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Articles

Light-Chain Phosphorylation and Cross-Bridge Conformation in Myosin from Vertebrate Skeletal Muscle†

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ABSTRACT: The effect of phosphorylation of the myosin light chains (LC-2) on cross-bridge conformation in synthetic myosin filaments from vertebrate skeletal muscle was studied by using chemical cross-linking and chymotryptic digestion methods. Phosphorylated and dephosphorylated myosin filaments, which were used in these experiments, had similar sedimentation coefficients, turbidities, and rates of growth from the respective minifilament structures. The proteolytic susceptibility at the heavy meromyosin–light meromyosin (HMM–LMM) junction was somewhat greater in the phosphorylated than in the dephosphorylated filaments at both pH 7.0 and pH 8.0. At the same time, the normalized rate of subfragment 2 (S-2) cross-linking to the filament surface, $k_{S\text{-}2}/k_{LMM}$, was reduced by phosphorylation of myosin. These results are consistent with partial release of cross-bridges from the thick filament surface in phosphorylated myosin filaments.

Phosphorylation of the Ca^{2+} binding subunits, LC-2,[1] in myosin, has been studied both in vitro and in vivo. According to the most popular view, light-chain phosphorylation regulates the ATPase activity and contraction in smooth muscle (Gőrecka et al., 1976; Chacko et al., 1977; Small & Sobieszek, 1977), and in more primitive motile systems (Kendrick-Jones & Scholey, 1981; Trotter & Adelstein, 1979; Scordilis & Adelstein, 1978). In vertebrate skeletal muscle the role of myosin light chain phosphorylation remains unclear in spite of an established link between LC-2 phosphorylation and the contractile cycle in the muscle. Both prolonged tetanic stimulation (Bárány et al., 1979) and, more importantly, low-frequency repetitive stimulation of skeletal muscle (Klug et al., 1981) result in significant LC-2 phosphorylation. These physiological correlations suggest that phosphorylation of myosin may play a regulatory role in the contraction of skeletal muscle.

Direct comparisons of actomyosin ATPase in phosphorylated and dephosphorylated myosin preparations have not revealed any significant differences between these two systems (Nairn & Perry, 1979). However, at least two recent studies showed that phosphorylated myosin had higher affinity for actin than the dephosphorylated protein (Pemrick, 1980; Michnicka et al., 1982), and a preliminary report indicated that LC-2 phosphorylation reduced the ATPase activity of intact myofibrils (Franks et al., 1982). The tighter binding of the phosphorylated myosin to actin is expressed both during ATP hydrolysis (Pemrick, 1981) and in the absence of nucleotides (Michnicka et al., 1982). According to Bárány et al. (1979, 1980), such modulating function of LC-2 phosphorylation on actomyosin interaction could originate from an ionic attractive force between the phosphoryl group on myosin and the actin-bound Ca^{2+}. Additional modulation of actomyosin interaction may arise from a net change in occupancy of the cation binding sites on LC-2 ensuing upon its phosphorylation (Kardami & Gratzer, 1982).

A potentially important role of light-chain phosphorylation in the contraction of skeletal muscle may be related to the altered charge balance on myosin cross-bridges. Cross-linking studies of Chiao & Harrington (1979) and Ueno & Harrington (1981a) showed that increasing the negative charge on myosin (through elevation of medium pH) resulted in the release of cross-bridges from the filament surface. Such release appeared to be associated with an increase in the proteolytic susceptibility at the HMM–LMM junction in myosin filaments and a substantial decrease in the rate of S-2 cross-linking to the filament backbone (Ueno & Harrington, 1981a). In this

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[1] Abbreviations: HMM, heavy meromyosin; LMM, light meromyosin; LC-2, Ca^{2+} binding 19 000 molecular weight subunit of myosin; S-1, subfragment 1; S-2, subfragment 2; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

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context, the observation of increased chymotryptic susceptibility at the HMM–LMM junction in phosphorylated filaments (Kardami et al., 1980, Ritz-Gold et al., 1980) merits special attention.

The aim of this work was to examine the effect of light-chain phosphorylation on cross-bridge conformation. Employing the procedures of Ueno & Harrington (1981a), we have compared the time course of chymotryptic digestion and the rate of S-2 cross-linking to the filament surface in phosphorylated and dephosphorylated filaments. Our results are consistent with partial release of S-2 from the phosphorylated filaments. Since in our experiments the rate of formation and the final size of phosphorylated and dephosphorylated filaments are similar, the structural effect of light-chain phosphorylation appears to be restricted to the myosin cross-bridge.

