Magnesium Adenosine 5′-Diphosphate Influences Proteolytic Susceptibility of Myosin in Myofibrils†

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ABSTRACT: The proteolytic susceptibility of the subfragment 2/light meromyosin junction [heavy meromyosin (HMM) junction] of myosin was employed as a probe of the cross-bridge conformation. The proteolysis was carried out in the myofibrils where myosin assembled in arrays typical of the in vivo organization. When subfragment 1 formation was inhibited by saturating the Nbs_2 [5,5′-dithiobis(2-nitrobenzoic acid)] light chains with Mg^{2+} ions, chymotrypsin attacked exclusively the HMM junction. The rate of this attack was assessed by measuring the rate of HMM formation by quantitative polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by following absorbance changes associated with the solubilization of myofibrillar suspensions. Under rigor conditions, the myofibrils were relatively resistant to the chymotryptic attack. The presence of MgAMP-PNP or $MgPP_i$ did not affect the rate of proteolytic attack. On the other hand, binding of MgADP had a powerful stimulating influence on the HMM site digestibility. The dissociation constant for the effect of MgADP was $10\ \mu M < K_d < 50\ \mu M$. MgADP did not exercise its unique effect through destabilization of myosin filaments or through dissociation of the actomyosin complex. These results are explained in terms of a change in the myosin cross-bridge conformation brought about by the binding of MgADP to the active site.

Most of the modern theories of contraction recognize that during muscle activity there exists a close relationship between substrate occupancy of the myosin active site and the spatial orientation of the cross bridge (Huxley & Simmons, 1971; Eisenberg & Hill, 1978). The coupling between enzymatic chemistry and spatial orientation implies the presence of "allosteric" effects in actomyosin where physiological ligands bound to the myosin active site impose on the cross bridge the precise spatial orientation with respect to actin. Morales & Botts (1979) have exemplified a mechanism in which a temporal sequence of occupants of the enzymatic site of myosin imposes (e.g., through polypeptide chain distortions) a temporal sequence of subfragment 1 (S-1)[1] attitudes resulting in mechanical activity. Experimentally, the early evidence for coupling between enzymatic and mechanical events came from the work of dos Remedios et al. (1972), who showed the dependence of the polarization of fluorescence (which is an attitudinal parameter of the cross bridge) on the nucleotide occupancy of the myosin active site. Electron microscope images of insect muscle fibers show that in the presence of MgAMP-PNP the cross bridges assume an orientation which is different from that in rigor (Marston et al., 1976; Beinbrech et al., 1976). Further, Tregear and his collaborators (Marston et al., 1979) recently clearly demonstrated that binding of a series of "mechanically effective" nucleotides is paralleled by the mechanical response of glycerinated insect muscle fibers.

If the specific ligand binding at the myosin active site is able to induce large conformational changes in the cross bridge, then it is likely that those changes will be also felt at the regions of myosin distant from the binding site. This paper presents evidence for the coupling between specific metal nucleotide binding at the active site and the proteolytic susceptibility of the distant flexible junction between subfragment 2 and light meromyosin of the myosin molecule (HMM junction). Two methods were employed in parallel to assess the efficiency of the proteolytic attack at this site: In the first method, the products of chymotryptic digestion were measured by densitometry of Coomassie Blue stained gels obtained by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$. In the second method, the rate of solubilization of myofibrils which proved to be proportional to the rate of proteolysis was measured by following changes in the absorbance of myofibrillar suspensions. Using these methods, it is shown that when the myosin active site is occupied by MgADP, but not either $MgPP_i$ or MgAMP-PNP, the susceptibility of the HMM junction, and hence presumably the conformation of the cross bridge, is altered.

Materials and Methods

Chemicals. α-Chymotrypsin was obtained from Worthington Biochemicals. ATP, ADP, AMP-PNP, P^1, P^5-diadenosine 5′-pentaphosphate (AP_5A), and phenylmethanesulfonyl fluoride (PMSF) were from Sigma. All other chemicals were of analytical grade.

Preparation of Myofibrils and Proteins. Myofibrils were obtained from fibers of rabbit psoas muscle which were glycerinated in a relaxing solution as described previously (Borejdo et al., 1979). A 0.3-g strip of muscle fiber was transferred from the relaxing glycerine solution to a solution containing 80 mM KCl, 2 mM EDTA, and 5 mM sodium phosphate buffer, pH 7.0, for 0.5 h at 0 °C. EDTA was used to inhibit contraction when MgATP in the relaxing glycerine solution is washed out. The muscle was cut with scissors into small pieces and homogenized in a Sorvall Omni-Mixer with 10 mL of a solution containing 80 mM KCl and 1 mM sodium phosphate buffer, pH 7.0, at setting 8 of speed control for 18 s in ice. Myofibrils were used on the day of preparation to prevent damage to the Z lines by the endogeneous proteases. PMSF when added at 0.1 mg/mL to myofibrils stabilized

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; HMM, heavy meromyosin; S-1, heavy meromyosin subfragment 1; S-2, heavy meromyosin subfragment 2; Nbs_2 light chain, 19000 molecular weight subunit of myosin dissociated by treatment with 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; K_d, dissociation constant; rms, root mean square.

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them for weeks, but in experiments on chymotryptic digestion, the inhibitor was not added. The concentration of myofibrils was measured by the biuret assay.

Myosin was prepared by the method of Tonomura et al. (1966). Heavy meromyosin (HMM) and heavy meromyosin subfragment 1 were prepared by the chymotryptic digestion of myosin according to Weeds & Pope (1977).

Solutions. The absolute stability constants for the complexes of EDTA, ATP, and ADP with H^+, Mg^{2+}, and Ca^{2+} under conditions prevailing in the present experiments were those quoted by Fabiato & Fabiato (1979). For the complexes of AMP-PNP, the data of Pettit & Siddiqui (1976) were used. The absolute stability constants for PP_i were obtained from Sillen & Martell (1971). The following equilibria were considered in computing the concentration of free Mg^{2+} or the free Mg–ligand complex: Mg–ligand, Mg–EDTA, Mg–Nbs_2 light chain. The K_{app} for Mg–light chain binding was an average of the two constants given by Werber et al. (1973). All computations were done on an Apple II microcomputer.

