Proteolytic Separation of the Actin-activatable ATPase Site from the Phosphorylation Site on the Heavy Chain of Acanthamoeba Myosin IA*

Hiroshi Maruta and Edward D. Korn

From the Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Previous work (Maruta, H., Gadasi, H., Collins, J. H., and Korn, E. D. (1978) J. Biol. Chem. 253, 6292-6300) had shown that phosphorylation of the heavy chain of Acanthamoeba myosin IA is required for actin activation of its Mg\(^{2+}\)-ATPase activity and that, like the phosphorylation site, the catalytic site and the actin binding site are also on the heavy chain. We now show that limited digestion of phosphorylated myosin IA by subtilisin allows separation of the catalytically active peptide fragment from the phosphorylated peptide without any significant loss of actin-activated Mg\(^{2+}\)-ATPase activity. A proteolytic fragment with full actin-activated Mg\(^{2+}\)-ATPase activity has also been isolated from subtilisin digests of nonphosphorylated myosin IA, which, before proteolysis, did not have actin-activated Mg\(^{2+}\)-ATPase activity. The simplest interpretation of these data is that, in its nonphosphorylated state, the phosphorylation site of Acanthamoeba myosin IA inhibits the catalytic site and that this inhibition can be reversed either by phosphorylation of the site or by proteolytically separating it from the catalytic site. Alternatively, phosphorylation and proteolysis may, by unrelated mechanisms, induce similar conformational changes in the myosin heavy chain that lead to activation of its actomyosin ATPase activity.

Phosphorylation of the heavy chains of single-headed Acanthamoeba myosins IA and IB is required for actin activation of their Mg\(^{2+}\)-ATPase activities (1, 2). In the accompanying paper (3), we showed that the ATPase catalytic site is on the heavy chain of several myosins including Acanthamoeba myosins IA and IB. Therefore, in contrast to vertebrate smooth muscle and non-muscle myosins which are regulated by phosphorylation of their 20,000 molecular weight light chains (4), both the regulatory and catalytic sites of Acanthamoeba myosins IA and IB, as well as the actin-binding site (2), are on the same (heavy) chain. The catalytic (3) and phosphorylation (5) sites of double-headed Acanthamoeba myosin II are also both on the heavy chains but with the important differences (5) that there are two phosphorylation sites per heavy chain and that dephosphorylation, rather than phosphorylation, is required for actin activation of the Mg\(^{2+}\)-ATPase activity of Acanthamoeba myosin II.

In a general sense, modification of the phosphorylation sites of myosins could regulate their catalytic sites by either activation or derepression. In this paper, we show that the phosphorylation and catalytic sites on the heavy chain of Acanthamoeba myosin IA can be separated by subtilisin digestion and that the reisolated catalytic site has complete enzymatic activity including actin-activated Mg\(^{2+}\)-ATPase activity in the absence of the phosphorylation site.

MATERIALS AND METHODS

Acanthamoeba myosin IA (6) and partially purified Acanthamoeba myosin I heavy chain kinase (1) were isolated as described previously. F-actin was prepared from acetone powders of rabbit skeletal muscle (7). Subtilisin was purchased from Sigma Chemicals. ADP-agarose (type IV) from P-L Biochemicals, Bio-Gel A-0.5m from Bio-Rad Laboratories, and [\(\alpha^{32}P\)]ATP and [\(\gamma^{32}P\)]ATP from New England Nuclear. All other chemicals were reagent grade.

Protein concentrations were measured by the procedure of Lowry et al. (8); sodium dodecyl sulfate polyacrylamide gel electrophoresis was as described by Laemml (9) and the gels were stained with Coomassie brilliant blue according to the method of Fairbanks et al. (10). ATPase assays were carried out by measuring the release of radioactive P from [\(\gamma^{32}P\)]ATP as described by Pollard and Korn (11). For (K\(^{+}\),EDTA)-ATPase activity, the buffer contained 20 mM Tris/chloride, pH 7.5, 1 mM ATP, 0.5 mM KCl, and 2 mM EDTA; for Mg\(^{2+}\)-ATPase activity the buffer contained 20 mM imidazole chloride, pH 7.5, 1 mM ATP, and 2 mM MgCl\(_{2}\). Protein-bound [\(\gamma^{32}P\)]phosphate was determined by the filter paper assay described by Pettit et al. (12).

