The Effect of Actin and Phosphorylation on the Tryptic Cleavage Pattern of Acanthamoeba Myosin IA*

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The Mg\(^{2+}\)-ATPase activity of Acanthamoeba myosin IA is activated by F-actin only when the myosin heavy chain is phosphorylated at a single residue. In order to gain insight into the conformational changes that may be responsible for the effects of F-actin and phosphorylation on myosin I ATPase, we have studied their effects on the proteolytic release of the myosin IA heavy chain by trypsin. Trypsin initially cleaves the unphosphorylated, 140-kDa heavy chain of Acanthamoeba myosin IA at sites 38 and 112 kDa from its NH\(_2\) terminus and secondarily at sites 64 and 91 kDa from the NH\(_2\) terminus. F-actin has no effect on tryptic cleavage of the 91- and 112-kDa sites, but does protect the 38-kDa site and the 64-kDa site. Phosphorylation (which occurs very near the 38-kDa site) has no detectable effect on the tryptic cleavage pattern in the absence of F-actin or on F-actin protection of the 64-kDa site, but significantly enhances F-actin protection of the 38-kDa site. Protection of the 64-kDa site is probably due to direct steric blocking because F-actin binds to this region of the heavy chain. The protection of the 38-kDa site by F-actin may be the result of conformational changes in this region of the heavy chain induced by F-actin binding near the 64-kDa site and by phosphorylation. The conformational changes in the heavy chain of myosin IA that are detected by alterations in its susceptibility to proteolysis are likely to be related to the conformational changes that are involved in the phosphorylation-regulated actin-activated Mg\(^{2+}\)-ATPase activities of Acanthamoeba myosins IA and IB.

Myosins IA and IB from Acanthamoeba castellani are monomeric proteins consisting of single heavy and light chains (140 and 17 kDa for IA, and 127 and 27 kDa for IB) whose actin-activated Mg\(^{2+}\)-ATPase activities depend on the phosphorylation of a single amino acid in the heavy chain (1-7). Myosins I cross-link actin filaments (8, 9), support superprecipitation of F-actin (8), and translocate latex beads (4) and organelles (10) along actin cables in \textit{vitro}. Similar enzymes have been isolated from Dictyostelium discoideum (11) and intestinal brush border (12, 13), and a related protein occurs in Drosophila photoreceptor cells (14).

Because the myosin I isoforms do not themselves form filaments, we have suggested that their ability to cross-link, precipitate F-actin, and translocate filaments derives from the fact that their single heavy chains contain two actin-binding sites (8, 9, 15, 16). Binding of F-actin to the site that is thought to be common to all myosins is weakened by MgATP and stimulates the Mg\(^{2+}\)-ATPase activity of myosin I (9, 15, 16). Binding of F-actin to the site that may be unique to myosins I is unaffected by MgATP and does not directly enhance the Mg\(^{2+}\)-ATPase activity, although it is responsible for the unusual triphasic actin dependence (activation, inhibition, and reactivation) of the enzymatic activity (9, 15, 16).

The MgATP-insensitive actin-binding site of the Acanthamoeba myosin I isoforms is located in the COOH-terminal segment of the heavy chain that can be released by controlled proteolysis (9, 16). The remaining NH\(_2\)-terminal portion of the molecule (112 kDa for myosin IA and 80 kDa for myosin IB) retains most of the actin-activated Mg\(^{2+}\)-ATPase activity of the intact molecule, but the dependence of the ATPase activity on F-actin concentration is hyperbolic (9, 16), as it should be when there is only one actin-binding site. Thus, the enzymatic properties of the NH\(_2\)-terminal fragments of Acanthamoeba myosins IA and IB are similar to those of the more extensively studied subfragment-1 of skeletal muscle myosin. The main difference is that the actin-activated Mg\(^{2+}\)-ATPase activities of the NH\(_2\)-terminal 112-kDa myosin IA and 80-kDa myosin IB fragments still depend on phosphorylation of a single amino acid residue in the heavy chain.

