Communication

Supramolecular Regulation of the Actin-activated ATPase Activity of Filaments of Acanthamoeba Myosin II*

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Acanthamoeba myosin II has three phosphorylation sites clustered near the end of the tail of each of its two heavy chains (six phosphorylation sites/molecule). Myosin II has little or no actin-activated ATPase activity when four to six of these sites are phosphorylated. Maximal actin-activated ATPase activity is obtained when all six sites are dephosphorylated. Under assay conditions, both phosphorylated and dephosphorylated myosin II form bipolar filaments. Filaments of dephosphorylated myosin II have larger sedimentation coefficients than filaments of phosphorylated myosin II but this difference does not explain the difference in their actin-activated ATPase activities. Heteropolymers, formed by mixing soluble dephosphorylated and phosphorylated myosins and then diluting the mixture into low ionic strength buffer containing MgCl₂, have sedimentation coefficients close to those of the homopolymer of phosphorylated myosin. The actin-activated ATPase activities of heteropolymers are, under most conditions, lower than the equivalent mixtures of homopolymers of dephosphorylated and phosphorylated myosins. It is concluded, therefore, that the phosphorylation of myosin tails regulates the actin-activated ATPase activity of Acanthamoeba myosin II by affecting the myosin filament as a whole rather than specifically affecting the heads of the phosphorylated myosin molecules only.

Acanthamoeba myosin II, one of three myosin isoenzymes in Acanthamoeba castellani (1, 2), contains two heavy chains of about 185,000 daltons and two pairs of light chains of about 17,500 and 17,000 daltons (2, 3). Each heavy chain contains three phosphorylatable sites (4) located within a peptide region no larger than about 9,000 daltons located very near the end of the tail of the heavy chain (5). At least four of these six sites can be phosphorylated in vivo (5-7). Myosin II with four to six sites phosphorylated has little or no actin-activated ATPase activity while the maximally dephosphorylated enzyme has maximal actin-activated ATPase activity (4, 6, 7). Dephosphorylation increases the Vₘₐₓ of the actomyosin II ATPase with little, if any, effect on the affinity of myosin II for F-actin (8).

It is difficult to imagine that the state of phosphorylation of sites at the end of the tail of the heavy chain could affect the catalytic site in the head region, which is at least 100,000 daltons away in linear sequence (5), unless the molecule were bent back upon itself, as has been shown for monomers of dephosphorylated smooth muscle myosin under specific circumstances (9). However, the strong positive cooperativity of the actin-activated ATPase activity of Acanthamoeba myosin II as a function of Mg²⁺ and myosin concentrations (7) suggested that myosin-myosin interactions are required for enzymatic activity. More recently, we observed (8) that both phosphorylated and dephosphorylated myosin II were present in the enzyme assay solutions as bipolar filaments (8). A myosin molecule could not be curled back upon itself in a bipolar filament.

These observations led us to suppose that regulation of the actin-activated ATPase of myosin II by phosphorylation might be exerted through intermolecular interactions within the filament. To test this hypothesis, we compared the actin-activated ATPase activities of homopolymers, of mixtures of homopolymers, and of heteropolymers formed from phosphorylated and maximally dephosphorylated myosin II. The data reported in this paper strongly indicate that regulation of the actin-activated ATPase activity of Acanthamoeba myosin II by phosphorylation does, in fact, occur through some generalized conformational effect on the filament as a whole.

MATERIALS AND METHODS

Rabbit skeletal muscle F-actin (10) was obtained from Dr. Lois F.
Greene (National Heart, Lung, and Blood Institute) and treated with
Dowex-1 to remove free ATP. Acanthamoeba myosin II was prepared
by slight modification of previous procedures (5, 7). Acanthamoeba
F-actin was added to the myosin fraction obtained by DEAE-
chromatography of the amoeba extract in the absence of ATP (1)
and the myosin was recovered from the actomyosin precipitate as
described (7). The purified myosin II had an actin-activated ATPase
activity of 0.57 pmol/min·mg, indicating a phosphohydrolysis rate
of about 2 mol/mol of myosin (6). One portion of the myosin was
incubated with Acanthamoeba myosin II heavy chain kinase and [γ-
³²P]ATP for 1 h at 35 °C as described (4), incorporating 1.9 additional
mol of phosphate/mol of myosin. Therefore, the phosphorylated
myosin contained about 4 mol of phosphate/mol. Its actin-activated
ATPase activity was only 0.02 pmol/min·mg consistent with this
level of phosphorylation (8). Another portion of the isolated myosin
was incubated with potato acid phosphatase for 2 h at 35 °C to remove
the protein-bound phosphate (4, 6). Its actin-activated ATPase
activity was 0.57 pmol/min·mg as expected (6) for fully dephosphorylated
myosin II. The phosphorylated and dephosphorylated myosins were
chromatographed on Bio-Gel A-1.5m (7) to remove the kinase and
phosphatase, respectively, dialyzed for 24 h against 10 mM imidazole,
ph 7.5, containing 10% sucrose (w/v) and 1 mM dithiothreitol, and
concentrated by dialysis against solid sucrose. The final solutions,
which contained 60% (w/w) sucrose by refractometry, were stored at
2 °C.

