Role of Magnesium Binding to Myosin in Controlling the State of Cross-Bridges in Skeletal Rabbit Muscle†

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ABSTRACT: The effect of Mg^{2+} on the disposition of myosin cross-bridges was studied on myofibrils and synthetic myosin and rod filaments by employing chymotryptic digestion and chemical cross-linking methods. In the presence of low Mg^{2+} concentrations (0.1 mM), the proteolytic susceptibility at the heavy meromyosin/light meromyosin (HMM/LMM) junction in these three systems sharply increases over the pH range from 7.0 to 8.2. Such a change has been previously associated with the release of myosin cross-bridges from the filament surface [Ueno, H., & Harrington, W. F. (1981) *J. Mol. Biol. 149*, 619–640]. Millimolar concentrations of Mg^{2+} block or reverse this charge-dependent transition. Rod filaments show the same behavior as myosin filaments, indicating that the low-affinity binding sites for Mg^{2+} are located on the rod portion of myosin. The interpretation of these results in terms of Mg^{2+}-mediated binding of cross-bridges to the filament backbone is supported by cross-linking experiments. The normalized rate of S-2 cross-linking in rod filaments at pH 8.0, $k_{S\text{-}2}/k_{LMM}$, increases upon addition of Mg^{2+} from 0.30 to 0.65 and approaches the cross-linking rate measured at pH 7.0 (0.75), when the cross-bridges are close to the filament surface. In rod filaments prepared from oxidized rod particles, chymotryptic digestion proceeds both at the S-2/LMM junction and at a new cleavage site located in the N-terminal portion of the molecule. Kinetic analysis of digestion rates at these two sites reveals that binding of Mg^{2+} to oxidized myosin rods has a similar effect at both sites over the pH range from 7.0 to 8.0. These results are consistent with the Mg^{2+}-induced association of cross-bridges with the filament backbone.

Until recently, the interest in the binding of divalent cations to myosin has been limited to the high-affinity binding sites located on the light chain 2 (LC-2)[1] subunits of this protein. The fact that in molluscan muscle the binding of Ca^{2+} to myosin regulates its interaction with actin (Lehman & Szent-Györgyi, 1975; Kendrick-Jones et al., 1976; Simmons & Szent-Györgyi, 1978) prompted extensive studies on the isolated (Werber et al., 1972; Morimoto & Harrington, 1974; Werber & Oplatka, 1974; Alexis & Gratzer, 1978) and in situ light chains (Morimoto & Harrington, 1974; Bagshaw, 1977; Weeds & Pope, 1977; Kardami et al., 1980; Oda et al., 1980; Pemrick, 1980; Borejdo & Werber, 1982; Cheung & Reisler, 1982; Wagner & Stone, 1983). In spite of these investigations, the function of LC-2 chains in vertebrate striated muscle is still disputed. In fact, Bagshaw & Kendrick-Jones (1979) have shown that the rabbit myosin LC-2 chains do not have specific Ca^{2+} sites (separate from Mg^{2+} sites) and are unlikely to regulate the actomyosin ATPase activity. Whether this lack of "Ca sensitivity" represents evolutionary or perhaps preparative loss (Lehman, 1978) is yet to be determined.

It now appears equally doubtful that the binding of Ca^{2+} to LC-2 chains has any effect on the disposition of myosin cross-bridges, i.e., any structure-mediated role in the regulation of the cross-bridge cycle (Haselgrove, 1975). The binding of divalent cations to the light chains does not change the rotational mobility and the angular disposition of myosin heads with respect to the thick filament backbone (Mendelson & Cheung, 1976; Sutoh & Harrington, 1977). Consistent with this, low concentrations of Ca^{2+} seem to have no effect on the hydrodynamic properties of myosin minifilaments (Cheung & Reisler, 1982), although previous studies on myosin filaments have detected minor Ca^{2+}-induced changes in the aggregated system (Morimoto & Harrington, 1974).

A new aspect of the possible involvement of divalent cations in cross-bridge action has been revealed in the recent findings of Borejdo & Werber (1982). Working with skeletal muscle myofibrils (at pH 8.5), these authors have noted that high concentrations (millimolar) of divalent cations inhibit the chymotryptic digestion at the HMM/LMM junction in myosin. They concluded that myosin contains a second set of metal binding sites, with association constants in the range of 10^3 M^{-1}, which are probably located in the rod portion of the molecule. Thus, under physiological conditions, these sites are at least partially occupied by Mg^{2+}, which in turn could have either a local effect on the HMM/LMM junction (sensed by chymotryptic digestion) or a major effect on the entire cross-bridge.

The goal of this work was to examine the state of myosin cross-bridges in myofibrils, myosin filaments, and rod filaments in the presence of millimolar concentrations of Mg^{2+}, and over the pH range from 7.0 to 8.5. By employing chymotryptic digestions and chemical cross-linking reactions, we show that in these three systems Mg^{2+} counters the charge-induced release of myosin cross-bridges from the filament surface. We note that in oxidized myosin rods a new proteolytic site is expressed in the N-terminal portion of these particles. The effects of Mg^{2+} on the cleavage reaction at this site are consistent with an overall change in the S-2 disposition, i.e., the "locking" of S-2 onto the filament surface. Our results suggest that physiological concentrations of Mg^{2+} promote tight association of cross-bridges with the thick filament backbone.