Unless otherwise indicated, all solutions contained, in addition to appropriate concentrations of $MgCl_2$, EDTA, and ligand, 80 mM KCl and 20 mM Tris buffer, pH 8.5. The pH at 8.5 was used throughout because the rate of digestion was accelerated and because Mg–ligand affinity was increased under these conditions. All solutions containing ADP as a ligand contained, in addition, 10 mM glucose and 100 μg/mL hexokinase to remove contaminating ATP as well as 100 μM AP_5A to inhibit the myofibrillar adenylate kinase from converting ADP to ATP (Abbott & Leech, 1973).

Chymotryptic Digestions. The digestions were carried out at room temperature in a solution containing 0.30 mg/mL myofibrils in an appropriate buffer. Chymotrypsin concentration was, unless otherwise stated, 25 μg/mL. The drop in turbidity was monitored in a Cary 118 spectrophotometer at 400 nm. The reference cell contained water. For quantitation of the formation of different fragments by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, 0.2 mL of myofibrils (1.5 mg/mL) in appropriate solution was digested with 12 μL of 2.5 mg/mL chymotrypsin. The reaction was stopped by adding 1 mM (final concentration) PMSF and 50 μL of $NaDodSO_4$-containing buffer. The aliquots were run on the two-phase resolving gel (3.0 and 7.5% acrylamide, unless otherwise indicated). The gels were scanned on a Gilford gel scanner, and the protein peaks corresponding to myosin, HMM, and S-1 were quantitated.

Myofibrils were digested during visual observation under the microscope by applying an appropriate digesting solution to one side of the microscope coverslip and sucking it with the filter paper on the other. The time of digestion was measured with a stopwatch.

Mechanical Experiments. These were done on single glycerinated fibers from rabbit psoas muscle. The muscle was mounted in an apparatus similar to the one described by Weis-Fogh & Amos (1972). The volume of the bath was 0.5 mL; solutions were changed by applying the solution at one end of the bath and sucking it out with the filter paper at the opposite end. The muscle fiber was mounted between one rigid and one compliant arm. The deflection of the elastic compliant arm to which one end of the muscle fiber was attached was a measure of the contractile force. This deflection was converted into voltage by a Kaman eddy current transducer (Model KD-2300-5 SU, Kaman Sciences Corp., Colorado Springs, CO) which was displayed on the recorder. Static stiffness was measured by slowly stretching one end of the fiber in steps of approximately 0.2% of its length and recording tension changes at the compliant arm.

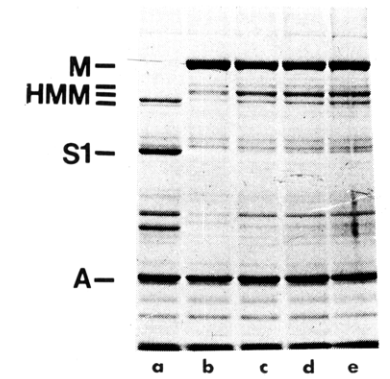

FIGURE 1: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of myofibrils after chymotryptic digestion at different free Mg^{2+} ion concentrations. The gel contained 7.5% acrylamide; the Tris–glycine buffer system was used according to the method of Laemmli (1970), employing stacking gel. The myofibrils (concentration 1.5 mg/mL) were in 80 mM KCl and 20 mM Tris-HCl, pH 8.5. Final chymotrypsin concentration was 30 μg/mL. Reaction time was 10 min at room temperature. Reaction was stopped by the addition of 0.17 mg/mL PMSF and the $NaDodSO_4$-containing buffer. Except in (a), free EDTA concentration was fixed at 10 μM. (a) 1 mM EDTA added; (b–e) free Mg^{2+} concentration fixed at 100, 10, 5, and 1 μM, respectively. The horizontal lines mark the position (from top to bottom) of heavy chain (hc) of myosin, hc of HMM, and hc of S-1 and actin. The log (molecular weight) vs. mobility graph was not strictly a straight line in this buffer system.

Optical Microscopy. The Zeiss (Oberkochen, West Germany) Axiomat microscope with phase contrast optics was used for visual observation of digestion of the myofibrils. The light source was a 100-W halogen lamp passed through a 5460-Å glass filter. The objective was Zeiss Planapochromat, NA (numerical aperture) = 1.3, oil immersion, focal length 90 μm, infinity corrected, phase. The intensity transmission ratio of the phase ring with respect to the complementary area of the diffraction plate was 0.1, and the optical path difference (OPD) between the phase ring and the complementary area was $\lambda/4$ (phase difference = $\pi/2$); the NA of the phase condenser was 0.9. Magnification of the image on the film was 320X. A built-in automatic 35-mm camera with Kodak Plus X film (125 ASA) was used throughout. The exposure time was ~1 s.

Results

Solubilization of Myofibrils by Chymotrypsin. In order to study the digestibility of the HMM junction, it was necessary to prevent proteolytic cleavage at the S-1/S-2 junction and to assure access of chymotrypsin to the HMM/LMM joint. These objectives were achieved by saturating the Nbs_2 light chains with Mg^{2+} ions (Weeds & Pope, 1977) and by working at high pH. Figure 1 shows the titration of the Nbs_2 light chains of myofibrillar myosin with Mg^{2+} at pH 8.5. It is clear that in myofibrils already in the presence of 1 μM free Mg^{2+} little S-1 is produced while HMM is generated at each free Mg^{2+} concentration. No attempt to obtain the in vivo affinity of Mg^{2+} for the Nbs_2 light chain has been made, but it was clear that the binding constant in myofibrils was equal to or greater than the Mg–Myosin binding constant given by Werber et al. (1973) (2.3×10^6 and 8.3×10^6 M^{-1}) and Watterson et al. (1979) (1.6×10^7 and 6.1×10^5 M^{-1}) and greater than the constants obtained by Beinfeld et al. (1975) and Holroyde et al. (1979). The formation of HMM at low ionic strength is due to the high pH which makes the HMM junction accessible to chymotrypsin. Thus, at pH 7.0 the rate of HMM formation was severalfold smaller, in agreement with

