Limited Proteolysis Reveals a Structural Difference in the Globular Head Domains of Dephosphorylated and Phosphorylated Acanthamoeba Myosin II*

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Phosphorylation at three sites in the tail of myosin II from Acanthamoeba castellanii inactivates the actin-activated Mg\(^{2+}\)-ATPase activity of filamentous myosin and the in vitro motility activity of both monomeric and filamentous myosin. To seek a structural explanation for these effects, we examined the susceptibilities of dephosphorylated and phosphorylated myosins II to endoproteinases. Endoproteinase Arg-C cleaved myosin II preferentially at two sites in the globular head, Lys-621 and Arg-638, producing an NH\(_2\)-terminal fragment of about 67,000 Da and a COOH-terminal fragment of about 112,000 Da. Dephosphorylated monomers and filaments were cleaved about 3 times more rapidly than their phosphorylated counterparts principally because of a much greater rate of cleavage at Arg-638; the ratio of cleavage at Arg-638:Lys-621 was about 3 for dephosphorylated myosin II cleaved more rapidly than their phosphorylated counterparts. These results demonstrate that phosphorylation at the tip of the tail of Acanthamoeba myosin II causes a conformational change in the globular head that contains the catalytic sites; therefore, this conformational change may be related to the different catalytic and motile activities of the dephosphorylated and phosphorylated enzymes.

As described more completely in the accompanying paper (1), Acanthamoeba myosin II is a conventional myosin composed of two heavy chains of Mr \(-172,000\) and two pairs of light chains of Mr \(-17,500\) and \(-17,000\). As in all myosins II, the amino-terminal region of the heavy chains forms two globular heads while their carboxyl-terminal tails interact to form a coiled-coil \(\alpha\)-helical rod through which the monomers can self-associate into bipolar filaments. A bend (hinge) occurs in the helical rod about 40% of the distance from the carboxyl terminus to the head-rod junction. Phosphorylation of three sites in a short, 29-amino acid, non-helical region at the tip of the tails of the heavy chains inactivates the actin-activated Mg\(^{2+}\)-ATPase activity of myosin II filaments but has no effect on the actin-activated Mg\(^{2+}\)-ATPase activity of myosin II monomers (1). These, and many more, data are consistent with the concept that the enzymatic activity of each myosin molecule in a filament is not determined by its own phosphorylation state but by the level of phosphorylation of the filament as a whole.

Despite the fact that phosphorylated monomeric myosin II has full actin-activated Mg\(^{2+}\)-ATPase activity, phosphorylation inhibits the ability of monomeric as well as filamentous myosin II to support movement of actin filaments in an in vitro motility assay (1). This result is surprising because the motility activity has been thought to be a property solely of the globular head (2), and it is difficult to imagine how this amino-terminal domain could be affected by phosphorylation at the tip of the tail.

In the present study, we report the results of limited digestion of Acanthamoeba myosin II by endoproteinase Arg-C. Both filamentous and monomeric myosin were cleaved preferentially within a limited region in the globular head, and both forms of dephosphorylated myosin II cleaved more rapidly than their phosphorylated counterparts. These results provide independent evidence that phosphorylation at the tip of the tail of monomeric Acanthamoeba myosin II affects the conformation of the globular head despite the fact that they are separated by approximately 90 nm of coiled-coil \(\alpha\)-helix.

**MATERIALS AND METHODS**

The preparation of dephosphorylated and phosphorylated Acanthamoeba myosin II and rabbit skeletal muscle F-actin, ATPase assays, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein assays are all described or referenced in the accompanying paper (1). Myosin was incubated with endoproteinase Arg-C from mouse submaxillary gland (Boehringer Mannheim) at 30 °C in buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and either 300 or 600 mM KCl (for monomers) or 5 or 10 mM MgCl\(_2\) (for filaments). Digestions were stopped by the addition of 1:1 tosylamido-2-phenylethyl chloromethyl ketone (50 pg/ml) and then heating at 100 °C for 3 min in the electrophoretic sample buffer. After electrophoresis, the gels (7.5%) were stained with Coomassie Blue and scanned in an LKB Ultrascan XL laser densitometer connected to an LKB 2400 Gelscan XL software package.