RESULTS AND DISCUSSION

To demonstrate the correlation between phosphorylation of Acanthamoeba myosin IA and the increase in actin-activated Mg\(^{2+}\)-ATPase activity, myosin was incubated with varying concentrations of kinase and either 0.25 mM [\(\gamma^{32}P\)]ATP or nonradioactive ATP under otherwise identical conditions. The concentration of kinase was varied, rather than the time of incubation, because this procedure minimized proteolysis of the myosin by proteases contaminating the partially purified kinase. The incubations with radioactive ATP were monitored for incorporation of protein-bound radioactivity and the corresponding nonradioactive samples were assayed for their actin-activated Mg\(^{2+}\)-ATPase activities (Fig. 1A). Phosphorylation reached a maximum value of 0.72 mol/mol of myosin and actin-activated Mg\(^{2+}\)-ATPase activity reached a maximum value of 1.52 pmol/min/mg of myosin. The ATPase activity was a direct linear function of the level of phosphorylation (Fig. 1B).

In preliminary experiments, to determine the physical relationship between the phosphorylation site and the ATP binding (catalytic) site on the myosin heavy chain, one sample of Acanthamoeba myosin IA was photofinity-labeled at the catalytic site with [\(\alpha^{32}P\)]ATP (3) and a second sample was labeled on the phosphorylation site by incubation with myosin I heavy chain kinase and [\(\gamma^{32}P\)]ATP. The two samples were then separately incubated with subtilisin under identical conditions and the digestion products were separated by sodium dodecyl sulfate polyacrylamide electrophoresis (Fig. 2).

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The original myosin IA preparation was either already slightly degraded, or not entirely free from impurities, as shown by the pattern of staining with Coomassie blue (Fig. 2, Lane A). Nonetheless, the radioactivity derived from photoaffinity labeling with [γ-32P]ATP was almost entirely restricted to the 130,000 molecular weight heavy chain (Fig. 2, Lane a). Upon digestion with subtilisin, radioactivity seemed to appear sequentially (Fig. 2, Lanes b to d) in a 28,000 molecular weight peptide and the gel front, which would contain the radioactive 16,000 molecular weight peptide identified in the accompanying paper (3). A 115,000 molecular weight peptide may have been the first detectable radioactive degradation product (Fig. 2, Lane b).

The 130,000 molecular weight heavy chain of Acanthamoeba myosin IA was also heavily labeled when the myosin was incubated with myosin I heavy chain kinase and [γ-32P]ATP (Fig. 2, Lane c), but other minor peptides, most probably derived from the partially purified kinase preparation, were also radioactive. Upon subtilisin digestion, the radioactivity originally in the phosphorylated heavy chain seemed to appear sequentially in a 115,000 molecular weight peptide, an 87,000 molecular weight peptide, and then, finally, in unidentified peptides that ran at the gel front (Fig. 2, Lanes f and g). One interpretation of the data in Fig. 2 is that a 115,000 molecular weight peptide contained both sites but an 87,000 molecular weight peptide contained only the phosphorylation site and a 28,000 molecular weight peptide contained only the catalytic site. From this analysis it appeared that subtilisin cleaves the heavy chain of Acanthamoeba myosin IA between the phosphorylation and catalytic sites and that, therefore, it might be possible to separate and isolate the peptides that contain the two sites and study their properties.

For this experiment, Acanthamoeba myosin IA was phosphorylated to the extent of 0.79 mol/mol of heavy chain by incubation with myosin I kinase and [γ-32P]ATP and then separated from radioactive ATP and myosin I heavy chain kinase by sequential chromatography on Bio-Gel A-0.5m and ADP-agarose (1, 6). The 32P-labeled myosin IA was digested with subtilisin as described in Fig. 3. About 95% of the original (K',EDTA)-ATPase activity was recovered after this limited proteolysis. The subtilisin digest was then fractionated by chromatography on ADP-agarose. About 95% of the applied (K',EDTA)-ATPase activity was recovered from the column: 20% of the enzymatic activity was eluted with 50 mM KCl and 80% of the enzymatic activity was eluted with 1 M KCl (Fig. 3).

About 98% of the applied radioactivity was also recovered from the ADP-agarose column but it was distributed between the two fractions differently than the enzymatic activity (Fig. 3): 70% of the radioactivity was eluted with 50 mM KCl and only about 30% was eluted with 1 M KCl. Therefore, the fraction that was eluted with 1 M KCl was about 3-fold enriched in functional catalytic sites relative to phosphorylation sites. Moreover, since the ratio of subtilisin to myosin IA (1:50) was greater than the maximum ratio used in the experiment described in Fig. 2 (1:200), the catalytic site and the contaminating phosphorylation site were almost certainly in separate peptides. Nevertheless, this fraction retained full actin-activated Mg2+-ATPase activity (Table I), even in the absence of added myosin I heavy chain kinase, indicating that the phosphorylation site is not required for actin-activated enzymatic activity.