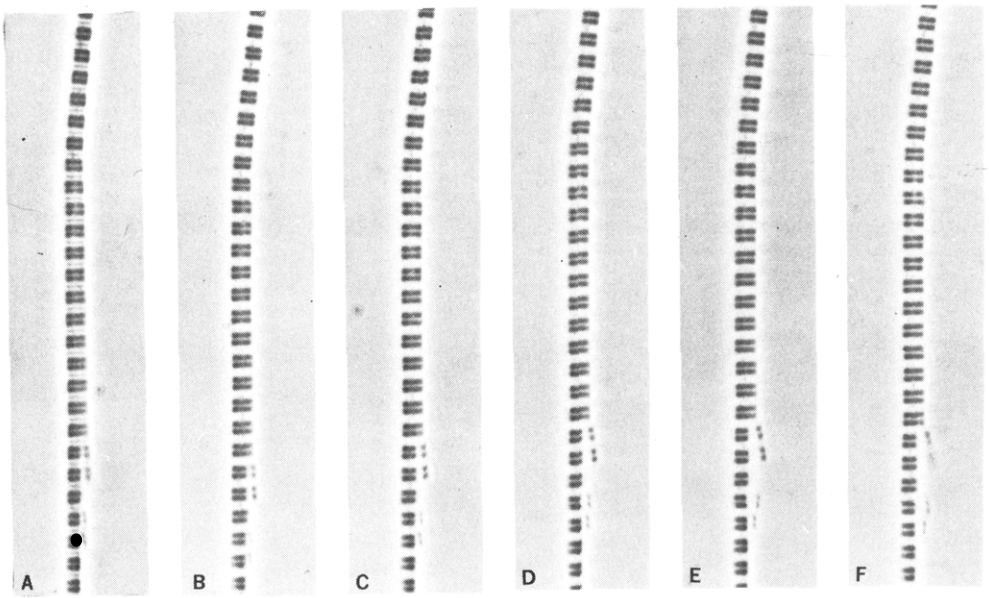

FIGURE 2: Photomicrograph of a myofibril in the absence of MgADP under attack by chymotrypsin. The myofibril was in 80 mM KCl and 20 mM Tris-HCl buffer, pH 8.5, with 10 μM free Mg and 50 μM free EDTA. (A) Myofibril before addition of chymotrypsin; (B) immediately after adding chymotrypsin; (C) 100 s after adding chymotrypsin; (D) 200 s; (E) 400 s; (F) 600 s. The horizontal bar marks 10 μm.

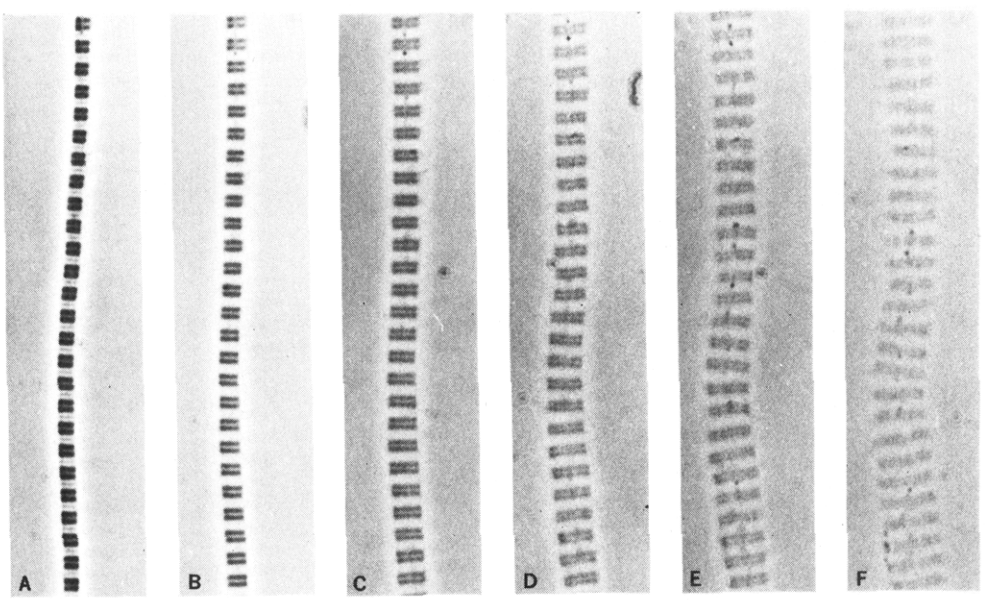

FIGURE 3: Photomicrograph of a myofibril during digestion by chymotrypsin in the presence of MgADP. Myofibril was in 80 mM KCl and 20 mM Tris-HCl buffer, pH 8.5, with 50 μM free EDTA, 10 μM free Mg, and 100 μM free MgADP. (A) Myofibril before addition of chymotrypsin; (B) the same myofilbril immediately after adding 30 μg/mL chymotrypsin; (C) 30 s after adding chymotrypsin; (D) 1 min after adding chymotrypsin; (E) 2 min; (F) 3 min. The horizontal bar marks 10 μm.

Ueno & Harrington (1981). An additional factor which may favor formation of HMM even at very low Mg^{2+} concentrations is the protecting effect of actin on the Nbs_2 light chains (Oda et al., 1980). Thus, in myofibrils, chymotrypsin preferably attacks the unprotected HMM junction. Even in the presence of EDTA a significant amount of HMM is formed (Figure 1a). In any case, in all experiments reported below, free Mg^{2+} concentration was fixed at or above 10 μM, well beyond the concentration necessary to prevent S-1 formation.