Materials and Methods

Distilled water and analytical-grade reagents were used in all experiments. Dimethyl suberimidate, α-chymotrypsin, trypsin, phenylmethanesulfonyl fluoride, soybean trypsin in-

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[1] Abbreviations: HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment 1; S-2, subfragment 2; LC-2, Ca^{2+}-binding 19000 molecular weight subunit of myosin; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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hibitor, diamide, and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Myofibrils and Proteins. Rabbit skeletal myofibrils were prepared as described by Kominz (1970) and stored at −20 °C in 50% glycerol, 0.02 M KCl, 0.01 M imidazole (pH 7.0), 1 mM DTT, and 0.1 mM sodium azide. Prior to their use, the myofibrils were washed (between 5 and 8 times) with 10 volumes of the final buffer and collected by centrifugation in a clinical centrifuge. The final buffer contained 80 mM NaCl and either 40 mM imidazole hydrochloride (pH 7.0–7.4) or 40 mM triethanolamine hydrochloride (pH 7.4–8.3). The protein concentration of a myofibrillar suspension was determined in 5% (w/v) SDS by assuming $E^{1\%}_{280nm} = 7.0$ (Sutoh & Harrington, 1977). Control chymotryptic digestions of myofibrils did not reveal any proteolytic differences between freshly prepared myofibrils and those stored in glycerol.

Rabbit myosin, rod filaments, light meromyosin, and subfragment 2 were prepared as described previously (Oriol-Audit et al., 1981; Sutoh et al., 1978). Unless stated otherwise, all rod preparations were carried out in the presence of 1 mM DTT. The purity of all proteins was verified on sodium dodecyl sulfate–polyacrylamide gels.

Myosin and rod filaments were prepared by dialyzing these proteins (in 0.5 M KCl and 10 mM sodium phosphate at pH 7.0) against 80 mM NaCl and 40 mM triethanolamine hydrochloride (pH 8.2) followed by dialysis against the final buffer (80 mM NaCl and either 40 mM imidazole or 40 mM triethanolamine).

Digestion of Myofibrils, Myosin Filaments, and Rod Filaments with α-Chymotrypsin. Myofibrils (3 mg/mL) in 80 mM NaCl and either 40 mM imidazole hydrochloride (pH 7.0–7.4) or 40 mM triethanolamine (pH 7.4–8.2) were digested with α-chymotrypsin (0.05 mg/mL) in the presence of 0.1 and 5.0 mM $MgCl_2$, at 25 °C. At given time intervals, aliquots were removed from the digestion system, the proteolysis was terminated by addition of phenylmethanesulfonyl fluoride (1 mM), and the samples were denatured and examined on polyacrylamide gels. BSA 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). The decay curves fitted a single exponential at all pH values. The first-order rate constants obtained from such plots were corrected for the intrinsic pH dependence of the proteolytic activity of α-chymotrypsin (Hess, 1971).

The digestion of myosin filaments (3 mg/mL) was carried out under the same conditions as those for myofibrils, employing the same buffer systems, the same amounts of α-chymotrypsin (0.05 mg/mL), and the same procedures for data collection and analysis.

The digestion of rod filaments (2 mg/mL) was carried out as above, except that the concentration of α-chymotrypsin varied in these experiments between 0.15 (at pH 7.0) and 0.03 mg/mL (at pH 8.2). The digested rods contained between four and five free sulfhydryl groups per rod as determined by DTNB titration.

Proteolytic digestions of filaments prepared from oxidized rods were conducted as above. Oxidized rods contained between 0 and 0.3 free sulfhydryl group per rod.

Oxidation of Rods. Myosin rods (4 mg/mL) were oxidized in their monomeric form in 0.5 M KCl and 20 mM Tris-HCl (pH 8.0) by incubation for 1 h with either a 100-fold molar excess of diamide (Kosower, 1969) or a 5-fold molar excess of $CuCl_2$. The oxidation by $CuCl_2$ was terminated by addition of 1 mM EDTA, and that by diamide was stopped by removing the reagent from the protein sample. Diamide, $CuCl_2$, and EDTA were removed from the respective samples by centrifugation through Sephadex G-50 centrifugation columns (Penefsky, 1977; Reisler & Liu, 1981).

Determination of the Free Sulfhydryl Groups in Rods. The number of free sulfhydryl groups in rods was determined by titration of the protein (1.0–2.0 mg/mL) with DTNB in the presence of 7 M urea (Ellman, 1958). The number of sulfhydryl groups was calculated by using an extinction coefficient of 13 600 M^{-2} at 412 nm.

Cross-Linking of Rod Filaments (Reduced Rods). Cross-linking, digestion, oxidation, and data analysis procedures were as described before (Reisler & Liu, 1982) and originally employed by Ueno & Harrington (1981). Rod filaments (1 mg/mL) suspended in 80 mM NaCl and either 40 mM imidazole hydrochloride (pH 7.0–7.4) or 40 mM triethanolamine hydrochloride (pH 7.4–8.2) were cross-linked with dimethyl suberimidate in the presence of 0.1 and 5.0 mM $MgCl_2$, at 20 °C. The concentration of dimethyl suberimidate varied in these experiments between 0.2 and 2.0 mg/mL. At various time points of the cross-linking reactions, aliquots were withdrawn from the mixtures and were digested for 4 min with α-chymotrypsin (0.05 mg/mL) in the presence of 0.5 M KCl (pH 8.0), to yield LMM and S-2. After termination of the digestion with phenylmethanesulfonyl fluoride, and neutralization of the remaining cross-linker with ethanolamine, the samples were oxidized with $CuCl_2$ (Ueno & Harrington, 1981), denatured, and run on polyacrylamide gels in the absence of β-mercaptoethanol. The oxidized LMM and S-2 were identified on gels as described previously (Reisler & Liu, 1982). Note that the cross-linking reactions were carried out with reduced rods. The oxidation step was introduced here after digestion of the sample in order to distinguish between intra- and intermolecular cross-linking of rods. The time course of S-2 and LMM cross-linking was determined by measuring the decay rate of the respective protein bands on sodium dodecyl sulfate–polyacrylamide gels as a function of cross-linking time.