Materials and Methods

Distilled water and analytical-grade reagents were used in all experiments. Dimethyl suberimidate, α-chymotrypsin, phenylmethanesulfonyl fluoride, and creatine phosphate were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Proteins. Rabbit skeletal myosin was prepared in the phosphorylated and dephosphorylated forms by the modified procedure of Kunz et al. (1980). The crude myosin extract was obtained in 0.3 M KCl, 0.15 M phosphate (pH 7.0), 0.2 mM phenylmethanesulfonyl fluoride, and 0.1 mM sodium azide. Full phosphorylation of the myosin light chains (LC-2) was achieved by incubating the crude extract for 30 min at 20 °C with 5 mM ATP, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and 0.5 mM pyrophosphate. Pyrophosphate was included in the incubation mixture in order to inhibit the action of light-chain phosphatase (Dr. R. S. Adelstein, personal communication). After a 30-min incubation, the phosphorylated myosin was rapidly precipitated with cold water. Subsequent purification of the precipitated myosin followed standard procedures. Dephosphorylated myosin was prepared either according to the procedure of Godfrey & Harrington (1970) or by incubating the crude myosin extract in 0.3 M KCl and 0.15 M phosphate for 1 h at 20 °C with 5 mM ATP, 5 mM EGTA, and 1 mM dithiothreitol. The extent of myosin phosphorylation was monitored on gel electrophoresis (7.5% polyacrylamide) in the presence of 6 M urea (Figure 1). Only fully phosphorylated myosin preparations were used in our experiments.

Cross-Linking of Phosphorylated and Dephosphorylated Myosin Filaments with Dimethyl Suberimidate. Cross-linking, digestion, and oxidation procedures follow closely those described by Ueno & Harrington (1981a) and Reisler & Liu (1982). Myosin filaments suspended in either 40 mM imidazole hydrochloride (pH 7.0) or 40 mM triethanolamine hydrochloride (pH 8.0) were cross-linked with dimethyl suberimidate at 20 °C in the presence of 80 mM NaCl and 1.0 mM $CaCl_2$. Whenever phosphate (10 mM) and creatine phosphate (10 mM) were present in the cross-linking reaction (at pH 7.0), the concentration of imidazole hydrochloride was reduced to 30 mM. The concentration of myosin was 1 mg/mL and that of dimethyl suberimidate 1.5 and 0.4 mg/mL at pH 7.0 and 8.0, respectively. At various time points of the reaction, which was allowed to proceed for up to 90 min, 1-mL aliquots were removed and digested with α-chymotrypsin (0.05 mg/mL) in the presence of 0.5 M NaCl and 1 mM $CaCl_2$, and at pH 8.0. After a 4-min digestion in the presence of Ca^{2+} to yield heavy meromyosin and light meromyosin, the samples were made up to 5 mM in EDTA, and the digestion was allowed to continue for additional 15 min. This latter digestion resulted in the formation of the high molecular weight S-2 (M_r 57 000)

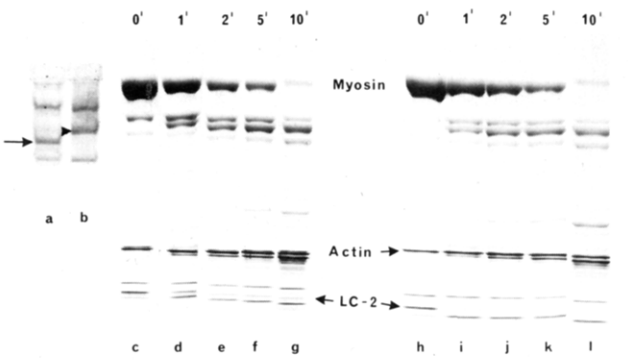

FIGURE 1: Electrophoresis of phosphorylated (a) and dephosphorylated myosin (b) and of chymotryptically digested phosphorylated (c–g) and dephosphorylated filaments (h–1). The first two lanes (a and b) demonstrate the electrophoretic behavior of LC-2 phosphorylated and dephosphorylated myosin on polyacrylamide gels in the presence of 6 M urea. Arrows next to lanes a and b indicate the position of LC-2. Note the greater mobility of the phosphorylated LC-2 in lane a. The time course of filament digestion at pH 8.0, at 20 °C, and in the presence of 1 mM Ca^{2+} is shown for the phosphorylated and dephosphorylated structures in lanes c–g and h–l, respectively. Digestion times are given at the top of these gels. Actin, which is an internal standard, was added to all samples after termination of proteolysis.