We have been studying the structure-function relationships of these enzymatically active NH\(_2\)-terminal regions in some detail (reviewed in Ref. 7). Although the 80-kDa fragment of myosin IB lacks the region corresponding to the NH\(_2\)-terminal 9 kDa of muscle myosin subfragment-1, the rest of the sequence is 55% similar to that of subfragment-1 (17). Also, several functionally important regions of the NH\(_2\)-terminal 80-kDa myosin IB (fragment and subfragment-1) are similar. For example, based on sequence, the trypsin cleavage site 62 kDa from the NH\(_2\) terminus of the myosin IB heavy chain (16) is homologous to the trypsin cleavage site 75 kDa from the NH\(_2\) terminus of subfragment-1 (18, 19), and both are protected from cleavage when F-actin is bound to the ATP-sensitive binding site (16, 18, 19). In both myosin derivatives, the region immediately to the COOH side of these sites is essential for the actin-activated Mg\(^{2+}\)-ATPase activity (16; for reviews, see Refs. 20 and 21). Therefore, the ATP-sensitive actin-binding site of myosin IB is most likely in the same location as in subfragment-1, \textit{i.e.} in the neighborhood, and presumably on both sides, of the protected 62-kDa site. Finally, the location of the ATP-binding site (purine ring) of myosin IB (about 12 kDa from the NH\(_2\) terminus (17, 22)) is consistent with the position of the ATP-binding site of subfragment-1 (20, 21, 23-25). Interestingly, the regulatory phosphorylation site of myosin IB probably lies between the ATP-binding and ATP-sensitive actin-binding sites (16, 26).

The amino acid sequence of the heavy chain of myosin IA is not known. The ATP-binding site of myosin IA has been localized only to the NH\(_2\)-terminal 27-kDa region (26), the

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regulatory phosphorylation site to between 38 and 58 kDa of the NH₂-terminal tail and the ATP-sensitive actin-binding site only to the NH₂-terminal 112 kDa (26). Furthermore, the smallest fragment of myosin IA shown to have actin-activated Mg²⁺-ATPase activity (112 kDa (9)) is appreciably larger than the active fragment prepared from myosin IB (80 kDa (16)).

The functional analysis of the structure of the heavy chain of Acanthamoeba myosin IA is significantly extended in this paper. We show (i) that the region of myosin IA that corresponds functionally to subfragment-1 can be restricted to the NH₂-terminal 80 kDa (as for myosin IB), (ii) that the ATP-sensitive actin-binding site of myosin IA is in a position similar to that of myosin IB and subfragment-1, and, perhaps most interesting, (iii) that phosphorylation of the amino acid residue that regulates the actin-activated Mg²⁺-ATPase activity of myosin IA causes a conformational change in a specific region of the heavy chain in an actomyosin IA rigor complex.

MATERIALS AND METHODS

Purification of Protein—Acanthamoeba myosin IA was purified as described previously (2, 3, 9) and stored in 50% glycerol, 1 mM dithiothreitol, 100 mM KCl, 0.01% sodium azide, 10 mM Tris·HCl, pH 7.5, at -20 °C for no longer than 5 days. Myosin I heavy chain kinase was purified according to Hammer et al. (6).

Rabbit skeletal muscle actin was purified according to Spudich and Watt (27) followed by gel filtration on Sephadex G-200. The actin concentration was calculated from its (K⁺,EDTA)-ATPase activity assuming a specific activity of 0.617 mg⁻¹·ml⁻¹·min⁻¹ (28). The myosin I concentration was calculated from its (K⁺,EDTA)-ATPase activity assuming a specific activity of 0 µmol min⁻¹·mg⁻¹ (16). The concentration of myosin IA fragments was estimated by scanning Coomassie Blue-stained gels using myosin IA as a standard.