Turbidity Measurements. The turbidity of myosin filaments was recorded at 310 nm with a Beckman Model 25 spectrophotometer. Prior to turbidity measurements, myosin solutions were allowed to equilibrate with 0.1 and 5.0 mM $MgCl_2$ at 4 °C for 24 h.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to the procedure of Laemmli (1970) using 7.5% (w/v) or composite 15% and 7.5% acrylamide gels. 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.

Results

Effect of Mg^{2+} on the Chymotryptic Digestion of Myofibrils. Limited chymotryptic digestion of myosin filaments in the presence of Ca^{2+} and Mg^{2+} leads to the formation of two major fragments, HMM and LMM (Weeds & Pope, 1977; Bagshaw, 1977; Borejdo & Werber, 1982). In order to obtain such a simple proteolysis pattern, the high-affinity binding sites for divalent cations on LC-2 have to be occupied. As shown in Figure 1, and demonstrated before by Weeds & Pope (1977) and Kardami et al. (1980), the full saturation of the metal sites on LC-2 is achieved in the presence of 0.1 mM Mg^{2+} (and below). Under such conditions, whether in myofibrils or in synthetic filaments, one cannot detect any significant formation of S-1. The obvious advantage of the localized proteolysis is

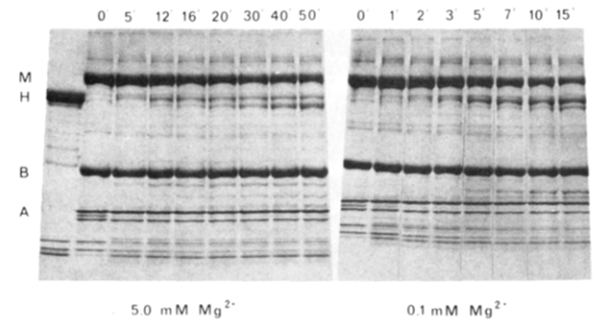

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gels showing the time course of chymotryptic digestion of myosin in myofibrils in the presence of 0.1 and 5.0 mM Mg^{2+}. Myofibrils (3.0 mg/mL) were digested at 25 °C in 80 mM NaCl and 40 mM triethanolamine hydrochloride (pH 8.2) with 0.05 mg/mL chymotrypsin and run on composite 7.5% and 15% (w/v) polyacrylamide gels. Digestion times are indicated on the top of each gel. The symbols on the left side refer to the following bands: M, myosin; H, HMM; B, BSA (which was added as an internal standard); A, actin.

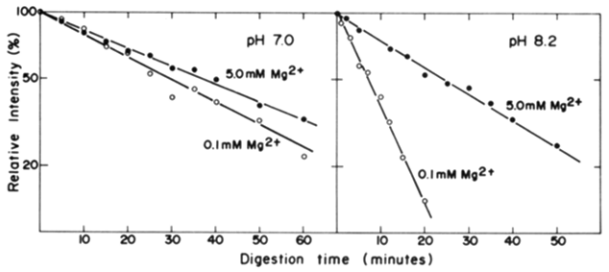

FIGURE 2: Relative densities of myosin heavy-chain bands as a function of the digestion time of myofibrils with α-chymotrypsin (at 25 °C) at pH 7.0 and 8.2, and in the presence of 0.1 and 5.0 mM Mg^{2+}.

that it becomes a convenient probe of the conformational state of the HMM/LMM junction. Thus, by monitoring the rate of myosin cleavage as a function of pH, it has been possible to correlate the large increase in the proteolytic susceptibility of the HMM/LMM junction (over the pH range from 7.0 to 8.2) with the release of cross-bridges from the filament surface (Ueno & Harrington, 1981; Reisler & Liu, 1982). According to Borejdo & Weber (1982), when the cross-bridges are released (at pH 8.5), the cleavage at the HMM/LMM junction in myofibrils is inhibited in the presence of millimolar concentrations of Mg^{2+} and Ca^{2+}. This observation is confirmed in Figure 1. Visual inspection of gels monitoring the chymotryptic digestion of myofibrils (at pH 8.2) in the presence of 0.1 and 5.0 mM Mg^{2+} indicates slower fragmentation of the myosin heavy chain and slower formation of HMM in the latter case. Since in the presence of both 0.1 and 5.0 mM Mg^{2+} we do not detect any direct proteolysis of myosin into S-1, the respective rates of heavy-chain degradation are pertinent to the events occurring at the HMM/LMM junction. Figure 2 shows in a quantitative manner the Mg^{2+}-induced inhibition of myosin digestion at pH 8.2. In order to check whether this inhibition is related to the state of myosin cross-bridges, we have examined the rate of myosin digestion in myofibrils at several pH values, i.e., at different average dispositions of cross-bridges. We note that at pH 7.0, when the cross-bridges reside primarily at (or next to) the filament surface (Chiao & Harrington, 1979), the proteolytic susceptibility of the HMM/LMM junction is but little affected by the binding of Mg^{2+} to the low-affinity sites on myosin (Figure 2). The two slopes for digestion of myosin in the presence of 0.1 and 5.0 mM Mg^{2+} are quite similar.