as described by Ueno & Harrington (1981a). The proteolytic reaction was terminated by addition of phenylmethanesulfonyl fluoride to a final concentration of 1 mM. The excess of dimethyl suberimidate was neutralized by adding 0.2 volume of 2 M ethanolamine to the digested samples.

The myosin digestion products, LMM and S-2, were identified as described previously (Reisler & Liu, 1982). The oxidation of the SH groups in LMM and S-2 (Ueno & Harrington, 1981a; Reisler & Liu, 1982) was carried out in order to distinguish between the intramolecular and intermolecular cross-links formed in the reaction with dimethyl suberimidate (Ueno & Harrington, 1981a).

The time course of cross-linking S-2 and LMM was determined by measuring the decay rate of these protein bands (in the oxidized form) on sodium dodecyl sulfate–polyacrylamide gels as a function of cross-linking time. Bovine serum albumin was used as a standard protein to calibrate the band densities of all myosin fragments. The rate constants of cross-linking S-2 and LMM were obtained from semilogarithmic plots of relative intensities of the corresponding bands on sodium dodecyl sulfate–polacrylamide gels vs. time.

Chymotryptic Digestion of Phosphorylated and Dephosphorylated Myosin Filaments. Myosin filaments (3 mg/mL) in 80 mM NaCl, 1 mM $CaCl_2$, and either 40 mM imidazole hydrochloride (pH 7.0) or 40 mM triethanolamine hydrochloride (pH 8.0) were digested with α-chymotrypsin (0.05 mg/mL) at 20 °C. At given time intervals aliquots were removed from the digestion system, the proteolysis was terminated as above, and the samples were denatured and examined on polyacrylamide gels. Actin was used as a standard calibration protein. Digestion rates were obtained by monitoring the decay in the intensity of the myosin heavy chain as a function of digestion time (Reisler & Liu, 1982).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to the procedure of Laemmli (1970) using 7.5% (w/v) acrylamide gels. Oxidized samples were run in the absence of β-mercaptoethanol. The optical densities of protein bands and the appropriate mass distributions were determined with a Helena Quick Scan R & D gel scanner equipped with an integrator.

Measurements of Myosin Association. Myosin minifilaments were prepared in 10 mM citrate–Tris, pH 8.0, as de-

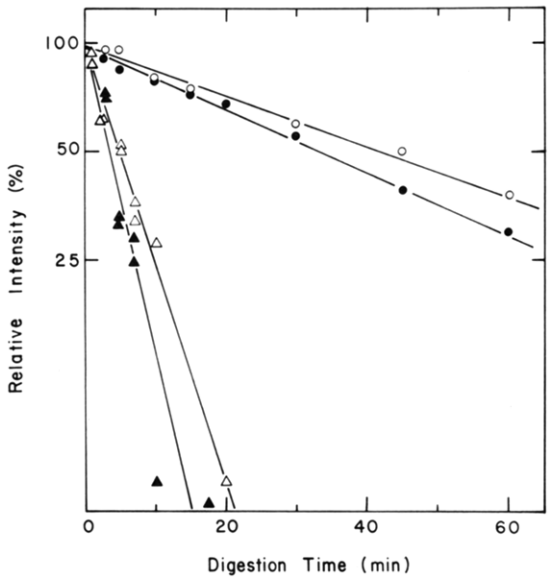

FIGURE 2: Kinetics of digestion of phosphorylated (●, ▲) and dephosphorylated (○, △) myosin filaments at pH 7.0 (○, ●) and 8.0 (△, ▲). Myosin filaments (3 mg/mL) were digested with α-chymotrypsin (0.05 mg/mL) at 20 °C. The products of digestion were analyzed as described under Materials and Methods. Relative densities of the myosin heavy chain are plotted vs. digestion time.

scribed previously (Reisler et al., 1980). The turbidity of phosphorylated and dephosphorylated minifilaments was recorded at 310 nm with a Beckman Model 25 spectrophotometer. The formation of filaments, induced by addition of 0.1 M KCl to minifilaments (Reisler et al., 1982), was followed by recording the increase in the optical density of myosin solutions at 310 nm. Turbidities of filaments prepared by directly dialyzing myosin in 0.5 M KCl solvent into the low-salt solvent (80 mM NaCl and 40 mM imidazole hydrochloride and pH 7.0) were measured in the same manner.

