Functional Consequences of the Proteolytic Removal of Regulatory Serines from the Nonhelical Tailpiece of *Acanthamoeba* Myosin II

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ABSTRACT: The actin-activated Mg^{2+}-ATPase activity of myosin II from *Acanthamoeba castellanii* is regulated by phosphorylation of 3 serines in its 29-residue, nonhelical, COOH-terminal tailpiece, i.e., serines-1489, -1494, and -1499 or, in reverse order, residues 11, 16, and 21 from the COOH terminus. To investigate the essential requirements for regulation, myosin II filaments in the presence of F-actin were digested by arginine-specific submaxillary gland protease. Two-dimensional peptide mapping of purified, cleaved myosin II showed that the two most terminal phosphorylation sites, serines-1494 and -1499, had been removed. Cleaved dephosphorylated myosin II retained full actin-activated Mg^{2+}-ATPase activity (with no change in V_{max} or K_{app}) and the ability to form filaments similar to those of the native enzyme. However, higher Mg^{2+} concentrations were required for both filament formation and maximal ATPase activity. The one remaining regulatory serine in the cleaved myosin II was phosphorylatable by myosin II heavy-chain kinase, and phosphorylation inactivated the actin-activated Mg^{2+}-ATPase activity, as in the case of the native myosin II. Also as in the case of the native myosin II, phosphorylated cleaved myosin II inhibited the actin-activated Mg^{2+}-ATPase activity of dephosphorylated cleaved myosin II when the two were copolymerized. These results suggest that at least 18 of the 29 residues in the nonhelical tailpiece of the heavy chain are not required for either actin-activated Mg^{2+}-ATPase activity or filament formation and that phosphorylation of Ser-1489 is sufficient to regulate the actin-activated Mg^{2+}-ATPase activity of myosin II.

Myosin II from *Acanthamoeba castellanii* is a conventional myosin, containing a pair of heavy chains of about 17 000 daltons and two pairs of light chains of about 17 500 and 17 000 daltons (Maruta & Korn, 1977; Pollard et al., 1978; Maruta et al., 1979). The NH_2-terminal half of each heavy chain folds to form a globular head domain with associated light chains (Atkinson & Korn, 1986). The COOH-terminal halves of the two heavy chains form a 90-nm, coiled-coil helical tail domain (Pollard, 1982; Hammer et al., 1987) ending in a 29-residue, nonhelical tailpiece (Cotē et al., 1984; Hammer et al., 1987). The nonhelical tailpiece of each heavy chain contains three phosphorylatable serines at positions 1489, 1494, and 1499 or, in reverse order, residues 11, 16, and 21 from the COOH terminus (Collins et al., 1982a; Cotē et al., 1984; Hammer et al., 1986). Phosphorylation of these three serine residues reversibly inactivates the actin-activated Mg^{2+}-ATPase activity of myosin II but has no effect on its Ca^{2+}-ATPase or K^+,EDTA-ATPase activities (Collins & Korn, 1980; Collins et al., 1982a,b; Cotē et al., 1981; McClure & Korn, 1983). Removal of the 66 carboxyl-terminal amino acids of each heavy chain, i.e., the last 37 residues of the coiled-coil helix and the 29-residue, phosphorylatable nonhelical tailpiece, by limited chymotryptic digestion abolishes the ability of myosin II to form filaments and also its actin-activated Mg^{2+}-ATPase activity (Kuznicki et al., 1985; Wijmenga et al., 1987). Its Ca^{2+}- and K^+,EDTA-ATPase activities are unaffected (Kuznicki et al., 1985). These results indicate that all or some of the 66 amino acids at the end of each heavy chain are required for bipolar filament formation and that filaments are necessary for actin-activated Mg^{2+}-ATPase activity. This hypothesis is supported by the observations that myosin II is filamentous under all conditions in which it expresses actin-activated Mg^{2+}-ATPase activity (Collins et al., 1982b; Kuznicki et al., 1983, 1984) and that monoclonal antibodies that bind to the end of the tail of the heavy chain (Kiehart & Pollard, 1984a) inhibit both filament formation and actin-activated Mg^{2+}-ATPase activity (Kiehart & Pollard, 1984a,b; Kiehart et al., 1984). Interestingly, polyclonal antibodies raised against a synthetic peptide corresponding in sequence to the last 19 residues of the coiled-coil region inhibit both filament formation and actin-activated Mg^{2+}-ATPase activity whereas polyclonal antibodies raised against a synthetic peptide with the sequence of the first 21 amino acids of the nonhelical tailpiece inhibit neither (Atkinson et al., 1988).