**Active Site Labeling of Myosin IA and its Fragments**—Myosin IA (0.7–1.5 mg/ml) was phosphorylated by myosin I heavy chain kinase (10 µg/ml) at 30 °C for 10 min in 5 mM Tris·HCl, pH 7.5, containing 50 mM KCl, 5 mM MgCl₂, 0.2 mM ATP, 0.5 mM dithiothreitol, and 25% glycerol. For fluorography, [γ-³²P]ATP was added up to 0.06 µCi/ml. Proteolytic fragments of myosin were phosphorylated by incubating the digest mixture in buffer T with added myosin I heavy chain kinase and [γ-³²P]ATP. Since the concentration of the proteolytic fragments was at least 10-fold less than the concentration of intact myosin IA, when it was the kinase substrate, different conditions were used to maximize phosphorylation of the fragments: ATP was used at twice the specific radioactivity and 10 times the concentration, and the kinase concentration and incubation time were doubled. Myosin IA and its fragments were labeled with [5,6-³²H]UTP according to Maruta and Korn (31) except that the specific activity of [5,6-³²H]UTP used for labeling the fragments was 10 times higher. Proteins labeled with [³²P] and [³⁵S] were visualized by fluorography according to Laskey (32) after separation by SDS-PAGE.

**Actin-binding Assay**—The digest mixtures containing the fragments of myosin IA and F-actin were centrifuged for 60 min at 30 p.s.i. (165,000 × g) in a Beckman airfuge at room temperature. Binding was assessed by SDS-PAGE analysis of samples before and after centrifugation.

**Reagents**—ATP, l-1-tyosylamide-2-phenylethyl chloromethylketone-trypsin, soybean trypsin inhibitor, and PMSF were from Sigma. [γ-³²P]ATP was from Du Pont-New England Nuclear and [5,6-³²H]UTP from ICN. Electrophoresis reagents and molecular weight standards were from Bio-Rad. All other materials were reagent grade.

RESULTS

**Localization of F-actin-protected Tryptic Cleavage Sites in Myosin IA**—Previously, we localized an ATP-sensitive actin-binding site in myosin Acanthamoeba myosin IB to a position about 62 kDa from the NH₂-terminal residue by demonstrating that F-actin protects against tryptic cleavage at this site in the absence, but not in the presence, of MgATP (16). This corresponds to a similar site 75 kDa from the NH₂-terminal of subfragment-1 (17, 18). In this section, we present evidence that F-actin protects myosin IA against tryptic cleavage at sites 38 and 64 kDa from its NH₂-terminal.

In the absence of F-actin, trypsin initially cleaves the heavy chain of phosphorylated and unphosphorylated myosin IA at sites 38 and 112 kDa from its NH₂-terminal (26). These two cleavages produce a COOH-terminal 27-kDa peptide, and a

![FIG. 1. Effect of F-actin and heavy chain phosphorylation on tryptic digestion of Acanthamoeba myosin IA: SDS-PAGE peptide patterns and identification of peptides containing the phosphorylation site. The digestions were performed as described under "Materials and Methods" in the presence or absence of F-actin and with or without myosin heavy chain phosphorylation as indicated. The digestions were stopped by adding PMSF except for the experiment shown in E when soybean trypsin inhibitor was used. Lanes 1–9 correspond to samples removed after 0, 1, 3, 10, 20, 40, 60, 90, and 120 min of digestion, respectively. The positions of the myosin IA heavy chain (HC) and the tryptic fragments (in kilodaltons) obtained in the absence of F-actin are indicated on the left. The positions of actin and the tryptic fragments (in kilodaltons) obtained in the presence of F-actin are indicated on the right. A–C are Coomassie Blue-stained gels. D–F are ³²P-labeled fluorograms. When phosphorylated myosin IA was digested (A, C, D, and F) the myosin was phosphorylated before digestion using [γ-³²P]ATP and kinase. When unphosphorylated myosin IA was digested (B and E), the digestion mixtures (after stopping digestions with soybean trypsin inhibitor) were incubated with [γ-³²P]ATP and kinase. Phosphorylation conditions are described under "Materials and Methods". Autophosphorylation of the myosin I heavy chain kinase occurred under the conditions used to phosphorylate the tryptic fragments, and it can be seen in E. Lane K is a ³²P-labeled fluorogram of autophosphorylated kinase incubated with [γ-³²P]ATP in the absence of myosin IA and actin.
complex between the NH2-terminal 38-kDa and central 74-kDa peptides with associated light chain. This digestion pattern was confirmed in the experiment shown in Fig. 1A which shows the formation of the 38- and 74-kDa peptides. With the higher trypsin:myosin IA ratio used in these experiments the 27-kDa peptide was mostly degraded to smaller peptides. As shown before (26), the 74-kDa peptide contained the phosphorylation site (Fig. 1D), and the NH2-terminal 38-kDa peptide contained the ATP-binding site (Fig. 2A). Secondary cleavages occur at sites 64 and 91 kDa from the NH2 terminus (26). When the secondary cleavage was at the 64-kDa site, the 74-kDa peptide was converted to an unphosphorylated 48-kDa peptide (Fig. 1, A and D) and a phosphorylated 26-kDa peptide (Fig. 1D) that was barely detectable on the Coomassie Blue-stained gel (Fig. 1A). When secondary cleavage occurred at the 91-kDa site, the 74-kDa peptide was converted to an unphosphorylated 53-kDa peptide (Fig. 1, A and D) and a 21-kDa peptide that was further degraded (26) and not detected on polyacrylamide gels (Fig. 1A). These cleavage sites and the resulting peptides are illustrated diagrammatically in the upper portion of Fig. 4.