The sedimentation velocity experiments were performed at 20 °C in a Spinco Model E analytical ultracentrifuge by using the schlieren optical system. Myosin minifilaments were run at 30 000 rpm and the filaments were sedimented at 17 000 rpm. The calculated sedimentation coefficients were normalized to standard conditions of H_2O at 20 °C.

Results

Chymotryptic Digestion of Myosin Filaments. Recent studies have shown that proteolytic digestions of the HMM–LMM hinge region in myosin can be used as a sensitive probe for structural transitions in this part of the molecule (Ueno & Harrington, 1981a,b; Reisler & Liu, 1982). The rate of chymotryptic cleavage at the S-2–LMM junction in myosin (and rod) was found to increase sharply between pH 7.4 and pH 8.2, i.e., over the same region in which the cross-linking of S-2 to the filament surface decreased rapidly. Thus, it appeared that the increased proteolytic susceptibility of the S-2–LMM junction in myosin filaments was linked to the charge-induced release of cross-bridges from the filament surface. With this in mind, we have compared the proteolytic susceptibility at the HMM–LMM junction in phosphorylated and dephosphorylated myosin filaments.

Myosin filaments were digested with α-chymotrypsin at pH 7.0 and 8.0 in the presence of 1 mM Ca^{2+}. Under these conditions the cleavage at the head–rod junction in myosin is blocked (Weeds & Pope, 1977), and the proteolytic digestion proceeds exclusively at the HMM–LMM hinge. Consequently, the rate of chymotryptic digestion at the HMM–

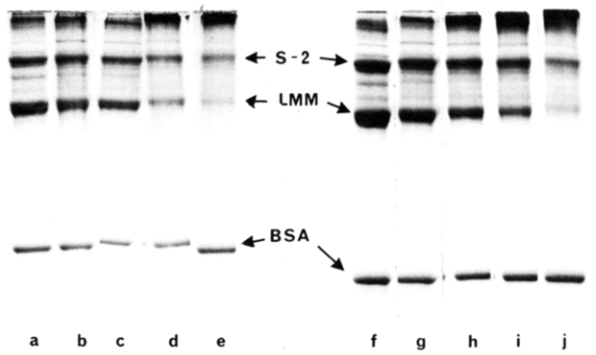

FIGURE 3: Electrophoresis of chymotryptically digested and then oxidized samples of cross-linked, phosphorylated (a–e), and dephosphorylated myosin filaments (f–j). Myosin filaments (1 mg/mL) were cross-linked with dimethyl suberimidate (0.4 mg/mL) at pH 8.0, at 20 °C, for 0, 5, 15, 30, and 90 min, respectively. Experimental details of the cross-linking, chymotryptic digestion, and oxidation reaction are given under Materials and Methods. Bovine serum albumin (BSA) served as the internal standard for quantitation of the S-2 and LMM bands.

LMM junction can be determined by monitoring the time course of the decrease in the intensity of the myosin heavy chain band on sodium dodecyl sulfate–polyacrylamide gels (Figure 1). The rate constants for digestion of myosin filaments were derived from semilogarithmic plots similar to those shown in Figure 2. In all experiments the decay curves followed an apparent first-order process. The phosphorylated filaments were digested at a somewhat faster rate (k_P) than their dephosphorylated counterparts (k_D). Both at pH 7.0 and at pH 8.0, the ratio of the respective rate constants, k_P/k_D, was consistently between 1.30 and 1.40. Obviously, this phosphorylation-induced acceleration of a hinge cleavage was significantly smaller than the previously observed effects of pH on the proteolysis of the HMM–LMM junction (Ueno & Harrington, 1981a; Reisler & Liu, 1982). Thus, for example, raising the pH from 7.0 to 8.0 increased the digestion rate approximately 9-fold, whereas light-chain phosphorylation caused an approximately 1.4-fold increase in this rate (Figure 2).