Additionally, it was found that phosphorylated myosin II inactivates dephosphorylated myosin II when they are in the same copolymer (Kuznicki et al., 1983) and that NEM-inactivated dephosphorylated myosin II activates phosphorylated myosin II when they are copolymerized (Atkinson et al., 1989). Thus, it seems likely that bipolar filament formation depends on some or all of the 37 amino acids at the end of the helical portion of the tail while the state of phosphorylation of three serine residues in the following 29-residue, nonhelical tailpiece regulates the actin-activated Mg^{2+}-ATPase of the filaments, possibly by global intermolecular interactions (Kuznicki et al., 1983; Atkinson et al., 1987) that alter the conformation around the hinge region that occurs about 40% of the distance between the end of the tail and the head–tail junction (Hammer et al., 1987; Wijmenga et al., 1987; Atkinson & Korn, 1987).

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The strictest interpretation of the preceding hypotheses predicts that proteolytic removal of the nonhelical tailpiece, while leaving the coiled-coil helical domain intact, would have little if any effect on filament formation. The presence or absence of actin-activated Mg^{2+}-ATPase activity in these filaments, which would be missing the phosphorylatable serines, might then provide further evidence on the mechanism by which phosphorylation regulates enzymatic activity.

The sequence (Cotē et al., 1984) of the nonhelical tailpiece is [1483]Pro-Ser-Ser-Arg-Gly-Gly-Ser*-Thr-Arg-Gly-Ala-Ser*-Ala-Arg-Gly-Ala-Ser*-Val-Arg-Ala-Gly-Ser-Ala-Arg-Ala-Glu-Glu[1509] (where Ser* indicates the sites of phosphorylation). The fact that there are no lysine residues in the nonhelical tailpiece suggested that the use of arginine-specific proteases might allow the tailpiece to be cleaved while minimizing cleavages elsewhere in the heavy or light chains. In the present study, we were able to remove two of the three phosphorylatable serines and show that the otherwise unmodified myosin II still formed bipolar filaments and had actin-activated Mg^{2+}-ATPase activity that was regulated by phosphorylation.

Materials and Methods

Acanthamoeba myosin II was purified as previously described (Cotē et al., 1981), dialyzed against 10 mM imidazole buffer, pH 7.0, 100 mM KCl, and 1 mM dithiothreitol, and concentrated against solid sucrose. Concentrated myosin II can be stored for several months at about 0 °C without loss of enzymatic activity. Rabbit skeletal muscle F-actin was purified by the method of Pardee and Spudich (1982). Partially purified *Acanthamoeba* myosin II heavy-chain kinase was prepared and used to phosphorylate myosin II according to the methods of Cotē et al. (1981). The amount of phosphate incorporated into the heavy chain was quantified by using [γ-^{32}P]ATP in the phosphorylation reaction and determining the amount of radioactivity incorporated into the heavy chains after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).[1] Myosin II was dephosphorylated as described by Collins and Korn (1980), and the extent of dephosphorylation was estimated by either the increase in specific activity of the actin-activated Mg^{2+}-ATPase activity or the loss of radioactivity when the myosin had been previously phosphorylated by [γ-^{32}P]ATP.