Actin-activated ATPase activity was measured as the difference in
the rate of release of ³²P, from [γ-³²P]ATP (8) in the presence and
absence of F-actin at 30 °C in buffer containing 10 mM imidazole, pH
7.5, 1 mM ATP, 0.1 mM CaCl₂, and other components as indicated in
the individual experiments. At myosin concentrations of 0.2-10 μg/ml,
2.1 mg of F-actin/ml were used and the samples were incubated at
30 °C for 30 min. At myosin concentrations of 60-240 μg/ml, 3.9
mg of F-actin/ml were used and the incubations were for only 3 min.
The rate of ATP hydrolysis was linear over the entire time courses
and proportional to the myosin concentration. All the components
of the reaction mixture were mixed at 0 °C with the myosin and F-actin
added last, in that order. To make myosin copolymers, the samples
were incubated for 30 min at 30 °C and then rapidly chilled on
ice.

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of soluble dephosphorylated and phosphorylated myosin were mixed as concentrated solutions in 60% sucrose before dilution in the incubation buffers.

Sedimentation coefficients were obtained on myosin samples prepared exactly as for the enzyme assays. After preparing the samples at 0 °C, they were warmed to 30 °C in a water bath and transferred to cells warmed to 30 °C. Analyses were carried out in a Beckman Model E analytical ultracentrifuge equipped with UV optics. The rotor was spun at 30 °C at 16,000–20,000 rpm for samples containing filaments and at 34,000 rpm for samples containing myosin monomer. Scans were obtained at 3- to 12-min intervals for 1 to 2 h after reaching the desired speed. A wavelength of 290 mm was used because of the presence of ATP, and the samples were read against blanks prepared in the same way except that 60% sucrose replaced the myosin. Measurements were made from the midpoint of the boundary. At the end of the experiments, samples were analyzed by polyacrylamide gel electrophoresis to show that no degradation had occurred.

RESULTS

We first compared filament size and actin-activated ATPase activity of phosphorylated and dephosphorylated myosin II as a function of MgCl2 concentration (Fig. 1). These experiments were carried out at a myosin concentration of 120 μg/ml, which was near the minimal concentration at which reliable sedimentation coefficients could be obtained and near the maximal concentration at which the enzymatic activity was proportional to the myosin concentration. Dephosphorylated myosin II was enzymatically inactive at 1 mM MgCl2, where it had a sedimentation coefficient of about 21 S, and fully active at 4 mM MgCl2, where it had a sedimentation coefficient of 31 S. With increasing concentrations of MgCl2 up to 8 mM, the filaments of dephosphorylated myosin II increased in sedimentation coefficient to about 120 S with no increase in enzymatic activity. The slight decrease in ATPase activity observed at the higher concentrations of MgCl2 may be due to effects on the F-actin or on the myosin (8). In contrast, phosphorylated myosin II was enzymatically active over the entire range of MgCl2 concentration although the filaments increased in sedimentation coefficient from 19 S in 1 mM MgCl2 to 57 S in 10 mM MgCl2. Therefore, although dephosphorylated and phosphorylated myosin II form filaments of different sizes under identical conditions, this difference does not account for the difference in their actin-activated ATPase activities. We do not, however, know if the increase from 21 to 31 S for dephosphorylated myosin II was causally related to its increase in enzymatic activity between 1 and 4 mM MgCl2. Monomeric myosin II in 0.6 M KCl, 5% sucrose was 4.8 S.

Table I

| Myosin species       | Myosin concentration | Sedimentation coefficient | Actin-activated ATPase activity |
|----------------------|----------------------|---------------------------|---------------------------------|
|                      | µg/ml                | S                        | nmol/min/mg                    |
| A. 4 mM MgCl2 and 10% sucrose |                   |                           |                                 |
| Dephosphorylated     | 60                   | 21                        | 49                              |
| Phosphorylated       | 60                   | 16                        | 2.4                             |
| Copolymer, 1:1       | 120 ND              | ND                        | 11                              |
| B. 4 mM MgCl2 and 5% sucrose |                   |                           |                                 |
| Dephosphorylated     | 60                   | 22                        | 0.37                            |
| Phosphorylated       | 60                   | 30                        | 46                              |
| Copolymer, 1:1       | 120                 | 20                        | 0.02                            |
| C. 7 mM MgCl2 and 10% sucrose |                   |                           |                                 |
| Dephosphorylated     | 120                 | 34                        | 51                              |
| Phosphorylated       | 120                 | 16                        | 3                              |
| Copolymer, 1:1       | 240                 | 20                        | 0.23                            |
| D. 7 mM MgCl2 and 5% sucrose |                   |                           |                                 |
| Dephosphorylated     | 120                 | 126                       | 49                              |
| Phosphorylated       | 120                 | 25                        | 1                              |
| Copolymer, 1:1       | 240                 | 38                        | 0.03                            |

* Sedimentation coefficients have not been corrected to standard conditions of 20 °C in H2O.

