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LIGHT CHAIN PHOSPHORYLATION ALTERS THE CONFORMATION OF SKELETAL MUSCLE MYOSIN

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SUMMARY: Using limited proteolysis by chymotrypsin or papain, we examined the effects of phosphorylation on the conformation of skeletal muscle myosin. In the absence of $MgCl_2$, phosphorylation of the 19,000 dalton light chain (LC_2) inhibited digestion of LC_2 by chymotrypsin or papain. Phosphorylation also suppressed chymotryptic conversion of the heavy chain to subfragment 1 and increased its conversion to heavy meromyosin. These results indicate that phosphorylation alters the conformation of the N-terminal segment of LC_2 and suggest that it also affects the heavy chain. In the presence of $MgCl_2$, phosphorylation inhibited the chymotryptic digestion of LC_2 but an effect on digestion of the heavy chain was not apparent. Thus, phosphorylation of LC_2 alters LC_2 conformation under physiological conditions.

INTRODUCTION

When skeletal muscle contracts, the 19,000 dalton light chain of myosin is phosphorylated by calcium-dependent light chain kinase (1-3). In rat skeletal muscle, the extent of phosphorylation was found to correlate with the degree of post-tetanic potentiation of isometric twitches (4). These findings suggest that phosphorylation might participate in regulating skeletal myosin. Since regulation by phosphorylation is known to be mediated by conformational changes in other proteins (5,6), conformational changes may also mediate a regulatory effect of phosphorylation in skeletal muscle. However, the effects of phosphorylation on the conformation of intact skeletal myosin have not been defined.

Since limited proteolysis is a sensitive tool for probing protein conformational changes (7), we investigated the effects of phosphorylation on myosin conformation by looking at changes in susceptibility to digestion at four sites in myosin that are especially sensitive to chymotryptic cleavage. Three of these sites are cleaved sequentially: cleavage at phe 18 within the N-terminal segment of LC_2 permits cleavage at a second site in LC_2 (phe 53) (8,9); this in turn permits degradation of LC_2 and cleavage of the myosin heavy chain at the flexible "swivel" site (10) joining the head to the rod, producing free heads [subfragment

Abbreviations used: LC_2, the 19,000 dalton light chain of skeletal muscle myosin; LC_2-P, the phosphorylated form of LC_2.

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1, (S_1)] and rods (9,11,12). Cleavage of the heavy chain at the fourth site, the flexible "hinge" within the rod (13), produces heavy meromyosin (HMM) and light meryomyosin (11).

Under physiological conditions, a divalent cation, either Mg^{2+} or Ca^{2+}, will always be bound to LC_2 (14). When a divalent cation is present during chymotryptic digestion of aggregated skeletal myosin, LC_2 is protected against cleavage at phe 53. This results in accumulation of a stable 17,000 dalton fragment of LC_2 and suppressed formation of S_1. In addition, formation of HMM is increased (9,12). These effects suggest that divalent cations alter the conformation of both LC_2 and the myosin heavy chain (9). We therefore compared the effect of phosphorylation on the chymotryptic digestion of aggregated myosin in the presence of a divalent cation with the effect in the absence of divalent cations to determine whether any of the effects of phosphorylation on myosin conformation would occur under physiological conditions.

Since the site of phosphorylation (ser 15) (8) is close to the first chymotrypsin-sensitive site in LC_2 (phe 18), a phosphate group at ser 15 could protect phe 18 from cleavage either by sterically shielding it or by stabilizing a conformation of the N-terminal segment of LC_2 in which this site would be less accessible to the protease (7). The latter mechanism would be revealed by resisance of the N-terminal segment to digestion by proteases of different specificities (7). Thus, we digested phosphorylated myosin with papain as well as with chymotrypsin to distinguish between these mechanisms.