For NH\(_2\)-terminal sequencing of the cleavage products, the polypeptide fragments were transferred from the acrylamide gels to polyvinylidene difluoride microporous membranes (Millipore) using buffer containing 50 mM Tris, 358 mM glycine, 0.1% sodium dodecyl sulfate, and 20% methanol at 4 mA/cm\(^2\) for 180 min (3). After the transfer was complete, the transfer membrane was stained with Ponceau S (Sigma), and segments of the bands of interest were sequenced in an Applied Biosystems model 470A gas-phase sequenator equipped with a model 120A on-line phenylthiohydantoin analyzer. The 470 Blott protocol supplied by the manufacturer was used with either the standard cartridge blocks or the Blott cartridge blocks (kindly loaned to us by M. Raum, Applied Biosystems). The background was markedly lower with the Blott cartridge blocks.

For sedimentation velocity analysis, monomeric phosphorylated and dephosphorylated myosin II were prepared by dialysis against buffer containing 50 mM Tris, pH 7.0, 300 mM NaCl, 1.0 mM dithiothreitol. Protein concentrations were adjusted to 0.95 mg/ml using an extinction coefficient of 0.56 cm\(^2\)/mg at 280 nm (4). The samples

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were centrifuged at 46,600 rpm at 20 °C in an AN-D rotor in a Beckman model E analytical ultracentrifuge. Difference scans were obtained at 4-min intervals using a schlieren optical system. A 2° deviation of light path in one cell allowed for simultaneous measurements of the two samples. Sedimentation coefficients were calculated using a buffer viscosity relative to water at 25 °C of 1.045, as measured in an Oswald viscometer, and a partial specific volume of 0.735 cc/g estimated from the amino acid composition (4).

RESULTS

When fully dephosphorylated and phosphorylated filamentous myosin II in 10 mM MgCl₂ were digested by endoproteinase Arg-C, the heavy chains were cleaved to two major products of approximately 112,000 and 67,000 Da (Fig. 1), as had been observed previously for cleavage by trypsin (5, 6). Notably, the initial rate of cleavage of dephosphorylated filaments was more than 3 times faster than for phosphorylated filaments (Fig. 1). The same results were obtained for filaments in 5 mM MgCl₂ (data not shown). Very similar results were obtained for endoproteinase Arg-C digestion of monomeric myosin II in 300 mM NaCl (Fig. 2) or 600 mM NaCl (data not shown); the same two major fragments were formed, and dephosphorylated monomers were cleaved more rapidly than phosphorylated monomers. The same relative rates of cleavage of dephosphorylated and phosphorylated myosin II were obtained when the buffer contained 1 mM ATP and when 8 μM F-actin was added to the filamentous myosin, although in the latter case the rates of digestion were appreciably lower.

By autoradiography of gels prepared from digests of ³²P-labeled phosphorylated myosin II, it was found that, as with limited trypsin digests (5, 6), the 112,000-Da polypeptide contained the phosphorylation sites (data not shown), i.e. it was derived from the carboxyl-terminal end of the heavy chain. Thus, the initial cleavage by endoproteinase Arg-C was well within the NH₂-terminal globular head about 67,000 Da. The extent of digestion was determined as described in the legend to Fig. 1. The first 5 lanes from left to right are the samples of dephosphorylated myosin taken at 0, 30, 60, 120, and 240 min. The 5 lanes from right to left are samples of phosphorylated myosin taken at 0, 30, 60, 120, and 240 min. This experiment has been repeated 6 times with essentially identical results. D, dephosphorylated myosin II; P, phosphorylated myosin II.