The rates of myosin heavy-chain cleavage in myofibrils, derived from plots similar to those shown in Figure 2, are summarized in Table I. In the presence of 0.1 mM Mg^{2+},

Table I: Rate Constants for Chymotryptic Digestion of the HMM/LMM Junction in Myofibrils in 80 mM NaCl and 40 mM Imidazole or Triethanolamine[a]

	rate constant (h^{-1})	
pH	0.1 mM $MgCl_2$	5.0 mM $MgCl_2$
7.0	0.58	0.50
7.4	0.88	0.75
7.8	1.70	0.88
8.2	2.76	0.73

[a] Rate constants for digestion of the myosin heavy chain at the HMM/LMM junction in myofibrils were derived from plots similar to those shown in Figure 2. In a given preparation of myofibrils, the rate constants were reproducible within 10%. The scatter between different preparations was somewhat greater. The presented rate constants are corrected for the intrinsic pH dependence of α-chymotryptic activity.

the digestion rate (corrected for the pH dependence of chymotrypsin activity) increased by 500% over the pH range from 7.0 to 8.2 whereas in the presence of 5.0 mM Mg^{2+} this increase is limited to about 50%. Thus, it appears that in the presence of millimolar Mg^{2+} concentrations the rate of proteolysis at the HMM/LMM junction does not change dramatically with pH and is comparable to that observed for myofibrils at pH 7.0, i.e., with the cross-bridges close to the filament surface.

Effect of Mg^{2+} on the Chymotryptic Digestion and Turbidity of Synthetic Myosin and Rod Filaments. A more detailed study of the pH-dependent changes in assembled myosin was carried out on synthetic myosin and rod filaments (reduced), which are easier to handle and amenable to more accurate data analysis than myofibrils. Following the same general procedures as used with myofibrils, we have determined the rates of chymotryptic cleavage at the HMM/LMM junction in these filaments in the presence of 0.1 and 5.0 mM Mg^{2+}. Each filament sample was prepared by dialyzing the protein against 80 mM NaCl and 40 mM imidazole or triethanolamine solvents at the appropriate final pH and was allowed to equilibrate with Mg^{2+} for 1 h. The rates of digestion at each pH were obtained by monitoring on SDS gels the cleavage of the heavy chain and then representing the derived data in the semilogarithmic plots similar to those shown in Figure 2. For digestion of myosin filaments in the presence of 0.1 mM Mg^{2+}, we observe (in Figure 3) the familiar sharp increase in the rate of proteolysis at the HMM/LMM junction over the pH range from 7.0 to 8.2 (Ueno & Harrington, 1981). A somewhat greater change with pH is noted for comparable digestions of rod filaments (Figure 3). In both synthetic systems of myosin and rod filaments, the charge-induced effects on chymotryptic digestion of the heavy chain are greater than in myofibrils. To show this, we have included in Figure 3 the normalized rates of myosin cleavage in myofibrils.

The pH-dependent changes in the proteolysis of the synthetic systems are likely to arise from superposition of two effects. The first one, related to cross-bridge disposition, operates in myofibrils as well. The second contribution, that of a pH-dependent change in the extent of myosin polymerization, is specific to the isolated filament systems. As shown by turbidity measurements in Figure 3, the size of myosin filaments decreases significantly between pH 7.0 and 8.0. This decrease in the turbidity of filaments is substantially reduced, but not abolished, in the presence of 5.0 mM Mg^{2+}. Sedimentation velocity experiments indicate that Mg^{2+} does not induce any "artifactual" or heterogeneous aggregation of myosin filaments. Schlieren patterns of myosin filaments at pH 8.2 in the presence of 0.1 and 5.0 mM Mg^{2+} are similarly sharp and show

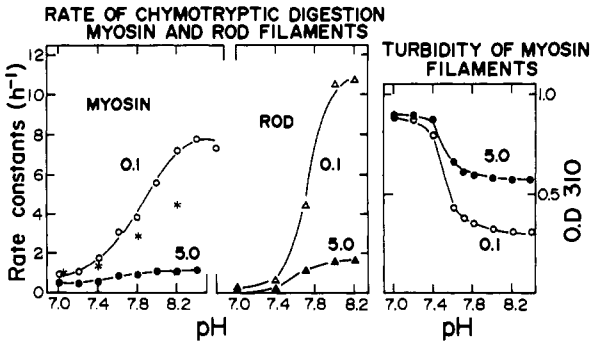

FIGURE 3: Effect of Mg^{2+} on the rate of digestion and turbidity of myosin and rod filaments. The left panel shows the rate of chymotryptic digestion of myosin (○, ●) and rod (△, ▲) filaments in the presence of 0.1 (○, △) and 5.0 (●, ▲) mM Mg^{2+} as a function of pH. Myosin and rod filaments were digested at 25 °C in 80 mM NaCl and 40 mM imidazole hydrochloride (pH 7.0–7.4) or 40 mM triethanolamine hydrochloride (pH 7.4–8.4). Digested products were analyzed as described under Materials and Methods. The apparent first-order rate constants for cleavage of myosin and rod at the HMM/LMM junction were derived at each pH value from plots similar to those shown in Figure 2. The presented rate constants are corrected for the intrinsic pH dependence of α-chymotryptic activity. Rate constants for digestion of myosin in myofibrils (*) are taken from Table I, normalized to the digestion rate of myosin filaments at pH 7.0, and shown here for comparison with the synthetic filament systems. The right panel shows the turbidity (optical density at 310 nm) of myosin filaments in the presence of 0.1 and 5.0 mM Mg^{2+} as a function of pH. Turbidities of myosin solutions (3.0 mg/mL) were measured after a period of 24-h equilibration with 0.1 and 5.0 mM Mg^{2+}. Essentially the same results were obtained after a much shorter, 30-min incubation of the myosin solution with Mg^{2+}.

