the enzyme is highly purified is strongly supported by SDS-PAGE, by the high specific activity of the purified kinase, and by the coincidence of the protein and activity peaks on gel chromatography. Myosin I heavy chain kinase is an approximately globular protein containing one polypeptide of Mr = 107,000. The kinase phosphorylates both myosin IA and IB at a high rate at one site within the heavy chain, which in the case of myosin IB is a serine residue. Although we have never had sufficient myosin I to measure it, the Vmax of myosin I kinase at excess substrate would almost certainly exceed 10 μmol/min·mg making this heavy chain kinase as active as the very active myosin light chain kinases purified from smooth (34), cardiac (35), and skeletal (36) muscles.

It was difficult to determine the yield and degree of purification of myosin I heavy chain kinase at each chromatographic step because of the low yield and lack of stability of the purified myosin I substrate. Several quantitative measurements of kinase activity in the DE-52 flow-through fraction (1 g of protein) were made, however, using purified myosin IB (0.7 μM) as substrate and determining the initial rate of phosphorylation by the filter paper assay. Myosin I kinase in these DE-52 fractions had a specific activity of about 2.5 nmol/min·mg. Based on the specific activity of purified kinase at the same substrate concentration (about 750 nmol/min·mg; see Fig. 8), the combination of phosphocellulose, red dye, and histone-Septarose chromatography provided about a 300-fold purification of kinase activity starting with the DE-52 eluate. It was not possible to measure the activity of myosin I kinase in the crude extract because of the contaminating myosin I. If, however, one arbitrarily assumes an 80% recovery of activity applied to the DE-52 column of which 85% was in the nonadsorbed flow-through fraction, then the DE-52 step would provide an additional 20-fold purification. With this assumption, a final yield of 0.5 mg of purified kinase would represent approximately an 11% yield of myosin I kinase from the extract with a 6000-fold purification. On this basis, 1 kg of cells would contain about 5 mg of myosin I kinase to about 150 mg of myosin IA and 100 mg of myosin IB (4, 18). Therefore, there would be sufficient kinase in the cell to phosphorylate all of the myosin I within about 5–10 s, assuming a rate of reaction equal to that in the assay in vitro and using the assumed recovery for the DE-52 chromatographic step.

Myosin I heavy chain kinase phosphorylates smooth muscle myosin light chains and we have recently found that the kinase also phosphorylates intact smooth muscle myosin and smooth muscle heavy meromyosin at high rates (at the same site as does smooth muscle myosin light chain kinase) and fully activates the actin-activated Mg2+-ATPase activity of heavy meromyosin (37). Smooth muscle light chain kinase does not phosphorylate Acanthamoeba myosin I (37). We have been unable to compare myosin I heavy chain kinase carefully to Acanthamoeba myosin I heavy chain kinase because of the poor yield and instability of the myosin II kinase. But myosin I kinase has no activity toward myosin II and partially purified myosin I heavy chain kinase has no activity toward myosin I.

We cannot be certain that the purified myosin I heavy chain kinase described in this paper is the only protein kinase in Acanthamoeba able to phosphorylate myosin I heavy chain and regulate its activity. The myosin I heavy chain kinase described by Maruta and Korn (16) was partially purified from the DE-52 fraction that contained myosin I and which we find accounts for about 15% of the total myosin I kinase activity recovered from the column. The two most prominent bands in SDS-polyacrylamide gels of this partially purified kinase were 95,000 and 58,000 Da (16). Also, as mentioned under "Results," we found a second kinase fraction, in addition to the one we purified, eluting from phosphocellulose. These other kinase fractions may contain different enzymes or, as we think more likely, they may be modified forms of the enzyme that has been purified. Myosin I heavy chain kinase is probably identical with the cofactor protein partially purified by Pollard and Korn (17) which was thought to have a molecular weight of about 100,000.

The mechanism by which myosin I heavy chain kinase is regulated in situ is not known. The isolated enzyme is not affected by Ca2+, Ca2+/calmodulin, or cAMP. In contrast, the
activities of myosin light chain kinases purified from muscle (34, 35, 38) and nonmuscle sources (39–41) are absolutely dependent on Ca influx/calcium. However, several of these myosin light chain kinases have been isolated as proteolytic products of the native enzymes that possess full activity in the absence of Ca (42–44). By analogy, the purified myosin I kinase we isolated might have been similarly deregulated by proteolysis but we have no evidence to suggest this. In preliminary experiments, we have found that at least 0.4 mol of phosphate can be incorporated per mol of myosin I kinase, probably by autophosphorylation. We do not know if this phosphorylation affects myosin I kinase activity. Both cAMP-dependent protein kinase (26) and cGMP-dependent protein kinase (26) undergo autophosphorylation and certain properties of both enzymes are altered by autophosphorylation.

The availability of highly purified myosin I heavy chain kinase with high specific activity has allowed us to study the effects of phosphorylation of the heavy chains of myosin IA and IB on their interaction with F-actin. Our initial studies are reported in the accompanying paper (18).

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