Myosin II (0.23 μM) was digested with submaxillaris protease, an arginine-specific protease, with a protease to myosin ratio of 1:30 (w/w), in 20 mM TES[1] buffer, pH 7.5, containing 15 mM $MgCl_2$ and 20% sucrose with or without 4.5 μM F-actin and 0.75 μM cytochalasin D. When F-actin and cytochalasin D were present, the reaction mixture was sonicated briefly, before addition of the myosin and protease, to obtain short actin filaments capped with cytochalasin D. After incubation for 18 h at 0 °C, the digestion was stopped by the addition of ~100 molar excess of TLCK. The digestion mixture was centrifuged at 4 °C for 2 h at 40000 rpm to pellet the actomyosin complex which was then dissociated and solubilized in 10 mM imidazole buffer, pH 7.5, containing 0.6 M KCl, 1 mM EDTA, 1 mM dithiothreitol, and 5 mM ATP. The dissociated F-actin was pelleted by centrifugation as before, and the cleaved myosin II in the supernatant solution was purified on a Sepharose CL-4B column equilibrated and eluted with 10 mM imidazole, pH 7.5, 0.6 M KCl, 1 mM EDTA, and 1 mM dithiothreitol. The fractions containing Ca^{2+}-ATPase activity were pooled, dialyzed against 10 mM imidazole buffer, pH 7.0, containing 0.1 M KCl and 1 mM dithiothreitol, and concentrated by dialysis against solid sucrose. The cleaved myosin II was stable for at least several months when stored at about 0 °C.

Ca^{2+}-ATPase and actin-activated Mg^{2+}-ATPase activities were assayed by incubating myosin II with [γ-^{32}P]ATP at 30 °C for 15 min under the conditions described by Collins and Korn (1980, 1981), and measuring the release of [^{32}P]P_i according to the procedure of Pollard and Korn (1973). Protein concentrations were estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard. SDS–PAGE was performed as described by Laemmli (1970) and urea–PAGE according to Perrie and Perry (1970); the gels were stained with Coomassie blue according to the method of Fairbanks et al. (1971). Two-dimensional peptide mapping on thin-layer cellulose-coated sheets of tryptic digests of ^{32}P-labeled myosin II and autoradiography of the peptide maps were performed as described (Cotē et al., 1981). Sedimentation analysis was carried out at 30 °C and 15000 rpm using an AN-G rotor in a Beckman Model E analytical ultracentrifuge equipped with UV optics.

For negative staining of myosin II, parlodion- and carbon-coated 400-mesh grids were glow-discharged for 15–30 s. Samples of cleaved and of uncleaved dephosphorylated myosin were diluted to about 20 μg/mL in the appropriate buffer (see Figure 4 legend). A drop of diluted sample was applied to the grid for about 60 s, and the grid was rinsed with 3 drops of buffer without ATP, followed by 3 drops of 2% uranyl formate (w/v in distilled water). The negative-stained myosin was photographed at 44400× in a Philips 410 electron microscope.

Potato acid phosphatase and ATP were from Sigma; imidazole and pepstatin from Fluka; ultrapure sucrose from ICN Biomedicals; [γ-^{32}P]ATP from New England Nuclear; Sepharose CL-4B from Pharmacia; submaxillaris protease, clostripain, thrombin plasmin, and kallikrein from Pierce; and cytochalasin D from Aldrich. All other chemicals were of reagent grade.

Results

Selective Cleavage of Myosin II by Submaxillaris Protease. Three products were formed when myosin II (Figure 1A, lane 2) was digested with submaxillaris protease in the absence of F-actin: a polypeptide with slightly faster mobility than the undigested heavy chain and two additional bands with mobilities corresponding to masses of 112 and 73 kDa (Figure 1A, lane 1). The latter two peptides are the NH_2-terminal and COOH-terminal fragments, derived from cleavage at a protease-sensitive site in the globular head, that were described previously (Kuznicki et al., 1984; Atkinson & Korn, 1986). When the digestion was carried out under identical conditions, except for the inclusion of F-actin at a myosin:actin molar ratio of 1:10, cleavage in the head domain was essentially totally inhibited, as it is for other myosins (Bertrand et al., 1989), and the 112- and 73-kDa peptides were not formed (Figure 1A, lane 3). Thus, in the presence of F-actin, the heavy chain was converted entirely to a single polypeptide of only slightly greater mobility than the uncleaved heavy chain (Figure 1A, lane 3). The two light chains were apparently unaffected (Figure 1B). Identical results were obtained with dephosphorylated and phosphorylated myosin II. The purified, cleaved myosin was free of actin (Figure 1C,D).