We show now that the cleavage pattern of unphosphorylated myosin IA is significantly different when the digestion is carried out in the presence of F-actin (Fig. 1B). Initially, a 112-kDa peptide was produced and it was then converted into a 91-kDa peptide. Neither of these two peptides was produced in significant amounts when the digestion was carried out in the absence of F-actin (Fig. 1A). Both the 112- and 91-kDa peptides contained the phosphorylation (Fig. 1F) and ATP-binding (Fig. 1E) sites. Thus, cleavage could not have occurred at the 38-kDa tryptic site, because it lies between the phosphorylation and ATP-binding sites. Because the mass of the intact myosin IA heavy chain is only 140 kDa, both the 112- and 91-kDa peptides must also have contained an uncleaved 64-kDa tryptic site. Thus, formation of these two fragments indicates that both the 38- and the 64-kDa tryptic sites were protected by F-actin.

Further digestion in the presence of F-actin resulted in the disappearance of the 91-kDa peptide (Fig. 1B), accumulation of the 38-kDa peptide (Fig. 1B) that contained the ATP-binding site (Fig. 2B), and the appearance of 53-, 42-, and 26-kDa peptides all of which contained only the phosphorylation site (Fig. 1E). Compared to digestions of myosin IA in the absence of F-actin, the 53-kDa peptide was more stable, less 26-kDa peptide (and no 48-kDa peptide) was produced, and a 42-kDa peptide which, as shown below, contained the 64-kDa tryptic site was formed (compare Fig. 1, B and E with Fig. 1, A and D). These observations indicate that F-actin protects the 64-kDa site against tryptic digestion more strongly than it protects the 38-kDa site.

The small amounts of 74- and 38-kDa peptides that were formed early in the digestion of myosin IA in the presence of F-actin (Fig. 1B) were almost certainly derived from myosin IA that was not bound to actin and, therefore, not protected against cleavage at the 64- and 38-kDa sites. The proportion of this myosin increased with the age of the myosin preparation indicating that at least some of the unbound myosin may have been partially denatured.

**Phosphorylation of the Heavy Chain of Myosin IA Enhances Actin Protection of the 38-kDa Tryptic Cleavage Site—Phosphorylation of the myosin IA heavy chain had no effect on its digestion by trypsin in the absence of F-actin but did affect the digestion pattern in the presence of F-actin. Cleavage at the 38-kDa site was still suppressed by F-actin (Fig. 1C), leading to initial formation of the 112-kDa peptide (Fig. 1C) that contains the phosphorylation (Fig. 1F) and ATP-binding sites (Fig. 2C). However, phosphorylation slightly increased the stability of the 91-kDa peptide formed in the presence of F-actin (compare Fig. 1, C and F with Fig. 1, B and E), and resulted in its cleavage to 80-kDa and 78-kDa peptides (seen