METHODS

Myosin from rabbit skeletal muscle (15) and myosin light chain kinase (16) were purified as described previously. Samples of phosphorylated aggregated myosin were prepared by incubating myosin (4 mg/ml) with kinase (5 μg/ml) at 25° for 45 min in 20 mM N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (pH 7.5), 0.1 mM $CaCl_2$, 15 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, and 5 mM ATP. Samples of nonphosphorylated aggregated myosin were prepared similarly but without kinase. The samples were then diluted with 1 mM dithiothreitol, incubated at 0° for 45 min to further aggregate the myosin, and centrifuged at 500 x g for 5 min. The extent of phosphorylation in each sample was estimated by densitometry of the LC_2 and LC_2-P bands after electrophoresis on 7.5% gels in the presence of 6 M urea and staining with Coomassie blue (1). The mean extent of phosphorylation was 87% for the phosphorylated samples and 3% for the nonphosphorylated ones.

A pair of myosin samples, one phosphorylated and one nonphosphorylated, were each resuspended to a concentration of 4 mg/ml in a digestion mixture containing 10 mM sodium phosphate (pH 7.0), 1 mM dithiothreitol, 120 mM KCl, and 2 mM EDTA. They were equilibrated at 22°, and α-chymotrypsin (myosin:α-chymotrypsin = 100-200:1, w/w) was added to start digestion. Two aliquots were taken from each digestion mixture before addition of chymotrypsin (controls) and after addition at times ranging from 1 to 300 min and were immediately treated with phenylmethylsulfonylfluoride (1 mg/ml final concn).

One aliquot was centrifuged at 90,000 x g for 10 min. The resulting supernatant was assayed for protein (17) to estimate the percentage of aggregated myosin released as soluble products. The relative amounts of S_1 heavy chains

and HMM heavy chains in the supernatant were determined by densitometry after electrophoresis on 7.5% gels in the presence of 0.1% sodium dodecyl sulfate and staining with Coomassie blue (18). Dye binding per μg of heavy chain was estimated by densitometry of known amounts of purified S_1 and HMM; binding to the HMM heavy chain was found to be 2.9 times that to the S_1 heavy chain. After being corrected for this difference, the relative amounts of S_1 and HMM heavy chains were multiplied by the amount of protein in the supernatant to obtain the percentage of myosin heavy chains converted to S_1 or HMM. The other aliquot was assayed for light chain composition by electrophoresis on 7.5% gels in the presence of 6 M urea (1). The relative amount of LC_2 in each aliquot was estimated by densitometry of the stained gel and normalized with respect to the amount of LC_1. This entire procedure was repeated on a second pair of samples containing 2 mM $MgCl_2$ instead of 2 mM EDTA.

A third pair of samples was digested with papain (myosin: papain = 100:1, w/w) in the presence of 2 mM EDTA at a KCl concentration of 500 mM. After times ranging from 1 to 60 min , digestion was terminated with iodoacetic acid (1 mM final concn). The digestion mixtures were then assayed for light chain composition as before, except that 2-mercaptoethanol was absent.

RESULTS

We first examined the effects of phosphorylation alone on the chymotryptic digestion of aggregated myosin ($-Mg^{2+}$, $\pm P_i$). As shown in Fig. 1, the rate of release of soluble products was slower for phosphorylated myosin that for non-phosphorylated, indicating that phosphorylation decreased the overall rate of digestion. Analysis of digestion products by gel electrophoresis showed, however, that, although phosphorylation inhibited digestion of LC_2 (Figs. 2A, 3A) and suppressed heavy chain conversion to S_1 (Figs. 2B, 3B), it increased heavy chain conversion to HMM (Figs. 2B, 3B).

We then examined the effect of 2 mM $MgCl_2$ alone ($+Mg^{2+}$, $-P_i$) and of 2 mM $MgCl_2$ together with phosphorylation ($+Mg^{2+}$, $+P_i$) on the chymotryptic digestion