The major effect of LC-2 phosphorylation was, as previously noted (Kardami et al., 1980; Ritz-Gold et al., 1980), the protection of this subunit from proteolytic degradation (Figure 1). The increase in the stability of the phosphorylated light chain could be detected even in the presence of 1 mM Ca^{2+}; i.e., the protective effects of divalent cations and phosphorylation appeared to be additive.

Cross-Linking of Phosphorylated and Dephosphorylated Myosin Filaments. Cross-linking experiments were carried out according to the procedure of Ueno & Harrington (1981a). After termination of cross-linking, the reacted samples were digested in two stages with α-chymotrypsin to yield LMM and S-2. LMM was obtained in the first part of the digestion, when HMM and LMM were generated in the presence of Ca^{2+}; S-2 was formed in the second, longer stage of digestion carried out in the presence of EDTA (Weeds & Pope, 1977). As before (Reisler & Liu, 1982), the oxidized products of such digestions were monitored on sodium dodecyl sulfate–polyacrylamide gels (Figure 3), and the rates of cross-linking S-2 and LMM to the filament backbone were determined from decay curves of the optical densities of the corresponding bands. Figure 4 shows such curves for the cross-linking of myosin filaments at pH 8.0. These and other curves followed an apparent first-order rate process and yielded the appropriate rate constants, $k_{S\text{-}2}$ and k_{LMM}, for the cross-linking of S-2 and

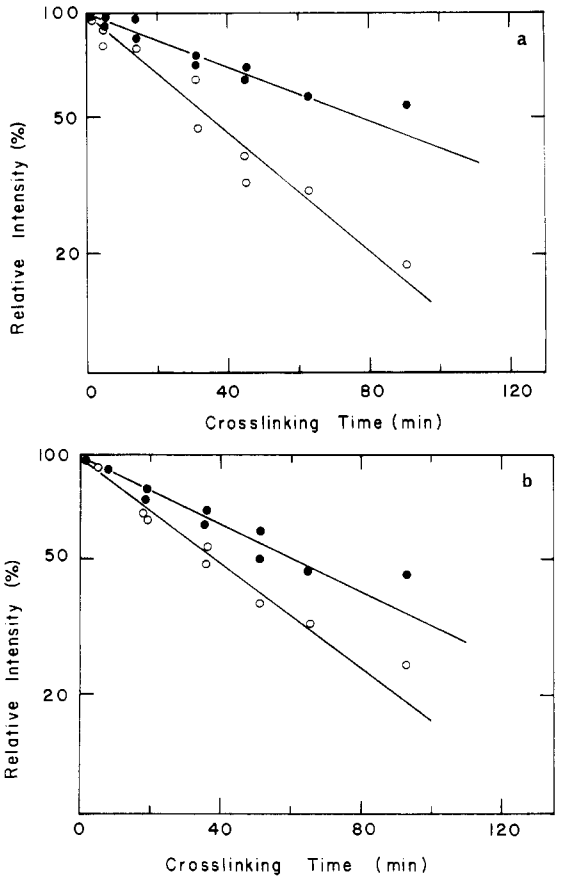

FIGURE 4: Typical time course of cross-linking S-2 (●) and LMM (○) in phosphorylated (a) and dephosphorylated (b) myosin filaments. The cross-linking was carried out with 1 mg/mL myosin and 0.4 mg/mL dimethyl suberimidate in 80 mM NaCl, 40 mM triethanolamine, and 1 mM Ca^{2+} (pH 8.0). As described under Materials and Methods, the cross-linked samples were digested with α-chymotrypsin to yield S-2 and LMM and subsequently oxidized and run on sodium dodecyl sulfate–polyacrylamide gels. Relative intensities of oxidized S-2 (●) and LMM (○) were obtained from densitometric scans of the appropriate protein bands on polyacrylamide gels. The slopes of the curves shown above yield the apparent first-order rate constants for cross-linking S-2 and LMM.