** ND, not determined.

Specific activity of the dephosphorylated molecules in the copolymer assuming the specific activity of the phosphorylated molecules are the same in the copolymer and the homopolymer.

Specific activity of the copolymer assuming phosphorylated and dephosphorylated molecules in the copolymer are equally active.
myosin in the copolymer is 22% that of the dephosphorylated myosin homopolymer. Very similar results were obtained in an experiment in 7 mM MgCl₂ and 10% sucrose (Table I, C).

The results of similar experiments carried out in 7 mM MgCl₂ and 5% sucrose (Table I, D) suggest that it may be more correct to calculate the specific activities of the copolymers based on their total myosin concentrations. Under these incubation conditions, the 1:1 copolymer was again much nearer in size to the phosphorylated homopolymer than to the dephosphorylated homopolymer, although all of the filaments were larger than in the previous conditions. However, actin-activated ATPase activity of the copolymer was greater in 7 mM MgCl₂ than in 4 mM MgCl₂ and, in fact, was equal to or higher than the sum of the activities of the homopolymers separately, 11% higher in the experiment reported in Table I, D. If the activity of the copolymer was due entirely to its content of dephosphorylated myosin, these molecules would have had an 18% higher specific activity in the copolymer than in their homopolymer, which is possible but quite unlikely. But, if we assume that all of the myosin molecules in the copolymer were equally active, they would have had only about 56% of the specific activity of the molecules in the homopolymer of dephosphorylated myosin.

We next compared the actin-activated ATPase activities of heteropolymers over a range of compositions to mixtures of homopolymers at the same ratios of dephosphorylated to phosphorylated myosin II and to the individual homopolymers at their concentrations in the heteropolymers (Fig. 2). The activity of the homopolymers of dephosphorylated myosin II was not affected by the presence in the incubation mixture of the homopolymers of phosphorylated myosin II but the heteropolymer was less active than the mixture of homopolymers at all ratios of phosphorylated myosin to dephosphorylated myosin. This establishes that the lower activities of the heteropolymers were not due to inhibitory substances in the solution of phosphorylated myosin II. The enzymatic data for the homopolymers and heteropolymers in Fig. 2 are replotted as specific activities in Fig. 3.

**DISCUSSION**

Because myosin II is filamentous under assay conditions (7, 8), the phosphorylation sites at the end of the tails are far removed from the catalytic site in the same polypeptide chain (4, 5), and it, therefore, seemed unlikely that phosphorylation could regulate the actin-activated ATPase activity intramolecularly, i.e. that the ATPase activity of a myosin head was a function of the state of phosphorylation of its own tail. Although phosphorylated and dephosphorylated myosin II do form different size filaments under a variety of conditions (Ref. 8 and Fig. 1), the data in Fig. 1 show that a difference in filament size cannot explain their different enzymatic activities. The data in Table I and Figs. 2 and 3 establish that the regulation is, in fact, intermolecular, i.e. fully phosphorylated myosin molecules inhibit the ATPase activity of fully dephosphorylated myosin molecules when they are contained within the same filament.

From the structure of the myosin II filaments (11) and the positions of the phosphorylation and catalytic sites within the myosin molecule (4, 5), however, it seems unlikely that the phosphorylated myosin molecules inhibit the activity of dephosphorylated myosin molecules by direct interaction. More probably, regulation by phosphorylation is exerted through some general conformational effect on the filament as a whole. In this case, it might well be that all the myosin heads in the heteropolymers have the same specific activity irrespective of whether they are attached to phosphorylated or to dephosphorylated tails. On this basis, the specific activity of each myosin molecule in the 1:1 copolymer in Fig. 3 would be about
25% of the specific activity of a dephosphorylated myosin molecule in the homopolymer and about 10 times greater than the specific activity of a phosphorylated myosin molecule in its homopolymer. The relative activities of myosin molecules in heteropolymers and homopolymers are significantly influenced by the compositions of the heteropolymer (Figs. 2 and 3) and the concentrations of MgCl₂ and sucrose (Table I).

Because we are as yet unable to prepare myosin uniformly phosphorylated at specific sites, it is impossible to determine at this time whether a homopolymer of 50% phosphorylated myosin would have the same specific activity as a 1:1 copolymer of fully phosphorylated and fully dephosphorylated myosin. Moreover, with six different phosphorylation sites available on each myosin molecule, and allowing for the possibility of random heteropolymers, the number of permutations and combinations of filament composition is very large indeed. But, irrespective of whether the enzymatic activity reflects the specific location of the phosphorylated residues or the overall state of phosphorylation, the data in this paper establish that the regulation of the enzymatic activity of *Acanthamoeba* myosin II by phosphorylation occurs at a supramolecular level within a myosin filament.

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