Two methods were used, in parallel, to measure the rate of chymotryptic attack at the HMM junction. In the first method (method I), myofibrils were digested with chymotrypsin, and the relative amounts of products were determined by quantitative $NaDodSO_4$–polyacrylamide gel electrophoresis. The fractional changes in the amount of HMM produced or, in some cases, the amount of myosin digested in a given time interval were taken as the rates of chymotrypsin attack. In the second method (method II), the proteolysis was carried out while monitoring the drop in optical density of the myofibrillar suspension in the spectrophotometer. Production of HMM results in solubilization of myofibrils as the thin filaments gradually become cutoff from the thick filaments. Figures 2 and 3 show the main features of the time course of the myofibrillar digestion. Following the application of chymotrypsin, there is a rapid digestion of the Z lines accompanied by the digestion of the M-line material. The particular susceptibility of these myofibrillar elements to proteolytic attack has been noted previously (Stromer et al., 1967; Garamvölgyi,

1968). After these components have been digested away, the only links holding myofibrils together are cross bridges (Borejdo & Oplatka, 1981). When the cross bridge is cleaved at the HMM junction, the myofibril solubilizes. The rate of the solubilization process, visible in Figure 3 as gradual swelling and solubilization, is then the reflection of the rate of attack at the HMM site. Indeed, we show below that the two methods give the same results. The chief advantage of method II is that it is much less time consuming and more reproducible than quantitative $NaDodSO_4$ gel analysis.

Effect of Ligands on HMM Junction Digestibility. In the following experiments, the free concentrations of various species were computed by considering the Mg^{2+}–EDTA and Mg^{2+}–ligand equilibria only. The effect of binding of Mg^{2+} to Nbs_2 light chains and of Mg–ligand to myosin was neglected because of the small concentration of total myosin. Mg–ligand concentrations were varied in three different ways of titration: (1) The first way is increasing the concentrations of total Mg^{2+} and ligand while keeping free Mg^{2+} concentrations fixed. This method has been adopted because it has been observed that Mg^{2+} ions, in the range of 1–100 μM, have some effect on the HMM junction digestibility. In fact, Figure 1 shows that an increase in the concentration of free Mg^{2+} ions suppressed the formation of HMM. The disadvantage of this method is that large quantities of total ADP are necessary to obtain MgADP concentrations above 50 μM. This is because Mg binds weakly to ADP, even at high pH (K_{app} at pH 8.5 = 985 M^{-1}). (2) The second way is keeping the ADP concentration constant and raising both total EDTA and Mg concentrations. The advantage of this method is that it eliminates the possible spurious effects of free ADP (see below). On the other hand, we have observed that Mg^{2+} in sufficiently high concentration can suppress HMM formation (see above). Because the concentration of free Mg^{2+} increases in parallel with the concentration of MgADP, at high [MgADP], the rate of HMM formation may be suppressed. (3) The third way is eliminating the use of Mg–EDTA buffers. This allows higher MgADP concentrations to be reached at a relatively low free ADP concentration, because it is now possible to fix the free Mg^{2+} concentration as high as 100 μM (this is not possible in titration 1 because total EDTA and Mg^{2+} concentrations then become too great). The contaminating divalent cations should be unimportant here because their concentration is much lower then the total Mg^{2+} concentration.

Figures 2 and 3 show the time course of digestion of myofibrils at different concentrations of MgADP. In Figure 2, no MgADP was present: the myofibrils are seen to lose the Z and M lines and to swell slowly. In Figure 3, the digestion was carried out in the presence of 100 μM free MgADP (titration 1). It is clear that the rate of swelling and solubilization is now much faster. At the same time, it is clear that at no stage is there a sign of myofibrillar shortening. This is not surprising because all the experiments have been carried out in the presence of myokinase inhibitor and hexokinase (see Materials and Methods).

The above observation was quantitated by both methods I and II. Figure 4 shows the time course of the digestion of myofibrillar myosin in the absence (Figure 4, top panel) and presence (bottom panel) of MgADP. In both cases, the digestion products are first 170 000-, 150 000-, and 135 000-dalton chains of HMM and then 75 000- and 60 000-dalton and lighter digestion products of LMM. With time, the 170K-dalton chain of HMM and the 75K-dalton chain of LMM degrade, enriching the 150K- and 135K-dalton chains of HMM and the 65K-dalton chain of LMM, respectively.

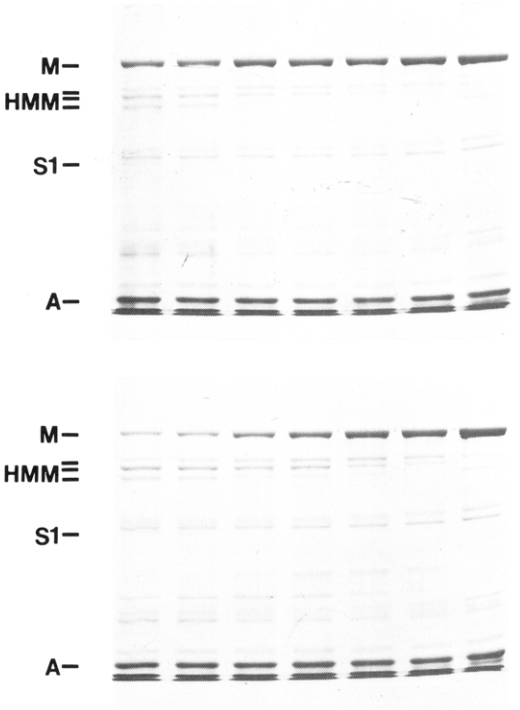

FIGURE 4: Time course of digestion of myofibrillar myosin in the absence (top panel) and in the presence (bottom panel) of MgADP. Myofibrils (1.6 mg/mL) were in 80 mM KCl and 20 mM Tris-HCl buffer, pH 8.5. Mg^{2+} (1.05 mM) and 1.32 mM AMP-PNP were added to myofibrils of the top panel to give 1 mM MgAMP-PNP and 50 μM free Mg^{2+}. Mg^{2+} (108 μM) and 1.06 mM ADP were added to myofibrils of the bottom panel to give 50 μM MgADP and 50 μM free Mg^{2+}. Chymotrypsin was added at time zero, and the reaction was stopped by the addition of 1 mM PMSF at the following times (from right to left): immediately and 0.5, 1, 2, 3, 5, and 7.5 min. The positions of heavy chains of myosin (M), HMM, S-1, and actin (A) are marked at the left.

