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 Pronase 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 frictional 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 nmol 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 phosphitin 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 immunochromatographic analysis (8, 9) support the conclusions that myosins IA, IB, and II are separate gene products and that the myosin II 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 chymotryptic peptide isolated from 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

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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 swelled packed gel (>88% 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); purified turkey gizzard smooth muscle light chains (Dr. James Sellers, National Institutes of Health); and bovine brain calmodulin (Dr. Claude Klee, National Institutes of Health). Casein (C-7659), phosvitin, the inhibitor of cAMP-dependent protein kinase (P-8140), ATP, cAMP, phosophoamino, phosphothreonine, TES, imidazole (grade II), pepstatin, leupeptin, 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-gelose 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. Myosin 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 nuclease.

Purification of Myosin I Heavy Chain Kinase, DE-52 Chromatography—Approximately 1 kg of Acanthamoeba castellanii was grown, named as described as described by Pollard and Korn (5). The cell pellet was disrupted (10 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 x 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 x g for 20 min. The supernatant was loaded onto a DE-52 column (5 x 80 cm) equilibrated with 10 mM Tris (pH 8.0), 10 mM KCl, and 1 mM dithiothreitol. The material collected during loading plus 1.5 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 x 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 (1.2 liters, 25 to 600 mM KCl), and fractions were assayed for protein and myosin I kinase activity (see below and Fig. 1).

Procion Red Dye Chromatography—The phosphocellulose peak eluting at 0.12 M KCl was dialyzed overnight against 2 liters of 20 mM imidazole (pH 7.5), 100 mM KCl, and 1 mM dithiothreitol, loaded onto a Procion red dye column (1.5 x 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 7.5), 75 mM KCl, 5% glycerol, and 1 mM dithiothreitol and applied to a histone-Sepharose column (1 x 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 Mg2+-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, 5% 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 ATPase assay mixture contained 15 mM imidazole (pH 7.5), 2 mM MgCl2, 1 mM EGTA, and 2 mM [γ-32P]ATP (6.5, 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 of 0.5 ml. ATPase activity was measured by the release of 32P, from the [γ-32P]ATP as described by Pollard and Korn (5) following a 10-min 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 250 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
Experimental Procedures.

Histone-Sepharose chromatography of myosin I—Histones were purified on histone-Sepharose and column fractions were assayed for protein concentration and for myosin I kinase activity (1 μl) as described under “Experimental Procedures.”

Phosphocellulose chromatography of the DE-52 fraction—Purification of 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 radii, ovalbumin (Rg = 2.85 nm), bovine serum albumin (Rg = 3.5 nm), and aldolase (Rg = 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 I (100 μg) by myosin I heavy chain kinase in the presence of [γ-32P]ATP, myosin was separated from ATP on a phosphocellulose 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 phosphorylserine and phosphothreonine standards, and analyzed by electrophoresis at pH 1.9 on cellulose thin layer sheets exactly as described by Cote et al. (13). 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.

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 KCl (4). As shown previously by Maruta and Korn (16), the myosin I peak contains some kinase activity. We found, however, that about 75% of the myosin I kinase 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 KCl and a smaller peak (PC2) at 0.36 M KCl (Fig. 1). PC1 usually contained 60–70% of the total activity eluted, was well...
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 °C, there was no detectable loss of myosin I heavy chain phosphate. Furthermore, incubation of the purified myosins with myosin I kinase (1:50 ratio of kinase to myosin, w/w) for 30 min at 30 °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 40 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 μM, 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 μM) 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 an pronounced optimum concentration

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**Table I**

Substrate specificity of myosin I heavy chain kinase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity a</th>
<th>Substrate concentration μM</th>
<th>Kinase concentration nM</th>
<th>μmol/min/mg</th>
<th>Substrate concentration μM</th>
<th>Kinase concentration nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin IB</td>
<td>2.4</td>
<td>0.05</td>
<td>4.64</td>
<td></td>
<td>2.4</td>
<td>0.05</td>
</tr>
<tr>
<td>Myosin IA</td>
<td>1</td>
<td>0.02</td>
<td>1.25</td>
<td></td>
<td>1</td>
<td>0.02</td>
</tr>
<tr>
<td>Histone 2A</td>
<td>67</td>
<td>1.0</td>
<td>3.03</td>
<td></td>
<td>67</td>
<td>1.0</td>
</tr>
<tr>
<td>Casein</td>
<td>42</td>
<td>3.0</td>
<td>0.15</td>
<td></td>
<td>42</td>
<td>3.0</td>
</tr>
<tr>
<td>Phosvitin</td>
<td>25</td>
<td>3.0</td>
<td>0</td>
<td></td>
<td>25</td>
<td>3.0</td>
</tr>
<tr>
<td>Myosin II</td>
<td>4.4</td>
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<td>0</td>
<td></td>
<td>4.4</td>
<td>4.0</td>
</tr>
<tr>
<td>SMLC</td>
<td>13</td>
<td>1.5</td>
<td>1.95</td>
<td></td>
<td>13</td>
<td>1.5</td>
</tr>
</tbody>
</table>