Characterization of the Cleaved Myosin II. Because the NH_2 terminus of proteolytically cleaved myosin II heavy chain remains blocked (Atkinson & Korn, 1986), it seemed most

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

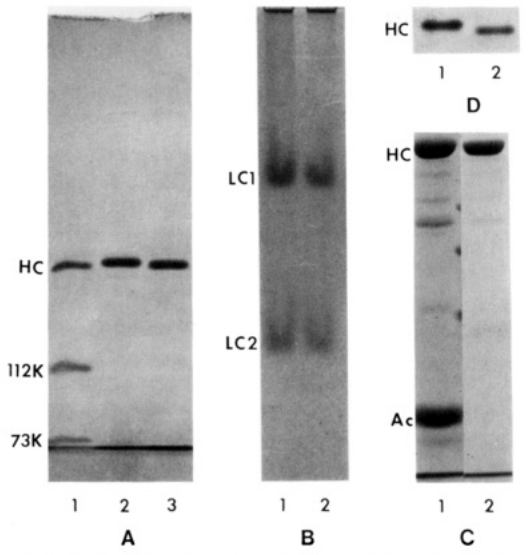

FIGURE 1: Effect of F-actin on the cleavage of *Acanthamoeba* myosin II by submaxillaris protease. Myosin II was digested with protease in the presence or absence of F-actin as described under Materials and Methods. (A) Samples containing 4 μg of myosin were analyzed on 5% SDS–PAGE. Lane 1, myosin II incubated with protease in the absence of F-actin; lane 2, control myosin II; lane 3, myosin II incubated with protease in the presence of F-actin. The myosin II light chains and actin (when present) were unresolved at the gel front. (B) Samples containing 30 μg of myosin were analyzed on 8% urea–PAGE. Lane 1, control myosin II; lane 2, myosin II incubated with submaxillaris protease in the presence of F-actin. (C) Samples containing 10 μg of myosin were analyzed on 7.5% SDS–PAGE. Lane 1, reaction mixture of digest; lane 2, purified cleaved myosin showing absence of actin. (D) Samples containing 2 μg of native myosin (lane 1) and purified, cleaved myosin (lane 2) were analyzed on 7.5% SDS–PAGE to show the difference in the size of the heavy chains. HC, myosin II heavy chain; LC1 and LC2, myosin II light chains; Ac, actin; 112K and 73K, principal proteolytic cleavage products of myosin II heavy chain in the absence of actin.

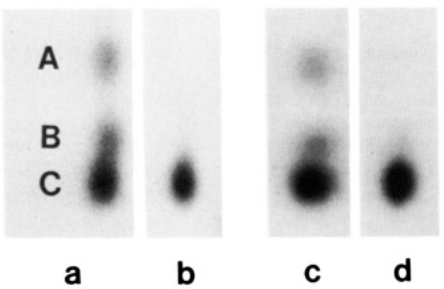

FIGURE 2: Autoradiogram of peptide maps of limit tryptic digests of ^{32}P-labeled native myosin II and protease-cleaved myosin II heavy chains. In one experiment, dephosphorylated myosin II (a) and cleaved dephosphorylated myosin (b) were phosphorylated by incubation with [γ-^{32}P]ATP and heavy-chain kinase and then digested with trypsin. In the other experiment, ^{32}P-labeled phosphorylated myosin II (c) and the cleaved ^{32}P-labeled cleaved phosphorylated myosin II (d) derived from it were digested with trypsin. Peptides A, B, and C each contain one phosphoserine corresponding to residues 1499, 1494, and 1489, respectively (Cotē et al., 1981, 1984). The protease-cleaved myosin II heavy chain contains only Ser-1489.