Eventually both degrade to lighter products. $NaDodSo_4$ gel electrophoresis on 12% acrylamide gels revealed that no predominant band with a molecular weight of less than 20K formed at this stage. With time, all higher molecular weight components are degraded into small fragments unresolvable even on 12% gels. No S-1 was produced during the first 3 min of digestion (the prominent band of 100K daltons migrating just above the S-1 heavy chain is most likely α-actinin). The trace amount of S-1 which appears at later times is a product of digestion at both soluble HMM and myosin. Under all experimental conditions, actin was not digested to a detectable extent, in agreement with Sutoh & Harrington (1977).

The most clear difference between the digestion patterns of the top and bottom panels of Figure 4 is the rate of disappearance of myosin and the rate of appearance of the heavy chains of HMM. The gels were scanned, and the intensity of the bands corresponding to the heavy chains of myosin, HMM, and S-1 was plotted as a function of time in Figure 5. It is seen that when myofibrils are digested in the presence of 50 μM MgADP the initial rate of digestion of myosin and the rate of production of HMM were increased by about 75% with respect to the amount digested/produced in the presence of 1 mM MgAMP-PNP used as control. The initial rate of digestion is defined here as the amount of protein digested or fragment produced during the first 2 min of proteolysis. At later times, the HMM undergoes further degradation. The same difference in digestion rates can be observed by following the rate of OD change (method II). Figure 6 shows the time course of the turbidity decrease under conditions identical with those of Figure 4. The initial rates of the OD change differ

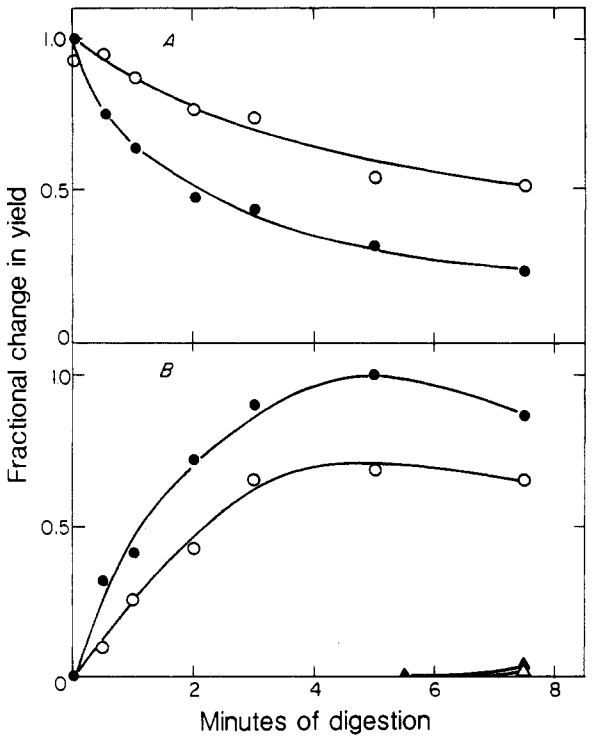

FIGURE 5: Time course of digestion of myofibrillar myosin (A) and of production of HMM (B). Densitometric scans of the data from Figure 4. (○) Digestions in the presence of 1 mM MgAMP-PNP; (●) digestions in the presence of 50 μM MgADP; production of S-1 in the presence of 1 mM MgAMP-PNP (△) and 50 μM MgADP (▲). The amount of myosin digested has been normalized in (A) to the amount present at time 0 in the myofibrils containing MgADP in (B) to the maximum amount of HMM produced in the myofibrils containing MgADP.

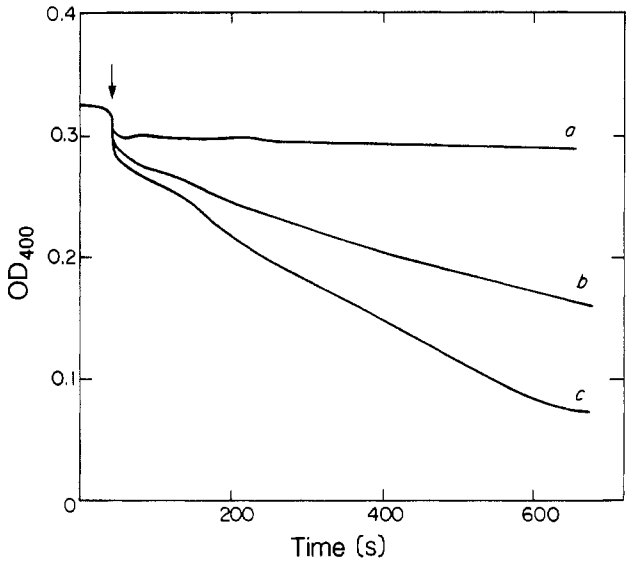

FIGURE 6: Solubilization of myofibrils during chymotryptic attack in the presence and in the absence of MgADP. Myofibrils (0.3 mg/mL) in 80 mM KCl and 20 mM Tris-HCl, pH 8.5, contained in addition the following: (a) 50 mM $MgCl_2$; (b) 1.05 mM $MgCl_2$ and 1.32 mM AMP-PNP to give 1 mM MgAMP-PNP and 50 μM free Mg^{2+}; (c) 108 μM $MgCl_2$ and 1.06 mM ADP to give 50 μM MgADP and 50 μM free Mg^{2+}. Chymotrypsin (30 μg/mL) was added at the arrow.

by about 75% in curves b and c.