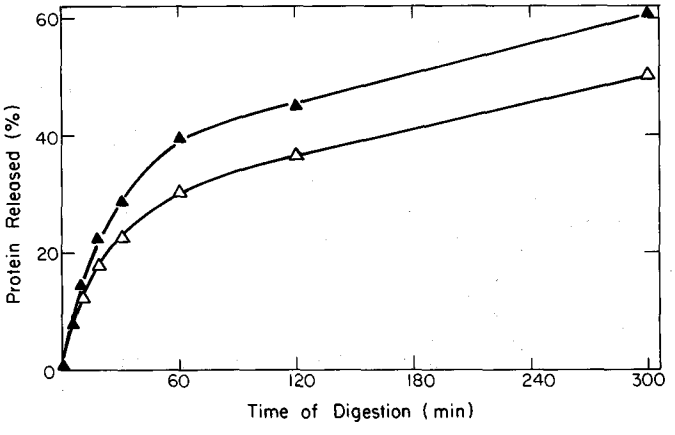

FIGURE 1. Percentage of myosin protein released as soluble products with time of digestion. Phosphorylated (△) and nonphosphorylated (▲) aggregated myosin samples were digested with α-chymotrypsin (myosin:α-chymotrypsin 100:1, w/w) in a mixture containing 2 mM EDTA as described in METHODS.

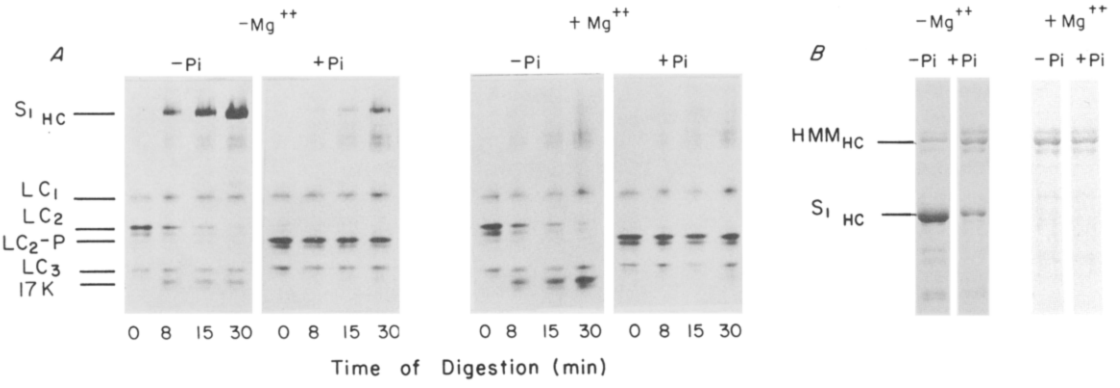

FIGURE 2. Polyacrylamide gel electrophoresis patterns of digestion products for phosphorylated and nonphosphorylated aggregated myosin samples digested with chymotrypsin as described in Fig. 1 in the presence of 2 mM EDTA ($-Mg^{2+}$) or 2 mM $MgCl_2$ ($+Mg^{2+}$). A) 7.5% gels containing 6 M urea showing the light chain composition of aliquots of phosphorylated ($+P_i$) and nonphosphorylated ($-P_i$) digestion mixtures after 0 to 30 min. B) 12% gels containing 0.1% sodium dodecyl sulfate showing the relative amounts of S_1 and HMM heavy chains in the supernatant obtained by centrifugation of the phosphorylated and nonphosphorylated myosin digestion mixtures after 70 min. of digestion.

of aggregated myosin. As found by others (9,12), with Mg^{2+} alone, LC_2 was rapidly cleaved to a stable 17,000 dalton fragment (Fig. 2A), S_1 formation was suppressed, and HMM formation was increased (Fig. 2B). As shown in Fig. 2B, S_1 formation was suppressed more by Mg^{2+} alone than it had been by phosphorylation alone. In the presence of Mg^{2+} together with phosphorylation, digestion of LC_2 was inhibited