Table I: Rate of Cross-Linking of Subfragment 2 in Phosphorylated and Dephosphorylated Myosin Filaments[a]

pH	ligand	$k_{S\text{-}2}/k_{LMM}$ dephosphorylated myosin	$k_{S\text{-}2}/k_{LMM}$ phosphorylated myosin
7.0		0.85 ± 0.06	0.73 ± 0.05
7.0	creatine phosphate, 10 mM	0.85 ± 0.06	0.70 ± 0.07
7.0	P_i, 10 mM	0.65	0.60
8.0		0.60 ± 0.05	0.45 ± 0.07

[a] Phosphorylated and dephosphorylated myosin filaments were cross-linked with dimethyl suberimidate either in 80 mM NaCl and 40 mM imidazole hydrochloride (pH 7.0) or in 80 mM NaCl and 40 mM triethanolamine hydrochloride (pH 8.0). When 10 mM ligand was present in the reaction, the concentration of imidazole hydrochloride was reduced to 30 mM. All cross-linking and digestion reactions were carried out at 20 °C. The cross-linking rates, which are averaged over three to four experiments carried out with different myosin preparations, were obtained as described for Figure 4; ± indicates range.

Table II: Comparison of the Assembled Forms of Phosphorylated and Dephosphorylated Myosin at pH 8.0[a]

assembly form	state of phosphorylation	$s_{20,w}$	OD_{310}
minifilaments	+	17.7	0.105
minifilaments	–	19.0	0.125
filaments	+	71.0	0.321
filaments	–	72.8	0.356

[a] Myosin filaments in 80 mM NaCl and 40 mM triethanolamine hydrochloride (pH 8.0) and minifilaments in 10 mM citrate–Tris (pH 8.0) were routinely measured at a concentration of 2 mg/mL. However, the sedimentation coefficients ($s_{20,w}$) of the phosphorylated (+) and dephosphorylated (–) structures were also similar at 1 and 3 mg/mL concentrations. Turbidity (OD_{310}) measurements were carried out at room temperature. The tabulated results are typical of the best (homogeneous) preparations. The differences between phosphorylated and dephosphorylated structures are within the range of variation between separate filament preparations (a total of five).

LMM to the filaments. The ratio of the cross-linking rates, $k_{S\text{-}2}/k_{LMM}$, can be viewed as a normalized measure of cross-bridge disposition (Ueno & Harrington, 1981a). Such presentation of cross-linking data corrects for variations in the reaction conditions and allows direct comparison of S-2 cross-linking in different systems. The results of cross-linking experiments are summarized in Table I. In all cases, the ratio $k_{S\text{-}2}/k_{LMM}$ was somewhat lower in the phosphorylated filaments than in dephosphorylated myosin. The phosphorylation-induced decrease in the rate of S-2 cross-linking was consistently detectable and always greater than the experimental error. Yet this decrease was significantly smaller than other charge-related changes in the myosin cross bridge. Both the elevation of pH from 7.0 to 8.0 (Ueno & Harrington, 1981a; Reisler & Liu, 1982) and the inclusion of 10 mM phosphate in the reaction medium resulted in a substantial drop in $k_{S\text{-}2}/k_{LMM}$ which could be attributed to the release of the S-2 segment from the filament surface. The physiologically relevant ligand, creatine phosphate, appeared to have no effect on the disposition of S-2.

It should be noted that all data presented in Table I refer to the behavior of long S-2 (60 000; generated during chymotryptic digestion of HMM) as revealed in our cross-linking experiments. However, similar results and conclusions regarding the relative effects of phosphorylation and pH on cross-bridge disposition were also obtained when the cross-linking rates of short S-2 and LMM were compared (not shown). So that short S-2 could be generated, the cross-linked samples were digested in two stages with α-chymotrypsin to yield long S-2 and LMM, which was followed by trypsin digestion to convert the long S-2 into its low molecular weight form (40 000).

Effect of Phosphorylation on Filament Formation. The sedimentation and turbidity properties of phosphorylated and dephosphorylated myosin filaments were compared in order to ascertain that the observed differences in their digestion and cross-linking did not stem from differences in their assembly. In agreement with Kardami & Gratzer (1982), we did not find any significant effect of light-chain phosphorylation on the formation of myosin filaments. The sedimentation coefficients of myosin minifilaments and filaments were virtually the same for the phosphorylated and dephosphorylated protein (Table II). Their respective turbidities were also similar (Table II), with somewhat higher values noted for the dephosphorylated structures. Not only did the final size of the phosphorylated and dephosphorylated filaments appear to be the same but also the rate of their formation was similar as well. When 0.1 M KCl was added to phosphorylated and dephosphorylated minifilaments in order to induce the growth of filaments

(Reisler et al., 1982), the turbidities of both systems increased at the same rate to yield similar final products (not shown).