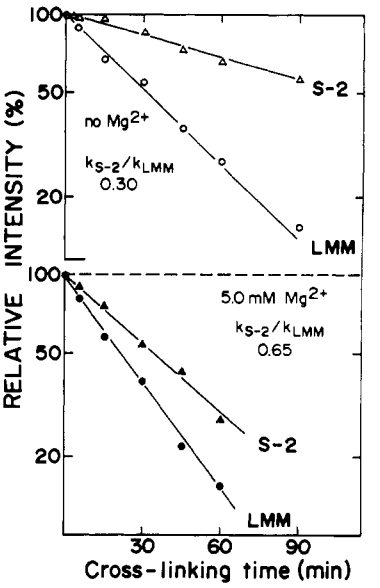

FIGURE 4: Time course of cross-linking LMM and S-2 in reduced rod filaments in 80 mM NaCl and 40 mM triethanolamine hydrochloride (pH 8.0) in the absence and presence of Mg^{2+} (5.0 mM). The cross-linked samples (1 mg/mL) were digested in the presence of 0.5 M NaCl to yield LMM and S-2. The products of digestions were oxidized and run on gels. Relative intensities of S-2 (△, ▲) and LMM (○, ●) were obtained from densitometric scans of sodium dodecyl sulfate–polyacrylamide gels. The slopes of the curves shown in this figure yield the apparent first-order rate constants for cross-linking S-2 and LMM. The ratio of these constants, k_{S-2}/k_{LMM}, is the normalized rate of S-2 cross-linking.

only a minor amount of monomer present but differ greatly in the sedimentation coefficients of the respective species (which is higher in the presence of 5.0 mM Mg^{2+}).

In analogy to myofibrils, the chymotryptic cleavage of myosin and rod filaments in the presence of 5.0 mM Mg^{2+} (or 3 mM Ca^{2+}) shows a rather limited pH dependence (Figure 3). Since myosin rods do not differ in this respect from myofibrils and myosin filaments, the low-affinity binding sites for Mg^{2+} must be located on the rod. The logical conclusion that can be drawn from the results presented in Table I and Figure 3 is that the binding of Mg^{2+} to the rod portion of myosin counters the pH-induced changes in the proteolytic susceptibility of the HMM/LMM junction in assembled protein. To the extent that such changes reflect the release of myosin cross-bridges from the filament surface, the present results may be taken to indicate that Mg^{2+} prevents or inhibits such release. However, an alternative explanation of the data shown above in terms of some as yet undefined local effects of Mg^{2+} on the HMM/LMM junction cannot be excluded on the basis of digestion experiments alone. In order to resolve between these two possible interpretations of the Mg^{2+} effect, we have carried out cross-linking experiments on rod filaments in the absence and presence of Mg^{2+} (5.0 mM).

Cross-Linking of Rod Filaments. Previous work on rod filaments (Reisler & Liu, 1982) has established that the relative rate of S-2 cross-linking in these structures decreases over the pH range from 7.0 to 8.2 in the same manner as in myofibrils and myosin filaments. This indicates the release of S-2 element from the filament surface at alkaline pH. We have now carried out similar cross-linking experiments in order to determine the state of cross-bridges in the presence of millimolar concentrations of Mg^{2+}. As before, the rod filaments were cross-linked with dimethyl suberimidate and then digested with α-chymotrypsin to yield LMM and S-2. The intensity of the corresponding bands on SDS gels (not shown here) was quantitated as previously described (Reisler & Liu, 1982). The cross-linking rates for these two fragments are determined from decay curves for the intensity of the respective bands (Figure 4). As discussed before (Ueno & Harrington, 1981; Reisler & Liu, 1982), the ratio of the cross-linking rates, k_{S-2}/k_{LMM}, i.e., the relative rate of S-2 cross-linking, is indicative of S-2 disposition with respect to the filament backbone. At pH 8.0, and in the absence of Mg^{2+}, we determine $k_{S-2}/k_{LMM} = 0.30$ (Figure 4), which is consistent with released state of S-2 (Reisler & Liu, 1982). However, the same cross-linking reaction carried out in the presence of 5.0 mM Mg^{2+} yields a much higher value, $k_{S-2}/k_{LMM} = 0.65$. This, in fact, is close to the relative rate of S-2 cross-linking at pH 7.0 (in the absence of Mg^{2+}, $k_{S-2}/k_{LMM} = 0.75$), when the cross-bridges are next to the filament surface. Mg^{2+} has only a minor effect on the rate of S-2 cross-linking at pH 7.0 (+5.0 mM Mg^{2+}, $k_{S-2}/k_{LMM} = 0.80$), well within the experimental error in these determinations ($k_{S-2}/k_{LMM} = \pm 0.10$). Nevertheless, we should note that the proteolytic digestions also reveal a small Mg^{2+}-induced inhibition of myosin cleavage at pH 7.0 (Figure 3). The clear implication of the cross-linking experiments is that the binding of Mg^{2+} to rod filaments counters the charge-induced release of S-2 and "positions" it next to the filament surface.