likely that the increase in electrophoretic mobility of the heavy chain was due to a loss of mass from the COOH terminus. The extent of this loss was evaluated by autoradiography of two-dimensional peptide maps of limit tryptic digests of cleaved myosin II that had been labeled with ^{32}P by incubation with [γ-^{32}P]ATP and heavy-chain kinase after cleavage (Figure 2b) or had been derived from ^{32}P-labeled myosin II (Figure 2d). Autoradiography of the maps of tryptic digests of ^{32}P-labeled phosphorylated native myosin II (Figure 2a) revealed the three spots (A, B, and C) that were shown previously (Cotē et al., 1981, 1984) to contain phosphoserine residues corresponding, respectively, to positions 1499, 1494, and 1489 (or positions 11, 16, and 21 from the COOH terminus) of the myosin II heavy chain. The autoradiograms of the peptide maps of cleaved myosin II that had been ^{32}P labeled after its preparation from dephosphorylated myosin II showed only one spot which corresponding exactly to peptide C (Figure 2b). Identical results were obtained when ^{32}P-labeled phosphorylated myosin II was cleaved with submaxillaris protease and then subjected to tryptic digestion and peptide mapping (Figure 2c,d). Analysis of the native proteins showed that the specific radioactivity of another sample of ^{32}P-labeled phosphorylated myosin II was reduced from 542 to 394 cpm/μg by cleavage with submaxillaris protease, consistent with the loss of sites B and C which are less intensely labeled than site A (Figure 2). This latter result indicates that the cleaved fragment dissociates from the native molecule.

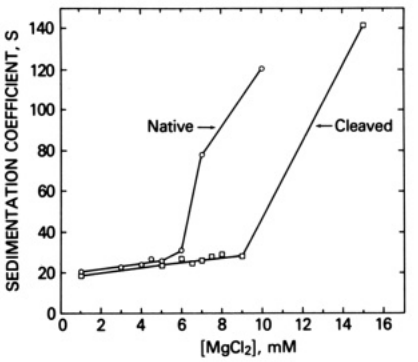

FIGURE 3: Sedimentation coefficients of native myosin II and protease-cleaved myosin II as a function of Mg^{2+} concentration. *s* values of 20–30 S indicate the presence of small bipolar filaments, and large *s* values are indicative of larger filaments or aggregates. Native myosin (○); cleaved myosin (□).

Thus, cleavage by submaxillaris protease removes the two terminal phosphorylation sites but not the third, most internal, site. This indicates that cleavage by this arginine-specific enzyme occurs at Arg-1491 (and probably also at Arg-1496) but not at Arg-1486. Attempts to cleave at this third potential site with other arginine-specific proteases (clostripain, thrombin, plasmin, and kallikrein), with trypsin, and with submaxillaris protease under a number of different conditions (including higher temperatures and mild denaturants) were all unsuccessful. Attempts to remove the remaining phosphorylatable serine by digesting the cleaved myosin with carboxypeptidases were also unsuccessful although the expected presence of arginine at the COOH terminus was confirmed.

Filament Formation by Myosin II. The effect of Mg^{2+} concentration on the sedimentation coefficients of native and cleaved myosin II is shown in Figure 3. In agreement with previous results (Kuznicki et al., 1983), native dephosphorylated myosin II had a sedimentation coefficient of about 20–30 S in the presence of 1–6 mM Mg^{2+} with an increase to about 130 S at higher Mg^{2+} concentrations. Similar results were obtained with cleaved dephosphorylated myosin II (Figure 3), except that the transition from small to large filaments required a higher concentration of Mg^{2+}, 9 mM instead of 6 mM. No significant differences between filaments of native and cleaved myosin II were discerned by electron microscopy (Figure 4) at Mg^{2+} concentrations that gave rise to *s* values of 20–30 S.

Enzymatic Activity of Cleaved Myosin II. The actin-activated ATPase activities of native and cleaved dephosphorylated myosin II at varying Mg^{2+} concentrations are shown