Discussion

In a series of recent studies, Harrington and his collaborators (Sutoh et al., 1978; Chiao & Harrington, 1979; Ueno & Harrington, 1981a) have shown that relatively small changes in the charge balance on myosin filaments produce considerable changes in the rates of their proteolytic digestion and cross-linking. These transitions are suggestive of the release of myosin cross-bridges from the filament surface (Ueno & Harrigton, 1981a). Such release could in turn lead to the generation of force through an α-helix–coil transition in the S-2 region, as proposed by Harrington (1979) and Tsong et al. (1979). It appears from our previous studies that the charge effects which are responsible for the release of cross-bridges operate directly in the S-2 segment. This is indicated by a ligand (pyrophosphate)-induced decrease in the α-helical content of rod minifilaments (Oriol-Audit et al., 1981) and the fact that rod and myosin filaments undergo similar charge-related transitions (Reisler & Liu, 1982). Assuming that these observations are relevant to muscle contraction, it is of considerable interest to test for physiological equivalent(s) of alkaline pH conditions, which promote cross-bridge release.

In this study we have examined the effect of light-chain phosphorylation on the disposition of myosin cross-bridges. It has been pointed out by Bárány et al. (1980) that the LC-2 phosphorylation, in analogy to alkaline pH, should increase the negative charge on the filaments and possibly result in the swinging of cross-bridges toward the thin filaments. The arrangement of myosin molecules in the thick filament and the presumed overlap of the LC-2 subunits of one myosin molecule with the S-2 region of the adjacent molecule are not inconsistent with such a scheme.

The experimental tools used in this work for comparing cross-bridge disposition in phosphorylated and dephosphorylated filaments, i.e., cross-linking and proteolytic digestion, require careful consideration of structural differences between these two types of filaments. Disparity in size or the form of their assembly could result in different cross-linking and digestion rates for such structures. Morphological studies of phosphorylated and dephosphorylated myosin filaments (from vertebrate striated muscle) did not reveal any significant differences between these systems (Koretz, 1982). Our sedimentation velocity and turbidity measurments confirm that the phosphorylated and dephosphorylated filaments used in this work are indistinguishable for all practical purposes. The small differences in their sedimentation coefficients and turbidities are within range of variation between separate filament preparations.

With the structural similarity of the phosphorylated and dephosphorylated filaments well established, the observed differences in their chymotryptic digestion and cross-linking gain importance. The rate of proteolytic cleavage at the HMM–LMM junction is about 40% faster in the phosphorylated myosin than in the dephosphorylated protein. Although this phosphorylation effect is rather small, in particular when compared to that caused by the increase in pH from 7.0 to 8.0, it has been noted in previous reports as well (Kardami et al., 1980; Ritz-Gold et al., 1980). The increased rate of digestion at the HMM–LMM hinge does not result from the phosphorylation-induced stabilization of the head–rod junction against proteolysis. Such possibility can be excluded because the rate of S-1 formation from the intact dephosphorylated myosin is negligible under our experimental conditions (80 mM NaCl and 1 mM Ca^{2+}). The interpretation of the digestion data in terms of phosphorylation-induced change in the HMM–LMM hinge is also consistent with the results of the cross-linking experiments. The decrease in the rate of S-2 cross-linking to the filament surface upon LC-2 phosphorylation can be explained, in analogy with previous studies (Ueno & Harrington, 1981a), by partial release of cross-bridges.

It is quite clear that on the scale of the pH (or phosphate)-induced effect, phosphorylation of myosin produces only a partial change in the properties of the myosin cross-bridge. This fact, or perhaps other factors, contributed to the observation that LC-2 phosphorylation does not affect the rotary mobility of myosin heads (Bárány et al., 1980). However, it should be realized that the pH effect may not necessarily be an appropriate yardstick or accurate measure of the in vivo transitions within the myosin cross-bridge. Furthermore, the significance of the phosphorylation-modulated partial release of cross-bridges should be evaluated in conjunction with the reports that actin has a higher affinity for the modified than the unmodified myosin. Both the cross-bridge release and changes in the affinity for actin facilitate actomyosin interactions. Thus, myosin phosphorylation may play an active role in the contraction of skeletal muscle even though it probably is not the sole or the main factor which determines cross-bridge disposition.