Proteolytic Cleavage Sites in Filaments Prepared from Oxidized Rods. In the previous sections, we have shown that Mg^{2+} inhibits (or counters) both the release of myosin cross-bridges and the chymotryptic digestion at the HMM/LMM junction. Yet, the precise relationship between these two effects of Mg^{2+} is not clear. The question in dispute is whether the observed charge-induced changes in the rate of cleavage at the HMM/LMM junction are related to its steric accessibility, which is a function of cross-bridge release, or whether they reflect changes in the secondary structure of that region which could be triggered by the release of cross-bridges.

An unexpected insight into the nature of the proteolytically sensed pH-induced changes in the HMM/LMM junction is

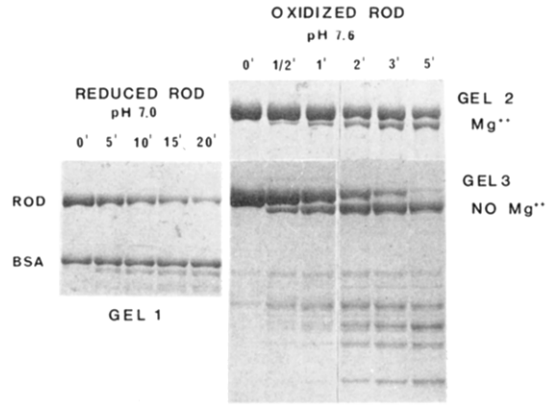

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gels showing the time course of chymotryptic digestion of reduced rod filaments at pH 7.0 (gel 1, left panel) and oxidized rod filaments at pH 7.6 in the absence (gel 3) and presence (gel 2, right panel) of 3.0 mM Mg^{2+}. Rod filaments were digested at 25 °C in 80 mM NaCl and 40 mM imidazole hydrochloride (pH 7.0) or 40 mM triethanolamine hydrochloride (pH 7.6). Reduced rod filaments were digested in the absence of Mg^{2+}. Times of digestion are indicated on the top of each gel. Other experimental details are given under Materials and Methods. Note the appearance of a new band corresponding to a short rod in digestions of rod filaments prepared from oxidized protein.

provided by studies on filaments of oxidized rods. We have noted that myosin rods can be oxidized under mild conditions by employing diamide or $CuCl_2$ (see Materials and Methods). The oxidized protein contains between 0 and 0.3 free sulfhydryl per rod and forms filaments whose turbidity and sedimentation velocity properties are similar to (though not identical with) those of reduced rod filaments. In spite of this similarity, the oxidized rod filaments show a distinct proteolysis pattern. Its main feature is the "expression" of a new cleavage site located about 10–11K from the N-terminal site of a rod, i.e., in the vicinity of the oxidized cysteine residues. As shown in Figure 5, the chymotryptic digestion of *reduced* rod filaments leads to the standard disappearance of the heavy-chain band on SDS gels with the progress of the proteolytic reaction. Similar chymotryptic (or tryptic, not shown here) digestion of the oxidized rod filaments produces a truncated "short" rod as well as LMM and S-2. Also, such short rods can be identified in some of the previous studies (Sutoh et al., 1978). This new cleavage site is affected by the binding of Mg^{2+} to the rod, since the formation of short rods is visibly inhibited in the presence of 3.0 mM Mg^{2+} (Figure 5).

The assignment of the new cleavage site to the N-terminal portion of the rod rests on the comparison of the LMM and S-2 fragments generated in chymotryptic digestion of purified short rods (in 0.5 M NaCl) with the fragments obtained from standard, reduced and monomeric, rods. LMM fragments made from short rods, and identified as such by their insolubility under low salt conditions (pH 6.5), comigrate on SDS gels with LMM made from standard rods (Figure 6). On the other hand, S-2 prepared from short rods has a higher mobility on SDS gels (about 10–11K) than the corresponding S-2 made from standard rods (Figure 6).

With the new cleavage site assigned by the above procedure to the N-terminal portion of oxidized rods, we can represent schematically their chymotryptic digestion as shown in Figure 7. The proteolysis may proceed at a rate k_1 at site 1 (CT-1) to yield short rods and at a rate k_2 at site 2 (CT-2, the S-2/LMM junction) to yield LMM and S-2. The short rods made in the first reaction can in turn be cleaved at a rate k_2' ($\approx k_2$) to give LMM and truncated S-2 (M_r = 46 000; this is different form the short S-2). The intuitively reasonable assumption that $k_2' \approx k_2$ is supported by the fact that the decay

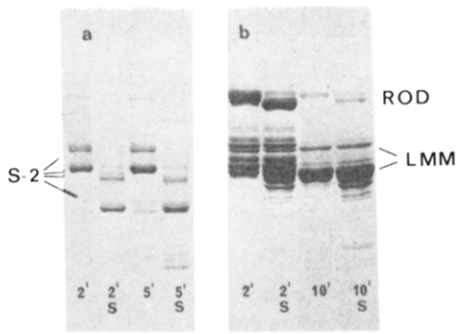

FIGURE 6: Electrophoresis of rod fragments prepared by chymotryptic digestion of standard and short rods in 0.5 M NaCl (pH 8.0). S-2 (panel a) was recovered from the soluble fraction of 2- and 5-min digestions of standard rods (2′ and 5′), and short rods (2′ S and 5′ S). LMM (panel b) was recovered from the insoluble fraction of 2- and 10-min digestions of standard (2′ and 10′) and short (2′ S and 10′ S) rods.