In a similar experiment, nonphosphorylated Acanthamoeba myosin IA was incubated with subtilisin and the products of digestion were fractionated by gel filtration on Bio-Gel A-0.5m (Fig. 4). All of the (K',EDTA)-ATPase activity was recovered in two broad, overlapping peaks: ST-1, which was eluted in the molecular weight range of about 17,000 to 68,000, and ST-2, which was eluted at a position corresponding to a molecular weight of less than 17,000. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5), ST-1 was found to contain no major peptide larger than molecular weight about 28,000 and ST-2 contained no peptide detectable by Coomassie blue staining of molecular weight greater than about 17,000. Aliquots of the two fractions, ST-1 and ST-2, were then photoaffinity-labeled with [α-32P]ATP (3) and separated by sodium dodecyl sulfate-polyacrylamide electropho-

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1 The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid.
Regulation of the Catalytic Site of Acanthamoeba Myosin IA

**Fig. 2.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis of limited subtilisin digests of [α-^32P]ATP-phosphorylated or [α-^32P]ATP photoaffinity-labeled Acanthamoeba myosin IA. Myosin was either photoaffinity-labeled with [α-^32P]ATP (3) or maximally phosphorylated by incubation with kinase and [γ-^32P]ATP (Fig. 1). The two preparations of radioactive myosins (20 μg) were incubated separately with 25, 50, or 100 ng of subtilisin for 15 min at 35°C in 0.05 ml of 10 mM Tris/chloride, pH 7.5, 2 mM MgCl₂, 1 mM dithiothreitol, and 1 mM EGTA. The samples were then heated at 95°C for 30 min in 2% sodium dodecyl sulfate, 40 mM dithiothreitol, and aliquots containing 10 μg of myosin or its digestion products were separated by electrophoresis on 11.3% polyacrylamide gels. The gels were stained with Coomassie blue, dried under vacuum, and the radioactive peptides were detected by autoradiography using Kodak X-ray film NS-2T.

**Fig. 3.** Separation of the [γ-^32P]phosphorylated fragment from the catalytic fragment following limited subtilisin digestion of phosphorylated Acanthamoeba myosin IA. Myosin (2 mg) was phosphorylated by incubation with myosin I heavy chain kinase (40 μg) and [γ-^32P]ATP in a total volume of 1 ml as described in Fig. 1. The [γ-^32P]phosphorylated myosin was separated from the radioactive ATP by chromatography on Bio-Gel A-0.5m in 0.3 M KCl, 10 mM Tris/chloride, pH 7.5, 1 mM dithiothreitol, and from the kinase by affinity chromatography on ADP-agarose (1). The reisolated [γ-^32P]-phosphorylated myosin (1 mg/ml) was incubated with subtilisin (20 μg/ml) for 15 min at 35°C in 0.5 ml of 10 mM Tris/chloride, pH 7.5, 2 mM MgCl₂, 1 mM dithiothreitol, 1 mM EGTA. The digest was then fractionated on a column of ADP-agarose (1.2 × 1.0 cm) by stepwise elution with 50 mM KCl followed by 1 M KCl + 2 mM EDTA in 15 mM Tris/chloride, pH 7.5, 1 mM dithiothreitol (1). Fractions of 1 ml were collected and assayed for (K⁺,EDTA)-ATPase activity (○) and radioactivity (●).

**Table I**

| Fraction Assayed | ATPase activity (μmol/min/mg) | Mg²⁺-ATPase activity (μmol/min/mg) |
|------------------|-------------------------------|---------------------------------|
| Phosphorylated myosin IA | 2.52 | 0.08 | 1.15 | 1.19 |
| Catalytic fragment | 2.40 | 0.04 | 1.02 | 0.95 |

resis. Only two radioactive peptides, with molecular weights of 28,000 and 16,000, were detected (data not shown). These radioactive peptides corresponded in electrophoretic mobilities to those observed when myosin IA was photoaffinity-labeled before digestion with subtilisin (3).