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Binding of Gizzard Smooth Muscle Myosin Subfragment 1 to Actin in the Presence and Absence of Adenosine 5′-Triphosphate†

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ABSTRACT: The binding of gizzard smooth muscle subfragment 1 (S-1) and skeletal muscle S-1 to skeletal muscle actin was compared at varying ionic strengths in the presence and absence of nucleotide. The nucleotides, AMP-PNP and ATP, weaken the binding of smooth muscle S-1 and skeletal muscle S-1 to actin to a similar extent. In both cases, the acto·S-1 association constant was reduced about 500-fold and 50 000-fold in the presence of AMP-PNP and ATP, respectively. On the other hand, the effect of ionic strength on the binding of S-1 to actin was found to be markedly different for smooth muscle S-1 and skeletal muscle S-1. Increasing ionic strength only slightly weakened the binding of smooth muscle S-1 to actin, whereas it greatly weakened the binding of skeletal muscle S-1. At low ionic strength ($\mu < 0.05$ M), skeletal muscle S-1 bound more strongly to actin than did smooth muscle S-1 both in the presence and in the absence of nucleotide, whereas at high ionic strength ($\mu > 0.05$ M), the reverse was true. The steady-state kinetics of the actomyosin ATPase were also examined with smooth muscle S-1. At low ionic strength ($\mu = 0.012$ M), the apparent binding constant obtained from the double-reciprocal plot of ATPase activity vs. actin concentration ($K_{ATPase} = 3.2 \times 10^4$ M^{-1}) was 4-fold larger than the binding constant obtained from direct measurement of the binding of smooth muscle S-1 to actin in the presence of ATP ($K_{binding} = 8.5 \times 10^3$ M^{-1}). This is similar to the difference between K_{ATPase} and $K_{binding}$ observed for skeletal muscle S-1. Therefore, at physiological ionic strength, the cross-bridges in smooth muscle may undergo a similar kinetic cycle as in skeletal muscle but may bind more tightly to the actin filaments.

The fundamental process which drives muscle contraction is the interaction of actin, myosin, and ATP. In skeletal muscle, these interactions have been well characterized by using the single-headed soluble fragment of myosin, subfragment 1. S-1[1] binds to actin with a binding constant of about 10^7 M^{-1} at physiological ionic strength (Marston & Weber, 1975; Highsmith, 1977; Margossian & Lowey, 1978; Greene & Eisenberg, 1980a), but this binding becomes considerably weaker in the presence of nucleotide. ADP weakens this binding about 30-fold, AMP-PNP weakens it about 500-fold, and ATP weakens it more than 5000-fold (Greene & Eisenberg, 1980a; Marston et al., 1979; Hofmann & Goody, 1978; Stein et al., 1979). In addition, with all of these nucleotides, as well as in the absence of nucleotide, increasing ionic strength weakens the binding of S-1 to actin (Highsmith, 1977; Margossian & Lowey, 1978; Greene & Eisenberg, 1978).

Studies have suggested that skeletal and smooth muscle myosins may differ in their strength of binding to actin in the presence of nucleotide. Krisanda & Murphy (1980) found the K^+-EDTA ATPase activity of smooth muscle (gizzard) myosin was inhibited by skeletal muscle actin at much lower concentrations than was the ATPase activity of skeletal muscle myosin. On the basis of this observation, they suggested that, at least at high ionic strength ($\mu \simeq 0.5$ M), smooth muscle myosin binds to actin more strongly than does skeletal muscle myosin. On the other hand, two studies conducted at much lower ionic strength suggest that smooth muscle myosin might bind to actin more weakly than does skeletal muscle myosin. Marston & Taylor (1980) measured the ATPase activity of both smooth muscle S-1 and skeletal muscle S-1 as a function of actin concentration. The values they obtained for the apparent binding constant (K_{ATPase}) suggested that in the presence of ATP, smooth muscle S-1 bound to actin more weakly than did skeletal muscle S-1. Similarly, Ikebe et al. (1981) found that, at low ionic strength in the presence of AMP-PNP, smooth muscle HMM bound more weakly to actin than did skeletal muscle HMM. To determine exactly how smooth and

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[1] Abbreviations: S-1, subfragment 1; acto·S-1, a complex of actin with S-1; AMP-PNP, adenyl-5′-yl imidodiphosphate; DTT, dithiothreitol; Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PEI, poly(ethylenimine); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.