![Fig. 2](image-url)
as a closely spaced doublet in Fig. 1C) that contained the phosphorylation (Fig. 1F) and ATP-binding sites (Fig. 2C). These two peptides were highly resistant to further digestion by trypsin but were eventually converted into the 53-, 42-, and 26-kDa phosphorylated peptides (Fig. 1, C and F). Because the 80- and 78-kDa subfragments of the 91-kDa peptide contained both the phosphorylation and the ATP-binding sites, they must both have contained an intact 38-kDa tryptic site. In contrast, the further digestion of the unphosphorylated 91-kDa peptide proceeded primarily at the 38-kDa site. Thus, phosphorylation of myosin IA further enhances protection of the 38-kDa tryptic site by F-actin. The slower formation of the 38-kDa peptide in the presence of F-actin (compare Fig. 2, B and C) was shown previously (using N-ethylmaleimide-labeled actin) to be derived from tryptic cleavage of F-actin (16). Although its formation did not require the presence of myosin, the 36-kDa peptide was formed from actin in higher yield (for unknown reasons) in the presence of myosin I heavy chain kinase (data not shown). That is why much smaller amounts were formed in the incubations with unphosphorylated myosin than with phosphorylated myosin (which still contained the kinase in the digestion mixture). Kinase (in the absence of ATP) had no effect on the tryptic cleavage of myosin IA (data not shown).

Two control experiments were carried out to determine whether the different cleavage patterns of unphosphorylated and phosphorylated myosin IA in the presence of F-actin might be explained by different affinities of the unphosphorylated and phosphorylated 112-, 91-, 80-, and 78-kDa peptides for F-actin. Results identical to those in Fig. 1 were obtained when 0.5 μM unphosphorylated or phosphorylated myosin IA was digested with trypsin in the presence of 30 μM F-actin (six times higher than the usual concentration). Also, all four peptides (both phosphorylated and unphosphorylated) remained bound to the F-actin during the digestion, as indicated by their quantitative co-sedimentation from the digestion mixtures (data not shown).

**MgATP Reverses Actin Protection of the 38- and 64-kDa Tryptic Cleavage Sites**—To determine whether the protection of myosin IA from tryptic digestion was due to the binding of F-actin to the ATP-sensitive or -insensitive site, the digestion was performed in the presence and absence of both F-actin and MgATP (Fig. 3). The addition of MgATP abolished the effect of F-actin on the tryptic digestion of myosin IA (compare Fig. 3, B and F with Fig. 3, D and H). The digestion of myosin IA in the presence of MgATP, regardless of the presence of F-actin (Fig. 3, A, B, E, and F), was similar to that obtained in the absence of both nucleotide and actin (Fig. 3, C and G). Therefore, the protection of myosin IA from trypsin is due to the binding of F-actin to the ATP-sensitive binding site on the myosin heavy chain. The effect of MgATP on the digestion in the absence of F-actin (compare Fig. 3, C and G with Fig. 3, A and E) will be described in detail elsewhere.

**Confirmation of the Origins of the 112-, 91-, and 80-kDa (78-kDa) Peptides**—The top half of the scheme shown in Fig. 4 illustrates the previously determined (26) alignment of the peptides obtained by tryptic digestion in the absence of F-actin. The locations of the tryptic cleavage sites are indicated at the top of the figure, the molecular masses (in kilodaltons) of the peptides produced by tryptic cleavage are indicated on the right, and the locations of the ATP-binding and phosphorylation sites in the native heavy chain and tryptic peptides are indicated by 3H (UTP photoaffinity-labeled) and 32P, respectively.

The bottom half of Fig. 4 illustrates the tentative alignment of the three major peptides obtained by tryptic digestion of phosphorylated myosin IA in the presence of F-actin. The reasons for these assignments are: (i) the 112-, 91-, and 80-kDa peptides contained the ATP-binding site (as well as the phosphorylation site). Thus, the NH2 terminus of all three peptides can be no further than 27 kDa from the NH2 terminus of myosin IA (26). (ii) the 80-kDa peptide produced by tryptic digestion of Acanthamoeba myosin IB in the presence of F-actin has been shown to originate from the NH2 terminus of myosin IB (16). It seems reasonable to make the same tentative assumption for the analogous derivatives from myosin IA.