Even though they were derived from myosin IA that had neither been phosphorylated nor exposed to ATP prior to the enzymatic assay, both ST-1 and ST-2 contained appreciable actin-activated Mg²⁺-ATPase activity in the absence of myosin I heavy chain kinase (Table II). Thus, these data show that Acanthamoeba myosin IA can be degraded by subtilisin
Regulation of the Catalytic Site of Acanthamoeba Myosin IA

**Table II**

Actin-activated Mg\(^{2+}\)-ATPase activity of the fractions isolated from the subtilisin digestion of nonphosphorylated Acanthamoeba myosin IA

| Fraction assayed | (K\(^+\), EDTA) | Mg\(^{2+}\) | ATPase activity |
|------------------|----------------|------------|-----------------|
| Myosin IA        | 2.4            | 0.10       | 0.21            |
|                  | 2.4            | 0.90       | 1.00            |
| ST-2             | 2.8            | 0.02       | 1.08            |
|                  |                |            | 1.32            |

Fig. 4. Isolation of the catalytic fragment following limited subtilisin digestion of nonphosphorylated *Acanthamoeba* myosin IA. Myosin (2 mg) was incubated with subtilisin (40 μg) at 35°C for 15 min in a total volume of 2 ml of 10 mM Tris/chloride, pH 7.5, 2 mM MgCl₂, 1 mM dithiothreitol, 1 mM EGTA. The digest was then fractionated on a Bio-Gel A-0.5m column (50 ml) in a buffer of 0.3 M KCl, 10 mM Tris/chloride, pH 7.5, 1 mM dithiothreitol. The eluate was assayed for (K\(^+\), EDTA)-ATPase activity. The elution position of undigested *Acanthamoeba* myosin IA (0) from the same column is also shown for purposes of comparison and the elution positions of smooth muscle myosin (500,000), bovine serum albumin (68,000), and myoglobin (17,000) are also shown.

Fig. 5. Sodium dodecyl sulfate polyacrylamide electrophoresis of the enzymatically active fragments obtained by subtilisin digestion of *Acanthamoeba* myosin IA. Intact myosin IA (A, 10 μg) and pooled fractions ST-1 (B, 5 μg) and ST-2 (C, 5 μg) from Fig. 4 were separated on 16% polyacrylamide gels after heating for 30 min at 95°C in 2% sodium dodecyl sulfate, 43 mM dithiothreitol. The gels were stained with Coomassie blue.

To enzymatically active fragments with molecular weights by gel filtration as low as approximately 20,000. The peptide that contains the catalytic site in these active fractions can have a molecular weight as low as 16,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The fact that proteolytic fragments of *Acanthamoeba* myosin IA can have full actin-activated Mg\(^{2+}\)-ATPase activity in the absence of phosphorylation by myosin I heavy chain kinase, and when separated from the peptide that contains the phosphorylation site, proves that the phosphorylation site is not required for enzymatic activity. Therefore, it seems most likely that, for intact *Acanthamoeba* myosin IA, phosphorylation of the heavy chain is necessary in order to overcome the inhibition of actin-activated Mg\(^{2+}\)-ATPase activity that occurs when the phosphorylation site is present in its nonphosphorylated form. According to this interpretation, the actin-activated Mg\(^{2+}\)-ATPase activity of the catalytic site can also be derepressed by proteolytic removal of the peptide that contains the phosphorylation site. Another possibility, which seems to us less likely, is that phosphorylation and proteolysis are alternative ways to induce the conformational changes necessary for actin-activated *Acanthamoeba* myosin IA Mg\(^{2+}\)-ATPase activity. Similar conclusions have been reached for the regulation of smooth muscle myosin (13-16) where the phosphorylation and catalytic sites reside on different peptides. One of the next tasks is to determine the nature of structural alterations in the myosin heavy chains induced by phosphorylation, dephosphorylation, and proteolysis.

**REFERENCES**

1. Maruta, H., and Korn, E. D. (1977) J. Biol. Chem. 252, 8329-8332
2. Maruta, H., Gadasi, H., Collins, J. H., and Korn, E. D. (1978) J. Biol. Chem. 253, 6297-6300
3. Maruta, H., and Korn, E. D. (1981) J. Biol. Chem. 256, 499-502
4. Adelstein, R. S., and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921-956
5. Collins, J. H., and Korn, E. D. (1980) J. Biol. Chem. 255, 8011-8014
6. Maruta, H., Collins, J. H., and Korn, E. D. (1979) J. Biol. Chem. 254, 3624-3630
7. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866-4871
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
9. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-688
10. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617
11. Pollard, T. D., and Korn, E. D. (1973) J. Biol. Chem. 248, 4692-4699
12. Pettit, F. H., Hamilton, L., Munk, P., Namihira, G., Eley, M. H., Wilms, C. R., and Reed, L. J. (1973) J. Biol. Chem. 248, 5282-5290
13. Sobieszek, A., and Small, J. V. (1976) J. Mol. Biol. 101, 75-92
14. Seidel, J. C. (1978) Biochem. Biophys. Res. Commun. 85, 107-113
15. Mrva, U., Troschka, M., Gross, C., and Katzinski, L. (1980) Eur. J. Biochem. 103, 415-419
16. Seidel, J. C. (1980) J. Biol. Chem. 255, 4355-4361