![Fig. 1. Time course of the digestion of filamentous phosphorylated and dephosphorylated myosin II by endoproteinase Arg-C. Myosin II (0.5 μM, 200 μg/ml) was incubated with endoproteinase Arg-C (proteinase:myosin = 1:25, w/w = 1:3, mol/mol heavy chain) in buffer containing 10 mM MgCl₂, at 30 °C, and samples were removed at the indicated times for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under "Materials and Methods." The inset shows the relevant portion of the Coomassie Blue-stained gels. The only major bands observed were the undigested heavy chain, the 112,000-Da COOH-terminal fragment, and the 67,000-Da NH₂-terminal fragment. The first 5 lanes from left to right are aliquots from the digestion of dephosphorylated myosin II removed at 0, 30, 60, 120, and 240 min. The first 5 lanes from right to left are the corresponding samples from the digestion of phosphorylated myosin II. The phosphorylated 112,000-Da fragment migrates more slowly than the dephosphorylated fragment. The percent of the heavy chain cleaved was calculated independently for each lane from gel scans assuming equal staining of the three bands per unit mass. This experiment has been repeated 3 times (and 2 additional times in 5 mM MgCl₂) with essentially identical results. D, dephosphorylated myosin II; P, phosphorylated myosin II.](image1)

![Fig. 2. Time course of the digestion of monomeric phosphorylated and dephosphorylated myosin II by endoproteinase Arg-C. Myosin II (0.5 μM, 200 μg/ml) was incubated with endoproteinase Arg-C (proteinase:myosin = 1:25, w/w = 1:3, mol/mol heavy chain) in buffer containing 300 mM NaCl at 30 °C. The extent of digestion was determined as described in the legend to Fig. 1. The first 5 lanes from left to right are the samples of dephosphorylated myosin taken at 0, 30, 60, 120, and 240 min. The 5 lanes from right to left are samples of phosphorylated myosin taken at 0, 30, 60, 120, and 240 min. This experiment has been repeated 6 times with essentially identical results. D, dephosphorylated myosin II; P, phosphorylated myosin II.](image2)
Phosphorylation Affects Proteolysis of Acanthamoeba Myosin II

The 112,000-Da bands produced after digestion of monomeric dephosphorylated (Dephos.) and phosphorylated (Phos.) myosin II with endoproteinase Arg-C for 4 h were subjected to NH₂-terminal sequencing as described under "Materials and Methods." Approximately 360 pmol of myosin II heavy chain were applied to each lane of the electrophoretic gel, but only a portion of the recovered peptides was sequenced. Two amino acids were detected at each cycle for each sample. It was apparent from the relative yields of each residue for the dephosphorylated sample that two sequences were present corresponding precisely to segments Ser-639 to Gly-646, produced by cleavage at Arg-638, and Ala-622 to Ala-629, produced by cleavage at Lys-621. The relative yields of the two fragments cannot be compared between the dephosphorylated and phosphorylated samples, but the relative yields of the two sequences within each sample individually are meaningful. The same two sequences were obtained at all time points for filamentous as well as monomeric myosins.

of phosphorylation on endoproteinase Arg-C digestion of myosin II was to reduce substantially the rate of cleavage at Arg-638 and perhaps increase slightly the rate of cleavage at Lys-621 with a resulting net decrease in the overall rate of cleavage.

Sedimentation velocity analysis gave s₂₀,₆₅ values of 7.87 for both phosphorylated and dephosphorylated myosins in 300 mM NaCl, in agreement with Sinard et al. (8) who reported values of 7.8 for myosin II heavy chain at concentrations of 50, 100, 200, and 400 μg/ml (0.12-1.0 μM) in buffer containing 300 mM NaCl. The percent heavy chain cleaved was calculated as described in the legend to Fig. 1. D, dephosphorylated myosin II; P, phosphorylated myosin II.

The two cleavage sites, Arg-638 and Lys-621, are well within the region of the 50-kDa/20-kDa tryptic cleavage site characteristic of myosins and which occurs at Arg-642 in Acanthamoeba myosin II heavy chain (8). Both sites are characteristic of myosins and which occurs at Arg-642 in Acanthamoeba myosin II heavy chain (8). Although, as expected, the initial rate of cleavage increased with protease concentration, it slowed appreciably when cleavage reached about 50% (Fig. 5). More extensive cleavage occurred when the molar ratio was increased to 4:1 (endoproteinase:myosin II heavy chain), but dephosphorylated myosin II was still cleaved much more rapidly than phosphorylated (data not shown).