To confirm this tentative alignment, myosin IA was first digested with trypsin in the presence of F-actin and absence of MgATP to produce the 112-, 91-, and 80-kDa peptides, and then MgATP was added at different times and the digestion continued. As shown in Fig. 3, the digestion of myosin IA in
the presence of F-actin and MgATP produces the same peptides as digestion in the absence of F-actin. Therefore, it is reasonable to assume that the same heavy chain sites that are cleaved by trypsin in the absence of F-actin would be cleaved in the 112-, 91-, and 80-kDa peptides formed in the presence of F-actin (in the absence of MgATP) after the addition of MgATP. The right side of the bottom half of Fig. 4 shows the results to be expected if this assumption and the tentative assignments in the left half of the figure are correct.

When the digestion in the absence of MgATP was stopped at a time when the 112-kDa peptide was the principal product (Fig. 5, A and E, lane 1), subsequent digestion in the presence of MgATP produced the predicted phosphorylated 74-, 53-, and 26-kDa peptides and unphosphorylated 48- and 38-kDa peptides (Fig. 5, A and E, lanes 2–4). When digestion in the absence of MgATP was allowed to continue until the principal products were the 91- and 80-kDa (78-kDa) peptides (Fig. 5, C, D, G, and H, lane 1), subsequent digestion in the presence of MgATP produced the predicted phosphorylated 53-, 42-, and 26-kDa peptides and unphosphorylated 38-kDa peptide (Fig. 5, C, D, G, and H, lanes 2–4). Because of the presence of F-actin, the 42-kDa tryptic peptide could not be resolved in the Coomassie Blue-stained gels. As expected from the scheme in Fig. 4, the relative amounts of the phosphorylated 53- and 42-kDa peptides formed in the second digestion (Fig. 5, F, G, and H, lanes 2–4) were proportional, respectively, to the relative amounts of 91- and 80-kDa peptides present at the beginning of that digestion (Fig. 5, F, G, and H, lane 1). Also consistent with the alignments in Fig. 4, neither the phosphorylated 74-kDa peptide nor the unphosphorylated 48-kDa peptide can be detected in Fig. 5, C, D, G, and H.

Thus, from the data in Fig. 5, we conclude that the scheme shown in Fig. 4 is correct and that the tryptic sites of myosin IA are cleaved in the sequences shown in Table 1. These data are consistent with our interpretation of the results shown in Figs. 1 and 2 and establish that the binding of F-actin to myosin IA strongly suppresses tryptic cleavage at the 38- and 64-kDa sites, suppression of the former being greatly enhanced by phosphorylation of the myosin. However, phosphorylation has little, if any, effect on F-actin suppression of tryptic cleavage at the 64-kDa site as evidenced by the similar absence of the 48-kDa peptide, formation of the 42-kDa peptide, and increased stability of the 53-kDa peptide when F-actin was added to unphosphorylated and phosphorylated myosins (Fig. 1, B, C, E, and F). On the other hand, the observation that the 80- and 42-kDa peptides were formed only in the presence of F-actin (compare Fig. 1, B, C, E, and F with Fig. 1, A and D) suggests that actin may enhance tryptic cleavage at a site 80 kDa from the NH₂ terminus.
Actin-activated Mg\(^{2+}\)-ATPase Activity of the NH\(_2\)-terminal 80 kDa of Myosin IA—Previously, we showed that the 80-kDa NH\(_2\) terminus of myosin IB and the 112-kDa NH\(_2\) terminus of myosin IA are functionally similar to muscle myosin subfragment-1 (9, 16). In this section, we show that the subfragment-1-like region of myosin IA can also be restricted to a segment no larger than 80 kDa. Phosphorylated myosin 80-, and 78-kDa peptides in approximately 1:1:1 ratio (Fig. 6A, inset). The 38-kDa tryptic peptide from actin was also present (not shown). This digest mixture had 93% of the initial NH\(_2\),EDTA-ATPase activity and 88% of the initial actin-activated Mg\(^{2+}\)-ATPase activity measured at 30 μM F-actin. Furthermore, the Mg\(^{2+}\)-ATPase activity of this mixture had a hyperbolic dependence on the concentration of F-actin (Fig. 6A) consistent with the removal of the second actin binding site that resides in the COOH-terminal 27-kDa region (9). We have shown previously that cleavage at the 38-kDa tryptic site destroys 97% of the actin-activated Mg\(^{2+}\)-ATPase activity (26). Thus, virtually all of the ATPase activity of the digest can be attributed to the 91-, 80-, and 78-kDa peptides. The linearity of the double-reciprocal plot of these data (Fig. 6B) suggests that the three NH\(_2\)-terminal peptides had similar catalytic activities. The \(V_{\text{max}}\) of 11 s\(^{-1}\) is identical to the value previously reported for the NH\(_2\)-terminal 112-kDa peptide from myosin IA (9) and is 81% of that of native myosin IA (3, 15). However the \(K_{\text{M}}\) for actin of 22 μM is three to four times higher than that of the 112-kDa peptide (9) and is closer to the \(K_{\text{M}}\) for actin of 30 μM obtained for the NH\(_2\)-terminal 80-kDa fragment of myosin IB (16). This suggests that removing the heavy chain segment lying between 80 and 112 kDa from the NH\(_2\) terminus weakens the interaction between F-actin and the ATP-sensitive actin-binding site.