MgADP was unique in its ability to accelerate the rate of digestion of myofibrils. The rate of digestion of myosin, the rate of production of HMM, or the rate of turbidity changes was the same in the presence of even millimolar concentrations

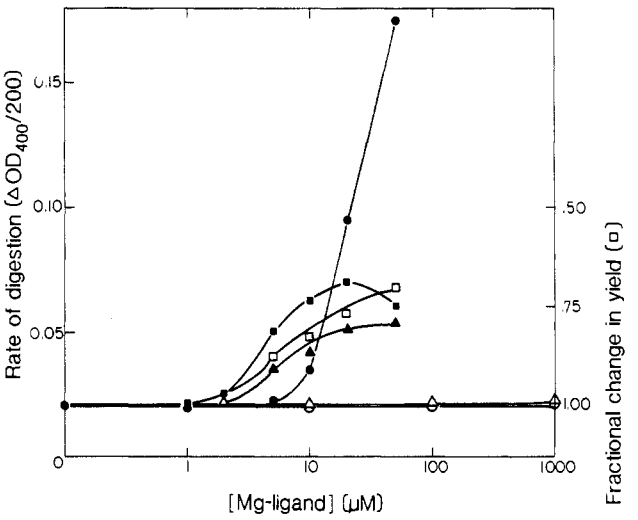

FIGURE 7: Rate of digestion of myofibrils in the presence of different ligands. Myofibrils (0.3 mg/mL) in 80 mM KCl and 20 mM Tris-HCl, pH 8.5, were digested by 25 μg/mL chymotrypsin. (●) 10 μM free Mg and 50 μM free EDTA, MgADP concentration increased by raising total Mg and ADP concentrations; (■) 2 mM free ADP and 10 μM free EDTA, MgADP concentration increased by raising total Mg^{2+} and EDTA concentrations; (▲) 50 μM free Mg^{2+} and no Mg–EDTA buffer used, MgADP concentration increased by raising total Mg^{2+} and ADP concentrations; (○) 10 μM free Mg^{2+} and 50 μM free EDTA, $MgPP_i$ concentration increased by raising total Mg and PP_i concentrations; (△) 10 μM free Mg^{2+} and 50 μM free EDTA, MgAMP-PNP concentration increased by raising total Mg and AMP-PNP concentrations; (□) amount of myosin digested during 7.5-min digestion of myofibrils in the presence of 50 μM free Mg^{2+}; no Mg^{2+}–EDTA buffer was used.

of other Mg–ligands such as $MgPP_i$ or MgAMP-PNP as in their absence (providing free Mg^{2+} concentration was the same). These effects are summarized in Figure 7 which shows the rates of digestion of myofibrillar myosin (by both methods) in the presence of increasing concentrations of Mg–ligands. The curves clearly distinguish between the effect of MgADP and that of other Mg–ligands. However, the shape of the MgADP titration curve depends on the way the MgADP concentration is raised. These differences arise because of three reasons: (1) Titration 1 (closed circles, Figure 7) overestimates the rate at high MgADP concentrations. It has been shown by Harrington & Himmelfarb (1972) that ATP, ADP, AMP-PNP, and PP_i all have the destabilizing effect on the myosin filament structure. Because these ligands, especially ADP, bind weakly to Mg^{2+}, even at pH 8.5, it is necessary to use high total ligand concentrations to obtain higher Mg–ligand concentrations. The resulting ADP exists then in sufficiently high concentration to destabilize the myosin filament structure. We have evidence (see below) to suggest that the effect is negligible below a total ADP concentration of 2 mM. Thus, the point corresponding to 50 μM MgADP is most likely an overestimation. (2) Titration 2 (closed squares, Figure 7) underestimates the rates at high MgADP concentrations. Under those conditions, free Mg^{2+} concentration becomes so high that it begins to influence the proteolytic attack at the HMM junction (see above). This method of titration also proves that ADP alone is ineffective in accelerating the proteolysis of the HMM junction and that Mg^{2+} is necessary for the effect. (3) Titration 3 (closed triangles and open squares, Figure 7) resembles the curve obtained by method II. The K_d for the effect of MgADP has been estimated from these data by a two-parameter fit in which all possible combinations of K_d and the maximum rate of digestion were examined by the computer for the fit to a simple binding curve. The one giving the smallest rms deviation corresponded