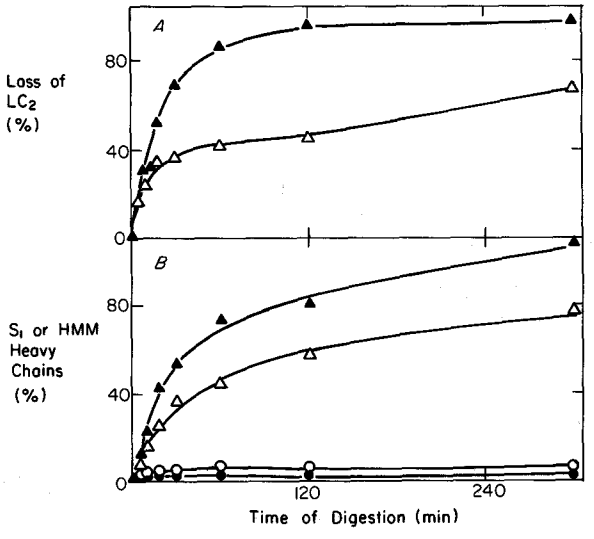

FIGURE 3. Percent loss of LC_2 and percentage of myosin heavy chains converted to S_1 or HMM with time of chymotryptic digestion in the presence of 2 mM EDTA as described in Fig. 1. A) Percent loss of phosphorylated (△) and nonphosphorylated (▲) LC_2. B) Percentage of myosin heavy chains converted to S_1 and HMM. (△) % S_1, phosphorylated myosin; (▲) % S_1 nonphosphorylated myosin; (○) % HMM, phosphorylated myosin; (●) % HMM, nonphosphorylated myosin.

to about the same extent as it had been by phosphorylation alone (Fig.2A); in addition, S_1 formation was suppressed and HMM formation increased to about the same extent as they had been by Mg^{2+} alone. (Fig. 2B).

Finally, we examined the effects of phosphorylation alone on the papain digestion of soluble myosin. Phosphorylation again inhibited digestion of LC_2; however, the heavy chain was fragmented in both phosphorylated and nonphosphorylated myosin and no differences were apparent in the heavy chain patterns after gel electrophoresis (not shown).

DISCUSSION

The protective effect of phosphorylation alone against digestion of LC_2 by either chymotrypsin or papain indicates that it stabilizes the N-terminal segment of LC_2 in a more compact and/or less flexible conformation (7). By analogy to glycogen phosphorylase a (19), the charged phosphate group may tether the N-terminal segment with an ionic bond.

The effects of phosphorylation alone on the digestion of the heavy chain suggest effects on heavy chain conformation like those proposed for divalent cations (9). These effects include protection of the swivel site and an apparent increase in susceptibility of the hinge site (7). The protection of the swivel may be achieved through preservation of the intact LC_2 structure, which then either shields the swivel sterically or stabilizes a more rigid conformation of the heavy chain at the swivel (7,12). The apparent increase in susceptibility of the hinge may be achieved through stabilization of a more flexible conformation of the heavy chain at the hinge (7). However, the extent to which the increased formation of HMM, suggesting increased hinge susceptibility, is due simply to the decreased formation of S_1 is not known. Preliminary analysis suggests that phosphorylation does not alter the susceptibility of the hinge site; however, more data and a rigorous kinetic analysis are needed to ascertain this.

The inhibition of chymotryptic cleavage of LC_2 at phe 18 by phosphorylation in the presence of Mg^{2+} indicates that the conformational changes induced by phosphorylaton in the N-terminal segment of LC_2 still occur in the presence of a divalent cation. On the other hand, phosphorylation evidently had little effect on digestion of the heavy chain swivel site when a divalent cation was present since the amount of S_1 formed was very similar when either phosphorylated or nonphosphorylated myosin were digested in the presence of Mg^{2+} (Fig. 2B). This lack of effect may be explained by the ability of divalent cations to stabilize the 17,000 dalton fragment of LC_2 against degradation, and to thereby strongly protect the swivel site from digestion (9,12). Thus, in the presence of a divalent cation, both LC_2 and LC_2-P would be stabilized by the divalent cation and the additional (weaker) protection conferred by phosphorylation against degradation of LC_2 would not be apparent. Phosphorylation also

had little apparent effect on digestion of the heavy chain hinge site when a divalent cation was present since the amount of HMM formed was very similar for both phosphorylated and nonphosphorylated myosin digested in the presence of Mg^{2+} (Fig.2B).

This lack of effect of phosphorylation may again be explained by a masking effect of the divalent cation. As in the case of phosphorylation, it is uncertain whether the increased formation of HMM by Mg^{2+} is simply due to the decreased formation of S_1.

In conclusion, this study demonstrates an effect of phosphorylation on the proteolytic digestion of intact skeletal muscle myosin. Although the most dramatic effects were seen in the absence of Mg^{2+}, some were still seen in its presence; this indicates that phosphorylation affects the conformation of LC_2 under physiological conditions. These effects could, in turn, mediate a regulatory effect on myosin function in vivo.

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