When the tryptic digestion was extended from 60 to 120 min, more than half of the 91-kDa peptide was converted to the 80- and 78-kDa peptides (Fig. 6A, inset) with virtually no loss of either NH\(_2\),EDTA-ATPase activity or Mg\(^{2+}\)-ATPase activity, at 30 μM F-actin (data not shown). This suggests that the NH\(_2\)-terminal 80-kDa segment of myosin IA is functionally homologous to the NH\(_2\)-terminal 80-kDa of myosin IB and the larger muscle myosin subfragment-1.

DISCUSSION

We have shown in this paper that the NH\(_2\)-terminal 80-kDa fragment of the Acanthamoeba myosin IA heavy chain has full actin-activated Mg\(^{2+}\)-ATPase activity. These results extend our earlier report (9) that the 112-kDa NH\(_2\)-terminal fragment of the myosin IA heavy chain is fully active and contains the catalytically involved ATP-sensitive actin-binding site, whereas the COOH-terminal 27-kDa fragment contains an ATP-insensitive actin-binding site that is not directly involved in ATP hydrolysis. Thus, the 80-kDa NH\(_2\)-terminal peptides derived from both Acanthamoeba myosins IA and IB (this paper and Ref. 16) are functionally analogous to muscle myosin subfragment-1.

The structural similarity between the amoeba myosins IA and IB and more conventional myosins includes the NH\(_2\)-terminal location of the purine part of the ATP-binding site (17, 22, 23-25, 26) and the location of an ATP-sensitive F-actin-protected tryptic cleavage site. This site lies 64 kDa from the NH\(_2\) terminus of myosin IA (this paper), 62 kDa for myosin IB (16), 75 kDa for skeletal muscle subfragment-1 (18, 19), between 68 and 78 kDa for gizzard (33), thymus (34), scallop (35), and frog (36) myosins and 68 kDa for Acanthamoeba myosin II. Proteolytic cleavage at this site drastically reduces the actin-activated Mg\(^{2+}\)-ATPase activities of myosin IB (37) and of its 80-kDa fragment (16) and of subfragment-1 (18, 19, 38, 39), while having minimal effects on their ATPase activities in the absence of F-actin. Furthermore, in both the 80-kDa fragment of myosin IB and muscle myosin subfragment-1, part of the actin-binding site (as well as the ATPase site) is located on the NH\(_2\)-terminal side of this proteolytic site (16, 40, 41). From these data and the results presented here, we conclude that the functionally important 80-kDa NH\(_2\)-terminal region of the Acanthamoeba myosin IA heavy chain must be similar, not only in function but also in structure, to the corresponding region of Acanthamoeba myosin IB and to the homologous regions of muscle myosins. Also, the protection of the 64-kDa site of myosin IA by F-actin is most likely due to the direct binding of F-actin in the neighborhood of this site.