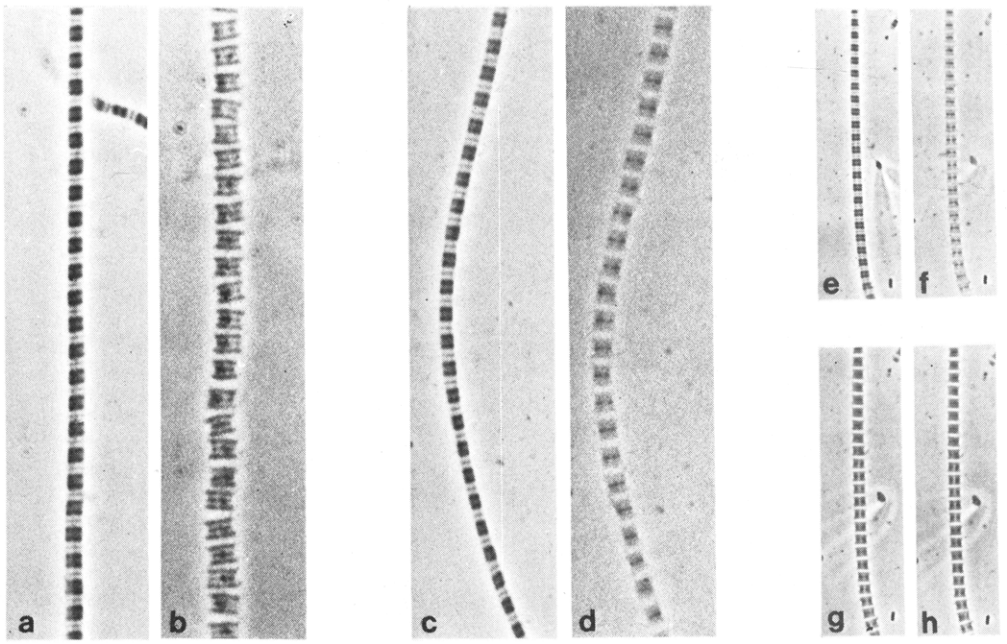

FIGURE 8: Photomicrographs of myofibrils in the presence of different ligands. In (a) and (c), the myofibrils were bathed in a solution containing 80 mM KCl and 10 mM Tris-HCl buffer, pH 8.5. In (b) and (d), myofibrils were perfused with a solution containing 500 mM KCl and 10 mM Tris-HCl buffer, pH 8.5. In (b), $MgCl_2$, EDTA, and ADP were added to give 10 μM free Mg^{2+}, 10 μM free EDTA, and 50 μM free MgADP. In (d), $MgCl_2$, EDTA, and AMP-PNP were added to give 10 μM free Mg^{2+}, 10 μM free EDTA and 50 μM free MgAMP-PNP. Magnification 1400× in (a)–(d). In (e), the myofibrils were bathed in 80 mM KCl, and 20 mM Tris-HCl, pH 8.5. (f) After removal of myosin; (g) after irrigation with 2.6 mg/mL S-1; (h) after 2-min wash with 3 mM MgADP. Magnification 686× in (e)–(h).

to 10 $\mu M < K_d < 50$ μM. A more accurate estimate of K_d was impossible because of the limited range of Mg–ligand concentrations.

An attempt has been made to assess the effect of MgATP under conditions of relaxation. However, application of even very low concentrations of chymotrypsin (e.g., 5 μg/mL) in the presence of MgATP and EGTA led to an instantaneous contraction of myofibrils. This effect most likely reflects an extreme proteolytic susceptibility of troponin, noted earlier (Drabikowski, 1975).

Digestion of Myosin Filaments. In an attempt to establish whether actin is required for the observed effect of MgADP, synthetic myosin filaments were digested under the conditions of the present experiments. Myosin filaments (1.5 mg/mL in 80 mM KCl and 10 mM Tris-HCl buffer, pH 8.5) were digested by chymotrypsin in a 1:300 up to 1:30 weight ratio. The 10-min digestion was done in the absence of actin and in the presence of 10 μM free EDTA and increasing concentrations of Mg–ligand. Under those conditions, mostly HMM and little S-1 were produced, in line with the results of Weeds & Pope (1977). The amount of HMM produced was the same in the presence of up to 20 μM MgADP, and 10 and 100 μM AMP-PNP and 10 μM $MgPP_i$.

Dissociation of Actomyosin by Mg–Ligands. It was interesting to ask whether the unique effect of MgADP on HMM junction digestibility was correlated in any way with the differences between various Mg–ligands in affecting actin–myosin binding. Several lines of evidence suggest that MgADP is indeed unique in rabbit muscle at room temperature in having little ability to dissociate actin and myosin. First, static stiffness of a single glycerinated muscle fiber was measured when the fiber was in rigor solution (80 mM KCl, 10 mM Tris-HCl, pH 8.5, and 4.21 mM EDTA) and when 4.22 mM $MgCl_2$ and 5.12 mM ADP were added to the above solution. No difference in static stiffness could be detected in agreement with Marston (1973). Both AMP-PNP and PP_i, on the other hand, gave a significant decrease in static stiffness. Next, the following experiment demonstrated that in vivo MgADP is a much weaker dissociating agent then either MgAMP-PNP or $MgPP_i$. Myofibrils were perfused with a solution containing 500 mM KCl, 10 mM Tris buffer, pH 8.5, and 50 μM free Mg–ligand, at fixed free $[Mg^{2+}] = 10$ μM and fixed free [EDTA] = 10 μM. As Figure 8 demonstrates, 50 μM MgADP at high ionic strength caused only partial removal of myosin from myofibrils. Myosin originally present in the nonoverlap zone of the A band diffused away due to disruption of the thick filaments by KCl, but it remained bound in the original overlap zone (Figure 8b). In contrast, 50 μM of either MgAMP-PNP (Figure 8d) or $MgPP_i$ (not shown) caused formation of myofibrillar "ghosts" devoid of myosin also in the overlap zone. Finally, the following experiment showed that MgADP is ineffective in comparison with $MgPP_i$ or MgAMP-PNP in dissociating S-1 from thin filaments also at low ionic strength: myofibril (Figure 8e) was treated with myosin-removing solution (Figure 8f) and irrigated with 2.6 mg/mL S-1 in rigor solution (Figure 8g). When MgADP (up to 3 mM) was added to such irrigated ghosts, no dissociation of S-1 occurred within 2 min, as judged by the phase contrast of the image (Figure 8h). Both $MgPP_i$ and MgAMP-PNP at the same concentration, on the other hand, caused dissociation as judged by the decrease in contrast of the image, corresponding to that present before application of S-1 (not shown).

Discussion

It has been shown above that MgADP has a potent stimulating effect on the rate of digestion of myofibrils. The digestion was shown to be mediated exclusively through the formation of HMM, and, therefore, the increase in the digestibility of myofibrils will be interpreted here solely on the basis of an increased accessibility of the HMM junction to proteolytic attack. The effect of MgADP is attributed to its binding to the active site of myosin, giving rise to the change in the local conformation of the cross bridge which in turn affects the HMM junction susceptibility. This conclusion is supported by the following evidence:

(1) The K_d for the effect of MgADP has been shown to lie between 10 and 50 μM. It has proven impossible to obtain a more accurate estimate of the K_d because of the difficulty in titrating myofibrils with high concentrations of MgADP. While this value is significantly lower than the K_d for ADP binding to acto-HMM ($\sim$500 μM; Stone, 1973), it is comparable to the dissociation constant for the displacement of nucleotide from acto-S-1 ($\sim$40 μM, Highsmith, 1976; $\sim$140 μM, Greene & Eisenberg, 1980). Most importantly, it is close to the K_d for the binding of MgADP to actomyosin in glycerol-extracted muscle fibers ($\sim$32 μM; Marston, 1973).