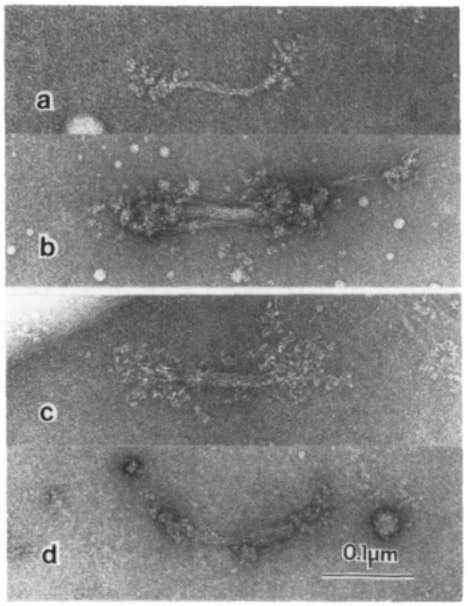

FIGURE 4: Negative-stained images of cleaved (a and b) and native (c and d) dephosphorylated myosin II showing typical bipolar filaments in both. The native myosin was originally in buffer containing 10 mM imidazole, pH 7.0, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, 1 mM ATP, and 3.5 mM $MgCl_2$, and the cleaved myosin was in the same buffer but containing 5.0 mM $MgCl_2$. Clumping of heads around the filament (as in panels b and d) was variable and appeared to occur when the heads did not interact with the substrate. The apparently thicker filaments (such as in panel b) may be side-to-side aggregates of thinner filaments, as described by Pollard (1982). Magnification is 117216×.

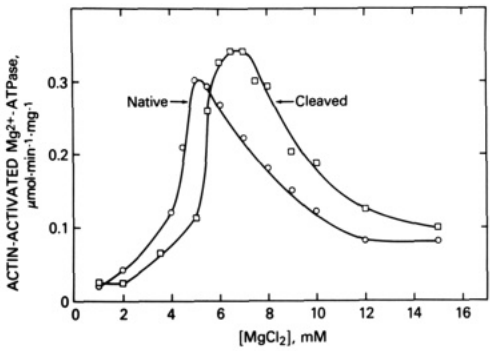

FIGURE 5: Actin-activated Mg^{2+}-ATPase activities of native and protease-cleaved myosin II as a function of Mg^{2+} concentration. The myosin concentration was 28 μg/mL, and the F-actin concentration was 528 μg/mL. Native myosin (○); cleaved myosin (□).

in Figure 5. As for filament formation, the optimal Mg^{2+} concentration was higher for cleaved myosin (7 mM) than for native myosin (5 mM). Double-reciprocal plots of the rates of ATP hydrolysis by native and cleaved dephosphorylated myosin II as a function of F-actin concentrations (each at its optimal Mg^{2+} concentration) produced similar values for V_{max} (0.42 and 0.37 μmol·min^{-1}·mg^{-1}) and K_{app} (5.0 and 4.4 μM), respectively.

Phosphorylation of Cleaved Myosin II. The peptide mapping data (Figure 2b) indicated that cleaved dephosphorylated myosin II has one remaining phosphorylatable serine on each heavy chain. However, a maximum of only 0.82 mol of PO_4/mol (0.41 mol/mol of heavy chain) was incorporated when cleaved dephosphorylated myosin II was incubated with heavy-chain kinase (Figure 6) under conditions in which native myosin II could be completely phosphorylated. This indicates either that it is difficult to phosphorylate the cleaved myosin or, more likely, that the myosin had not been completely dephosphorylated before it was cleaved with submaxillaris protease [we have never been able to remove all of the phosphate from native myosin II (Cotê et al., 1981)]. However, subjecting the cleaved dephosphorylated myosin II to a second incubation with phosphatase before incubation with heavy-chain kinase did not increase the extent of phosphorylation (data not shown).

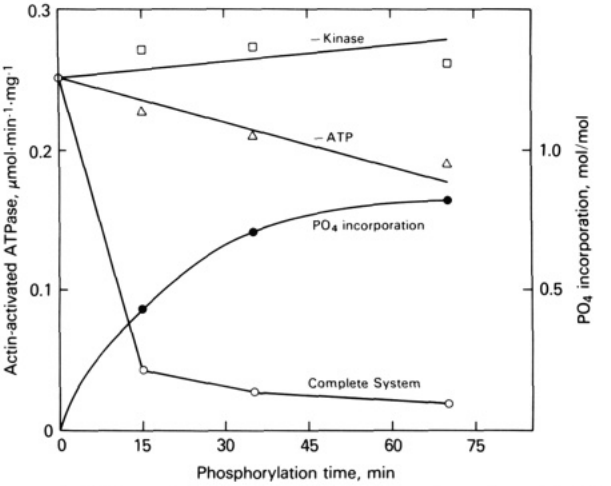

FIGURE 6: Effect of phosphorylation on the actin-activated Mg^{2+}-ATPase activity of protease-cleaved myosin II. Cleaved myosin II was incubated with [γ-^{32}P]ATP, and aliquots were removed at different times to determine the extent of phosphorylation (●) and the actin-activated Mg^{2+}-ATPase activity of the phosphorylated myosin (○). Control samples were incubated with kinase but no ATP (△) or with ATP but no kinase (□). Concentrations were 28 μg/mL myosin and 528 μg/mL F-actin.