In addition to protecting the 64-kDa site of myosin IA, we now find that F-actin also strongly affects the proteolytic susceptibility of the 38-kDa site of Acanthamoeba myosin IA. We have no evidence at this time that protection of the 38-kDa tryptic site is due to direct binding of F-actin at or near that site. Cross-linking studies and gel overlay studies with 2 V. Sathyamoorthy, M. A. L. Atkinson, and E. D. Korn, personal communication.
The principal sites of tryptic cleavage are 38-, 64-, and 80-kDa from the NH2 terminus. Phosphorylation, which occurs very near the 38-kDa site, has no effect on tryptic cleavage in the absence of F-actin. F-actin binds at or near the 64-kDa site and inhibits tryptic cleavage at the 64-kDa site, probably by steric blocking, and the 38-kDa site, probably by a conformational change in the heavy chain (although a steric effect cannot be ruled out). Phosphorylation of the myosin heavy chain has no effect on F-actin protection of the 64-kDa site, but it enhances F-actin protection of the 38-kDa site, probably by an additional conformational change in this region of the myosin IA heavy chain. The conformational changes that are detected by proteolytic susceptibility are probably related to the mechanisms by which F-actin and heavy chain phosphorylation activate the Mg\(^{2+}\)-ATPase activity of Acanthamoeba myosin IA.

The 38-kDa site of Acanthamoeba myosin I corresponds to the middle of the central 50-kDa region of muscle myosin subfragment-1 (more precisely about 45 kDa from the NH2 terminus of subfragment-1, according to the sequence similarities (43)). This 50-kDa domain is thought to couple the ATP-binding and actin-binding sites by ligand-induced conformational changes (for reviews, see Refs. 20 and 21). In contrast to the tryptic susceptibility at the 38-kDa site of myosin IA, the middle of the 50-kDa domain of native subfragment-1 is highly resistant to proteolysis. However, it does undergo proteolysis after mild heat denaturation (44), and F-actin protects this region against denaturation by both heat (44) and methanol (45). In addition, it has been reported recently (46) that an arginine residue in the NH2-terminal half of the 50-kDa domain (which would include the 38-kDa site in myosin IA) can be cross-linked to F-actin. Thus, it seems likely that the region of subfragment-1 that corresponds to the 38-kDa site of Acanthamoeba myosin IA also interacts with F-actin. Possibly, phosphorylation of the Acanthamoeba myosin I heavy chain activates its Mg\(^{2+}\)-ATPase activity by inducing an active conformation in the 38-kDa region in the actomyosin IA complex that is similar to the conformation of the homologous region of the native acto-subfragment-1 complex in the absence of phosphorylation. In a somewhat similar manner, phosphorylation of the light chain of gizzard myosin affects proteolysis of the heavy chain at a site that is also influenced by actin binding (33).

**Fig. 7.** Schematic representation of the effects of phosphorylation and F-actin on tryptic cleavage of Acanthamoeba myosin IA. The principal sites of tryptic cleavage are 38-, 64-, and 80-kDa from the NH2 terminus. Phosphorylation, which occurs very near the 38-kDa site, has no effect on tryptic cleavage in the absence of F-actin. F-actin binds at or near the 64-kDa site and inhibits tryptic cleavage at the 64-kDa site, probably by steric blocking, and the 38-kDa site, probably by a conformational change in the heavy chain (although a steric effect cannot be ruled out). Phosphorylation of the myosin heavy chain has no effect on F-actin protection of the 64-kDa site, but it enhances F-actin protection of the 38-kDa site, probably by an additional conformational change in this region of the myosin IA heavy chain. The conformational changes that are detected by proteolytic susceptibility are probably related to the mechanisms by which F-actin and heavy chain phosphorylation activate the Mg\(^{2+}\)-ATPase activity of Acanthamoeba myosin I.

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Effect of Phosphorylation on Actomyosin IA Conformation