(2) It has been possible to exclude the alternative explanation whereby the effect of MgADP is due to trivial destabilization of thick filaments and associated increased accessibility of the enzyme to the HMM junction: (a) ADP alone had no effect on the rate of digestion. Addition of Mg^{2+} accelerated the rate of digestion even though it had a stabilizing effect on myosin filaments (Harrington & Himmelfarb, 1972). (b) MgAMP-PNP (1 mM) or $MgPP_i$ (1 mM) had no effect on the rate of digestion even though both analogues are more effective destabilizers of thick filament structure than ADP (Harrington & Himmelfarb, 1972). (c) MgADP had no effect on the digestibility of synthetic myosin filaments.

(3) It is likewise impossible that MgADP exercises its action through a secondary effect such as acceleration of removal of Nbs_2 light chains by EDTA. This is because the concentration of free EDTA was kept constant, and MgADP concentration was increased by raising ADP and Mg concentrations (total). Also little effect of EDTA concentration is observed at room temperature on the light chain of skeletal myosin: Higher EDTA concentrations and 37 °C are required to remove the Nbs_2 light chains (Srivastava et al., 1980).

It is nevertheless impossible to completely exclude the possibility that the observed effect of MgADP is due to the nucleotide binding at or near to the HMM junction. For example, it has been suggested that purine disulfide analogues of ATP may bind to myosin at sites different than the active site (Wagner & Yount, 1975). On the other hand, the results of direct binding studies strongly indicate that myosin contains only two nucleotide binding sites [cf. Weber & Murray (1973)]. While our own data do not exclude the possibility of a direct effect, it is difficult to imagine a mechanism involving direct binding which would be specific to MgADP and which would require the presence of actin.

If the effect of MgADP is indeed achieved through binding at the active site, then it is possible to speculate upon the mechanism which allows binding at this site to influence the distant HMM junction. There are three ways in which the effect of MgADP could be felt at the HMM site: (a) binding of metal–nucleotide to the active site could have no effect on the gross conformation or orientation of the cross bridge and exercise its action through the polypeptide chain distortions; (b) binding at the active site could influence the accessibility to the HMM junction of the neighboring myosin molecule; and (c) binding of nucleotide to the active site could alter the conformation or orientation of the cross bridge and thus affect the environment of the protease-sensitive region.

The first of the above mechanisms would be consistent with the observation of Rodger & Tregear (1974) that the X-ray diffraction patterns from insect muscle fibers in the presence of ADP were indistinguishable from rigor patterns, suggesting that the angle of attachment was the same in rigor and in the presence of MgADP. [It should be mentioned, however, that Rodger & Tregear (1974) worked with glycerol-extracted insect muscle fibers at 1 °C and 45 mM KCl, i.e., under conditions quite different from those used here.] It is conceivable that the Nbs_2 light chain is involved in transmitting the polypeptide chain distortions from the active site to the HMM junction. Such an allosteric change in the heavy chain of myosin is not without precedent: Schaub et al. (1978) have shown that the SH group located on the 11 000-dalton chain residing most likely near the S-1/S-2 junction is preferentially alkylated during ATP hydrolysis. In spite of this, it seems improbable that an allosteric change in myosin could propagate over a distance as large as 500 Å.

The second scheme which could explain the effect of MgADP would be the one which presumes a close spatial proximity between the myosin head and the HMM junction of the *neighboring myosin molecule* [e.g., see Harrington (1979)]. In such a scheme, changes in the vicinity of the myosin active site could exert an influence on the HMM site of the adjoining molecule. It is difficult to see, however, why the MgADP would be ineffective in myosin filaments when the myosin head/HMM junction interactions still should occur.

The final and in my opinion the most likely mechanism of action of MgADP is the one which presumes that binding at the active site of myosin induces conformational or attitudinal change of the cross bridge and that this change is mechanically transmitted to the HMM junction, thus altering the accessibility of chymotrypsin to the proteolytic site. This mode of action is consistent with all of our data, in particular with the lack of effect of MgADP on synthetic myosin filaments. Of the two, it seems more likely that the nucleotide induces a local conformation change in S-1 rather than a gross attitudinal change of its orientation with respect to actin (cross-bridge rotation). Attitude change would require a large drop in rigor force and a change in the appearance of the X-ray diffraction patterns of rigor muscle upon application of MgADP, neither of which have been experimentally observed (Rodger & Tregear, 1974; Marston et al., 1979).

If the effect of MgADP is indeed due to the local conformational changes associated with Mg–nucleotide binding, then it is possible that it is related to the intrinsic fluorescence enhancement (Werber et al., 1972) or the relative motions of the SH_1 and SH_2 groups of myosin (Wells et al., 1979) provoked by ligand binding. The observation that MgADP is unique in inducing accessibility changes while all three ligands MgADP, MgAMP-PNP, and $MgPP_i$ induce fluorescence change and are efficiently trapped in the active site by cobalt phenanthroline and *N,N'-p*-phenylenedimaleimide (Wells et al., 1980) can then be explained by the fact that MgADP is unique in having no detectable effect on S-1–actin affinity in vivo. Since the digestibility changes at the HMM junction require the presence of both the ligand and actin, neither MgAMP-PNP nor $MgPP_i$ will be effective, because both seriously reduce the affinity of S-1 to actin. At the same time, each one may induce the local conformational change in S-1.

It is interesting to speculate that the conformational changes in the myosin head could be the result, rather than the cause, of accessibility changes in the HMM junction region, because the coupling between MgADP binding and the HMM junction must be reciprocal. In other words, binding of MgADP to the active site would cause changes in the accessibility of the HMM junction which in turn would result in the altered conformation of the cross bridges. The susceptibility changes could, in principle, be associated with the helix-to-coil transition. Harrington (1979) (Tsong et al., 1979) has speculated that just such a mechanism is in fact responsible for the muscular contraction.

Regardless of the detailed mechanism which couples binding of MgADP to changes in the susceptibility of the HMM junction, the very fact that MgADP is uniquely effective in inducing changes in a part of a molecule which must be somehow involved in the contractile process—whether through its inherent flexibility or through its ability to undergo the helix-to-coil transition—strongly suggests that the ternary complex actin–myosin–MgADP *is* an intrinsic part of the enzymatic cycle of actomyosin.

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