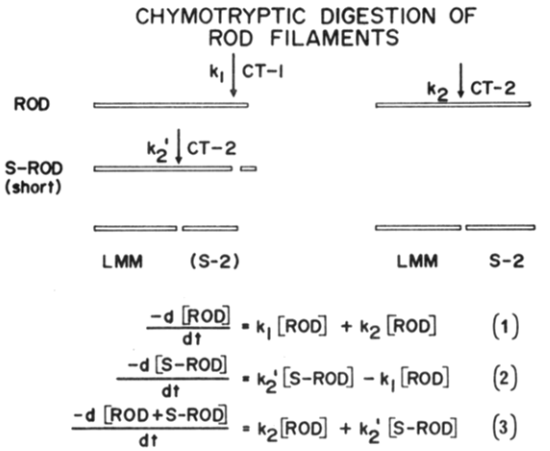

FIGURE 7: Schematic representation of chymotryptic digestion of rod filaments. In reduced rod filaments, the rate constant k_1 is very small, and the cleavage proceeds primarily at site CT-2 with a rate constant k_2. In oxidized rods, the digestion occurs at both sites CT-1 and CT-2 (the HMM/LMM junction). It can be assumed that k_2' is equal to k_2 (see text). Rate constants k_1 and k_2 are determined according to eq 1–3. The decay in the intensity of the original rod bands yields $k_1 + k_2$ (eq 1). The decay in the combined intensity of original and short rods determines k_2 (eq 3).

of the combined fractions of standard and short rods during chymotryptic digestion (eq 3 in Figure 7) fits a single exponential (not shown here).

Analysis of Chymotryptic Digestion of Oxidized Rod Filaments. The attractive feature of oxidized rods is that in each digestion experiment we can obtain separately $k_1 + k_2$ (by following the decay in the amount of the original rod species, eq 1 in Figure 7) and k_2 (according to eq 3 in Figure 7, since $k_2 \approx k_2'$). Thus, we can determine the effect of Mg^{2+} on the rate of cleavage (k_2) at the S-2/LMM junction and on the rate of proteolysis (k_1) at site CT-1, which is distant from this junction. The rate data presented in Table II indicate that the ratios $k_{1,Mg}/k_1$ and $k_{2,Mg}/k_2$ are indeed similar at all pH values tested. This suggests that both cleavage sites sense the same Mg^{2+}-induced change. It should be noted in this context that the rate of cleavage at the S-2/LMM junction in oxidized rods, k_2, varies with pH in the same manner as shown for reduced rods (Figure 3).

The kinetic analysis of rod digestions and the use of eq 1–3 in Figure 7 for deriving the rate constants k_1 and k_2 have been tested by following the cleavage of rods in the presence of 3 mM MgATP. Initially, we have found that MgATP does not have any effect on the rate of cleavage (k_2) in standard, re-

Table II: Effect of Mg^{2+} on the Rates of Chymotryptic Digestion of Oxidized Rod Filaments at Two Distinct Sites[a]

pH	$k_{1,Mg}/k_1$	$k_{2,Mg}/k_2$
7.0	0.40	0.43
7.4	0.42	0.27
7.7	0.19	0.17
8.0	0.13	0.14

[a] Rod filaments were prepared by dialyzing oxidized rod against 80 mM NaCl and 40 mM imidazole or triethanolamine buffer. They were digested at a concentration of 2 mg/mL, at 25 °C, in the absence or presence of 3.0 mM Mg^{2+}. The concentration of α-chymotrypsin varied from 0.15 mg/mL at pH 7.0 to 0.03 mg/mL at pH 8.0. Rate constants k_1 and k_2 (and $k_{1,Mg}$ and $k_{2,Mg}$) for cleavage at sites CT-1 and the HMM/LMM junction were determined as described in the text. The effect of Mg^{2+} on the digestion rate of rod filaments at these two sites is given in the form of ratios of digestion rates in the presence and absence of Mg^{2+}, i.e., $k_{1,Mg}/k_1$ and $k_{2,Mg}/k_2$. The experimental accuracy of rate determinations (two or three experiments per each rate constant) is ±10%.

duced rods (in which site CT-1 is not cleaved) and in short oxidized rods (which lack site CT-1). Yet, MgATP inhibits about 2-fold the rate of fragmentation of standard oxidized rods ($k_1 + k_2$) at all pH values tested (7.0–8.2). Analysis of the digestion reactions according to eq 1–3 revealed, in agreement with the direct measurements of k_2, that MgATP inhibits k_1 but not k_2. This set of experiments provided a direct test and support for the analysis of digestion rates in oxidized rod filaments.

Discussion

The detachment and movement of myosin cross-bridges from the thick filament surface is an important element of current models for muscle contraction (Huxley & Simmons, 1971; Harrington, 1979). Under in vitro conditions, the release of cross-bridges can be easily achieved by raising the medium pH from 7.0 to 8.2 (Mendelson & Cheung, 1976; Sutoh & Harrington, 1977) and can be "assayed" in proteolytic and cross-linking experiments (Ueno & Harrington, 1981). It is of considerable interest to elucidate the factors and conditions which govern cross-bridge disposition. The binding of Ca^{2+} to the LC-2 subunits in myosin is unlikely to fulfill such a role in striated skeletal muscle (Mendelson & Cheung, 1976; Sutoh & Harrington, 1977; Cheung & Reisler, 1982). Phosphorylation of the light chains promotes cross-bridge release but does not have a major effect on their disposition (Mrakovčič-Zenic & Reisler, 1983). In contrast to these, the binding of divalent cations to the recently detected low-affinity binding sites located on the myosin rod (Borejdo & Werber, 1982) appears to determine the conformational state of cross-bridges. Although Ca^{2+} is somewhat more effective in this respect than Mg^{2+}, we have been mainly concerned with the physiologically significant binding of Mg^{2+} to myosin.