a SMLC (smooth muscle light chains) were a mixture of the 17,000- and 20,000-Da light chains from turkey gizzard smooth muscle myosin in the molar ratio of 4:6.
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 \mu M) varied by less than 10% between incubations containing 0.1 mM EGTA (4.36 \mu mol/min-mg), 20 \mu M free Ca\(^{2+}\) (4.68 \mu mol/min-mg), 20 \mu M free Ca\(^{2+}\) plus 2 \mu M calmodulin (4.28 \mu mol/min-mg), 50 \mu M cAMP (4.11 \mu mol/min-mg), and 20 \mu g/ml of cAMP-dependent protein kinase inhibitor (4.70 \mu mol/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 \mu M to 2 mM ATP (Fig. 6). The results obeyed classical Michaelis-Menten kinetics and when plotted by Lineweaver-Burk analysis yielded a \(K_m\) for ATP of 43 \mu M (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 1-13). Similarly, no \(^{32}\)P was incorporated into the 17,000-Da light chain of myosin IA.

Phosphoamino acid analysis of maximally phosphorylated myosin IA maximally phosphorylated by purified myosin I heavy chain kinase and separated on a 6–15% polyacrylamide gradient gel (molecular weight standards are phosphorylase b, 93,500; bovine serum albumin, 67,000; ovalbumin, 43,000; \(\alpha\)-chymotrypsinogen, 25,700; \(\beta\)-lactoglobulin, 18,400; lysozyme, 14,300; cytochrome c, 12,300; bovine trypsin inhibitor, 6,200; and insulin, 3,000). Lane 13, corresponding autoradiogram showing no \(^{32}\)P was incorporated into the 25,000-Da light chain. Lanes 14 and 15, phosphorylation of the 130,000-Da heavy chain of myosin IA. Lane 14, Coomassie blue stain of myosin IA maximally phosphorylated by purified myosin I heavy chain kinase and separated on a 6–15% polyacrylamide gradient gel (molecular weight standards are phosphorylase b, 93,500; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,300). Lane 15, corresponding autoradiogram showing no \(^{32}\)P incorporated into the 17,000-Da light chain.

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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 assays were performed with myosin IB varied from 0.3 to 2.4 \(\mu\)M (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

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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 vary-
ability, and it may be that the type of assay performed under-
estimates the phosphorylation rate when the protein concen-
tration 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 V_{max} 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 Iα and Iβ
was compared with its activity toward the general phosphate-
accepting proteins, histone 2A, casein, and phosvitin, and
toward Acanthamoeba myosin I (Table I). As stated above,
we were limited in the concentration of myosin I we could
use. Nevertheless, even at these low myosin IB concentrations
(1–2 μM), the specific activity was on the order of 2–4 μmol/ml
min mg. Myosin I kinase phosphorylated the myosin Iα iso-
enzyme at essentially the same rate as myosin Iβ. Myosin
I kinase also phosphorylated histone 2A at a high rate, but
phosphorylated casein at a very low rate, and phosvitin not at
all. Absolutely no phosphorylation of Acanthamoeba
myosin I by myosin I kinase was observed, even at very high
myosin I kinase concentrations (40 nM). In separate experi-
ments, no phosphorylation of myosin Iα or Iβ by a partially
purified Acanthamoeba myosin I 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 phosphoryl-
ated isolated light chains from turkey gizzard smooth muscle
myosin at a significant rate.

**DISCUSSION**

After the discovery that heavy chain phosphorylation regu-
lates the actin-activated Mg^{2+}-ATPase activity of Acan-
thamoeba 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 phosphoryl-
ation inhibits the actin-activated ATPase activity), and
Physarum myosin (30) (where phosphorylation activates the
actin-activatable ATPase activity). Heavy chain phosphoryla-
ton also occurs in liver macrophage (31), lymphocyte (32),
and brain (33) myosins, although the stoichiometry and con-
sequences of these phosphorylations are unknown. The puri-
ification 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 M_{r} = 107,000. The
kinase phosphorylates both myosin Iα and Iβ at a high rate
at one site within the heavy chain, which in the case of myosin
Iβ is a serine residue. Although we have never had sufficient
myosin I to measure it, the V_{max} 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 purifi-
cation of myosin I heavy chain kinase at each chromatog-
ographic step because of the low yield and lack of stability of
the purified myosin I substrate. Several quantitative meas-
urements of kinase activity in the DE-52 flow-through frac-
tion (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 phospho-
cellulose, red dye, and histone-Sepharose chromatography
provided about a 300-fold purification of kinase activity start-
ing 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 purifi-
cation. On this basis, 1 kg of cells would contain about 5 mg
of myosin I kinase, which is about 150 mg of myosin Iα and 100 mg
of myosin Iβ, a large amount 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 Mg^{2+}-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 I kinase. But myosin
I kinase has no activity toward myosin Iα and partially purified
myosin I heavy chain kinase has no activity toward myosin Iα.

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 addi-
tion 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
(proteolytic or phosphorylated products, for example) of
the enzyme that has been purified. Myosin I heavy chain kinase
is probably identical with the cofactor protein partially puri-
fied 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 Ca^{2+}, Ca^{2+}/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\(^{2+}\)/calmodulin. 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\(^{2+}\) (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- and cGMP-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).

REFERENCES
Purification and characterization of a myosin I heavy chain kinase from Acanthamoeba castellanii.
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