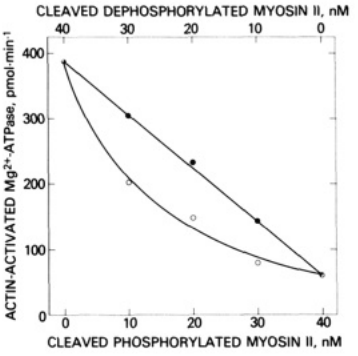

FIGURE 7: Effect of phosphorylated protease-cleaved myosin II on the actin-activated Mg^{2+}-ATPase activity of dephosphorylated protease-cleaved myosin II. Cleaved dephosphorylated myosin was rephosphorylated, and the enzymatic activity of cleaved dephosphorylated myosin in copolymers containing increasing quantities of rephosphorylated cleaved myosin was determined (○). These activities are compared to the sum of the activities of the homopolymers assayed individually (●). Concentrations were 528 μg/mL F-actin, 5 mM Mg^{2+}, and myosin as indicated.

As with native myosin II, phosphorylation of the cleaved dephosphorylated myosin II inactivated its actin-activated Mg^{2+}-ATPase activity (Figure 6). Thus, incorporation of more than 0.5 mol of phosphate into the remaining phosphorylatable serine by incubation with kinase and ATP almost completely inactivated the enzyme while incubations of the cleaved dephosphorylated myosin II with either kinase or ATP alone had little effect. Also as observed for native myosin II, phosphorylated cleaved myosin II inhibited the actin-activated Mg^{2+}-ATPase activity of dephosphorylated cleaved myosin II when the two were present in the same copolymers (Figure 7); i.e., the activity of the copolymers was much less than the sum of the activities of equivalent concentrations of the two proteins assayed separately [cf. Kuznicki et al. (1983) for more details].

Discussion

We have shown that in the presence of F-actin arginine-specific submaxillaris protease selectively cleaves the heavy chain of filamentous myosin II, removing 18 amino acids (including 2 of the 3 phosphorylatable serines) from the end of the COOH-terminal nonhelical tailpiece. The inability of the protease to cleave at Arg-1486 suggests that this third and final potential cleavage site in the nonhelical domain is protected either by its proximity (three to six residues) to the coiled-coil helical domain or because it has its own secondary structure. The ability of the cleaved myosin II to form apparently normal filaments indicates that filament formation depends only on interactions between the helical domains. Thus, the inability of chymotryptic-cleaved myosin II to form antiparallel associations that was demonstrated previously (Kuznicki et al., 1985; Wijmenga et al., 1987) is due to the loss of the terminal 46 amino acids that may all be within the helical domain.

The normal actin-activated Mg^{2+}-ATPase activity of the submaxillaris protease cleaved dephosphorylated myosin II shows that at least most of the nonhelical tailpiece including two of the three regulatory serines is not required for activity and that phosphorylation of the one remaining serine is sufficient for inactivation. It is possible, but as yet unproven, that the third serine is also not required for actin-activated Mg^{2+}-ATPase activity, or for filament formation, and that the sole function of the three phosphorylatable serines, and the nonhelical tailpiece in which they occur, is to provide a mechanism for regulating the enzymatic activity of the filaments. The fact that the optimal Mg^{2+} concentrations for both filament formation and enzymatic activity are altered by removal of a portion of the tailpiece containing two of the regulatory serines is consistent with the proposal that regulation is effected through a change in filament conformation.

Acknowledgments

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Registry No. Mg-ATP, 1476-84-2; ATPase, 9000-83-3; L-serine, 56-45-1.

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