Our chymotryptic digestions of myofibrils at pH 8.2 confirm and quantitate the original observation of Borejdo & Werber (1982) on the Mg^{2+}-induced inhibition of proteolysis at the HMM/LMM junction. Measurements of the rates of myosin digestion in myofibrils in the presence of 0.1 and 5.0 mM Mg^{2+}, and in the pH range from 7.0 to 8.2, offer a clear correlation between the Mg^{2+} effect and the state of cross-bridges. The inhibition of proteolysis is minimal at pH 7.0 and 7.4, when the cross-bridges are mainly at the filament surface, and relatively large at pH 7.8 and 8.2, when the cross-bridges are released. The net effect of Mg^{2+} binding appears to be that of countering or reversing the charge-induced changes in the HMM/LMM junction. The small but reproducible inhibition of digestion at pH 7.0 can be understood in terms of additional "tightening" of cross-bridge–backbone interactions.

After establishing the correlation between Mg^{2+}-induced inhibition of myosin digestion and the state of cross-bridges in myofibrils (in which the size of myosin filaments does not change with pH), we have extended our studies to the synthetic systems of myosin and rod filaments. As in myofibrils, the binding of Mg^{2+} to myosin reduces the proteolytic susceptibility of the HMM/LMM junction at all pH values to approximately that observed at pH 7.0. The fact that Mg^{2+} also reverses the charge-induced changes in rod filaments supports the suggestion of Borejdo & Werber (1982) that the low-affinity binding sites for divalent cations are located on the rod portion of the molecule. From the most recent studies of Ueno et al. (1983), it appears that Mg^{2+} binds to the long S-2 and does not bind to the short S-2 element. The former yields paracrystalline arrangements in the presence of high concentrations of Mg^{2+} whereas the latter shows no tendency to aggregate upon addition of divalent cations. Proteolytic digestions of rod filaments carried out by Ueno et al. (1983) agree well with our own results and reveal the same picture of Mg^{2+}-induced reversal of charge effects in myosin.

Since the binding of Mg^{2+} to myosin rods seems to occur at or near the S-2/LMM junction, the consequent inhibition of proteolysis may be viewed as either due to local changes in this junction or to its steric inaccessibility after locking in cross-bridges to the filament surface. Cross-linking experiments provide strong evidence in favor of the latter possibility. They supplement the above-discussed correlation between the binding of Mg^{2+} to myosin and the rate of its digestion with a direct link between Mg^{2+} binding and cross-bridge disposition. We may conclude that in the presence of millimolar Mg^{2+} concentrations the cross-bridges are on the filament surface (even at pH 8.2) and the proteolytic digestion at the LMM/HMM junction is inhibited. Therefore, it seems unlikely that the two effects of Mg^{2+} are causatively unrelated and that chymotryptic digestion of myosin should sense only local events at the junction. Obviously, we are tempted to question the nature of the transitions occurring in the HMM/LMM junction in greater detail. Is the rapid proteolysis of the junction (observed upon cross-bridge release at alkaline pH) related to the α-helical–coil transition postulated by Harrington (1979) or to more prosaic steric accessibility considerations? The digestions of oxidized rods are quite illuminating in this context. They show that the rate of cleavage at another site of the rod (CT-1), which is only marginally exposed to chymotrypsin in the reduced protein, is affected in the same manner and to the same extent by the binding of Mg^{2+} as the S-2/LMM junction. Keeping in mind that the CT-1 site is located in the N-terminal portion of the rod, which apparently does not bind Mg^{2+} (Ueno et al., 1983), the cation-induced inhibition of its proteolysis must stem from the tight association of cross-bridges with the filament backbone. Since the inhibitions of digestion at the CT-1 site and the S-2/LMM junction are closely similar, the argument that steric accessibility of the cleavable residues determines their digestion rate can be extended to the junction as well.

Although changes in the proteolysis of the HMM/LMM junction do not necessarily point to its melting upon cross-bridge release, such a connection appears to be established in the recent studies on myosin minifilaments (Applegate & Reisler, 1983). In this work, we have shown that myosin and rod minifilaments have a lower α-helical content at pH 8.0 (released cross-bridges) than at pH 7.0.

In our turbidity measurements, we detect that binding of Mg^{2+} to the rod portion of myosin filaments affects the filament size. Presumably, locking of cross-bridges onto the filament surface allows more ordered packing of myosin and contributes toward filament growth. The general implication of this result is that chemical and structural events which occur on myosin cross-bridges and affect their disposition may be expected to influence the stability of myosin filaments.

In summary, our results demonstrate that the binding of Mg^{2+} to the low-affinity sites on the myosin rod determines cross-bridge disposition and affects filament assembly. They indicate that physiological concentrations of Mg^{2+} promote the tight association of cross-bridges with the filament backbone. This factor alone may be sufficient to "secure" the return of cross-bridges to the filament surface upon dissociation of the actomyosin complex in a muscle fiber.

Registry No. Magnesium, 7439-95-4.

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