DISCUSSION

The two cleavage sites, Arg-638 and Lys-621, are well within the subfragment 1-like globular head of myosin II and fairly distant from the head-tail junction which is conventionally assigned to Pro-847 but which Rimm et al. (9) suggest may be near residue 900 in Acanthamoeba myosin II. Both sites are in the region of the 50-kDa/20-kDa tryptic cleavage site characteristic of myosins and which occurs at Arg-642 in Acanthamoeba myosin II. The selectivity of cleavage by both trypsin and endoproteinase Arg-C is interesting given that there are 3 Lys and 3 Arg residues between Lys-621 and Lys-629.

1 M. A. L. Atkinson, personal communication.
phosphorylated and dephosphorylated monomers have the back on the heads, is similarly affected by phosphorylation.

Phosphorylation of one of the two pairs of light chains of smooth muscle myosin and certain vertebrate non-muscle myosins converts monomers from a folded 10 S conformation (in which the tail interacts with the head) into an extended 6 S conformation (11-13), which is the immediate precursor of filaments. The 6 and 10 S conformations also show different susceptibilities to protease digestion (14, 15). A similar conformational change cannot, however, be the basis of the different susceptibilities of dephosphorylated and phosphorylated myosin II to endoproteinase Arg-C cleavage because phosphorylated and dephosphorylated monomers have the same sedimentation coefficient and because endoproteinase digestion of filaments, in which the tails cannot be folded back on the heads, is similarly affected by phosphorylation.

Recent electric birefringence studies (16) have shown that filaments of dephosphorylated myosin II are much more rigid than filaments of phosphorylated myosin II. This has been rationalized on the assumption that the hinge region in each monomer in the filament lies near the phosphorylation sites of other molecules in the filament (8, 17), and thus the state of phosphorylation at the tips of the tails could influence the conformation around the hinge. The observations reported in this paper must have a different explanation, however, because phosphorylation affects the rate of endoproteinase Arg-C digestion of monomeric as well as filamentous myosin II, and the phosphorylation sites and the hinge region within the same molecule are too far apart for one to directly affect the other.

Thus, if, as seems most likely, there is a single explanation for the very similar results obtained for monomeric and filamentous myosin II, it would seem that it can be neither a large change in shape (such as the 6 S = 10 S interconversion) nor a more subtle change in conformation at the hinge region in the rod. It is as if the consequences of phosphorylation at the tip of the tail of Acanthamoeba myosin II are projected through the coiled-coil, helical tail to the globular head. Whatever the specific mechanism, it may be caused by the significant change in charge that results from the addition of 3 phosphate groups (6 negative charges) to a region of the molecule that contains 4 arginine residues and 2 glutamic acids (7). Recent differential scanning calorimetry studies of Acanthamoeba myosin II (18) have revealed a highly cooperative transition (unfolding) of both the dephosphorylated and phosphorylated molecules, indicative of strong interactions between the head and rod domains, that was not seen with rabbit skeletal muscle myosin.

In all the experiments, the rate of endoproteinase Arg-C cleavage of both monomers and filaments slowed dramatically when approximately 50% of the heavy chains were cleaved. This was not due to heterogeneity in the myosin preparation nor was it affected significantly by changes in either the myosin or endoproteinase concentrations. These results could indicate that cleavage, particularly at Arg-638, is facilitated by interaction between the two heads and that once either head in a monomer is cleaved the other becomes much more resistant. The slower rate of cleavage of phosphorylated myosin II would occur if the putative head cooperativity were greatly reduced by phosphorylation. Inhibition by phosphorylation of cooperative interactions between the heads might also explain why monomeric phosphorylated myosin II is inactive in the in vitro motility assay although it has full actin-activated Mg²⁺-ATPase activity (1).

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