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Interplay of actin, ADP and Mg^{2+} interactions with striated muscle myosin: Implications of their roles in ATPase

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Abstract

The effects of Mg^{2+} on the interaction between ADP, a product of the ATPase reaction, and striated muscle myosin-subfragment 1 (S1) were investigated with both functional and spectroscopic methods. Mg^{2+} inhibited striated muscle myosin ATPase in the presence of F-actin. Significant effects of Mg^{2+} were observed in both rate constants of NOE build-up and maximal intensities in WaterLOGSY NMR experiments as F-actin concentration increased. In the absence of F-actin, myosin S1 with Mg^{2+} bound to a fluorescent ADP analog about five-times tighter than without Mg^{2+} . In the presence of F-actin, the affinity of myosin S1 toward the ADP analog significantly decreased both with and without Mg^{2+} . The equilibrium titration of myosin-S1 into F-actin revealed that in the presence of ADP the apparent dissociation constant (K_d) without Mg^{2+} was more than five-fold smaller than with Mg^{2+} . Further, we examined effects of F-actin, ADP and Mg^{2+} binding to myosin on the tertiary structure of myosin-S1 using near UV circular dichroism (CD) spectroscopy. Both in the presence and absence of ADP, there was a Mg^{2+} -dependent difference in the near UV CD spectra of actomyosin. Our results show that Mg^{2+} affects myosin-ADP and actin-myosin interactions which may be reflected in myosin ATPase activity.

Keywords

striated muscle; myosin ATPase; actin filament

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AUTHOR CONTRIBUTIONS

MK designed and performed experiments, and wrote the manuscript; BER contributed analytic tool and contributed to the manuscript writing and discussion; CMW provided protein samples and contributed to the manuscript writing and discussion.

The abbreviations used are: S1, myosin subfragment-1; NMR, nuclear magnetic resonance; WaterLOGSY, water ligand-observed gradient spectroscopy; NOE, nuclear Overhauser effect; CD, circular dichroism; Tn, troponin; Tm, tropomyosin; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; PCA, perchloric acid; mantADP, *N*-methylanthraniloyl ADP

SUPPORTING INFORMATION

There are supporting data (a gel picture, additional NMR data, kinetic data) available for this manuscript in a single PDF file.

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Muscle contraction results from the relative sliding between myosin filaments (thick filaments) and actin (thin filaments) converting chemical energy from ATP into mechanical energy. A divalent metal cation, Ca^{2+} plays an essential role in regulation of the striated muscle. Although it is the conventional view that the intracellular Mg^{2+} concentration is rather static and that it does not contribute to regulating cellular events as much as the intracellular Ca^{2+} does, it was found that more ADP molecules are uncomplexed with Mg^{2+} compared with ATP which mostly exists in the form of the MgATP complex in the cytosol (1). This example suggests that free Mg^{2+} concentration may fluctuate inside the cell depending on conditions such as intracellular ATP and ADP concentrations. Potential evidence indicates that intracellular Mg^{2+} concentration fluctuates in striated muscles (2, 3, 4). Moreover, either high or low serum Mg level is linked to adverse effects on muscle function (5, 6). While it has been known for quite some time that non-muscle myosin such as myosin V can be regulated by Mg^{2+} (7, 8, 9, 10), in comparison striated muscle myosin II modulation by Mg^{2+} -dependency, has been understudied.

It has been suggested that ADP release (product release) from myosin V is affected by Mg^{2+} concentration (Scheme I) and that Mg^{2+} is released before ADP dissociates from the myosin head, thus resulting in two ADP states, i.e., strong and weak (11).

Several studies pinpoint that conformations of the nucleotide binding pocket (NBP) are linked to determination of duty ratio (12, 13, 14). Of those studies, for example, residues such as S217 and Y439, located in switch-1 and switch-2 regions, respectively, were shown to be important for myosin V to maintain a high duty ratio. Striated muscle myosin II is considered to have a low duty ratio and to have a replacement at the residue corresponding to Y439 in switch-2 myosin V. According to Nagy *et al.*, the alanine replacement Y439A results in reduction of actin activated ADP release in the presence of Mg^{2+} , while it maintains a similar rate in the absence of Mg^{2+} (14), somewhat suggestive of differential Mg^{2+} -modulation over different myosin motors. Another more recent study reported that ADP release of skeletal muscle myosin was very fast compared with those of the other myosin motors and that the authors were not able to detect Mg^{2+} -dependency in ADP release. (13).

Therefore, we aimed to shed light on Mg^{2+} -dependent modulation of myosin – ADP interaction to better understand the ATPase cycle of striated muscle myosin II in this study. We employed spectroscopic approaches in addition to functional studies to look for an indication of structural changes associated with Mg^{2+} -dependent modulatory mechanism.

MATERIALS AND METHODS

Materials and Muscle Proteins

A 1 M MgCl_2 stock solution and ATP were purchased from Sigma. ATP stock solutions were made from the acid powder and the pH was adjusted into the neutral range by adding NaOH. N-(1-pyrenyl)iodoacetamide (PIA), N-methylanthraniloyl ADP (mantADP) and phalloidin were purchased from Invitrogen. Actin and myosin subfragment 1 (S1) were natively purified from rabbit skeletal tissue as previously described (15,16). The two essential light chains were co-purified with the S1 preparation (Figure S1). Briefly, actin was

extracted from the acetone-dried powder in the form of G-actin, then other contaminating muscle proteins including tropomyosin (Tm) and troponin (Tn) were removed by a series of ultracentrifugation after removal of unwanted materials and subsequent polymerization of actin via high salt treatment. The purified actin was stored at 4 °C as monomeric form and used within 1–1.5 months. Cardiac Tm and Tn components were purified according to the methods described elsewhere (17).

Solution S1-ATPase assays

In order to assess a change in the myosin S1-ATPase enzymatic rates, phosphate (a product of the reaction) release was measured by the malachite green procedure (18). Throughout the reaction the temperature was maintained at 25°C. The reaction was initiated by addition of the pH-adjusted ATP solution (to the final concentration of 1.25 mM). The buffer condition of the assays was 20 mM MOPS, pH 7.0, indicated amount of MgCl₂ and 35 mM NaCl plus if needed additional NaCl to keep the ionic strength constant. The concentrations of F-actin and myosin S1 were 5 µM and 0.2 µM, respectively. For samples with either Tm or both Tm and Tn, the final concentrations of Tm and Tn were 2 µM and 1.8 µM, respectively.

NMR measurements

All the water ligand-observed gradient spectroscopy (WaterLOGSY) data were collected at 298K on Bruker Avance 900MHz spectrometer equipped with a cryogenic probe housed in the Center for Structural Biology on the campus of the University of Illinois at Chicago (UIC RRC NMR facility). All the protein samples were prepared in a solution containing 150 mM NaCl, 10 mM MOPS, and 0.01 % NaN₃, pH 7.0. The concentration of myosin S1 was 7.6 µM. Then D₂O was added to 12 % for the field-frequency lock. ADP was added to 1.5 mM, and for the samples with Mg²⁺, MgCl₂ was added to 5.4 mM. A pulse program for the WaterLOGSY experiment was described elsewhere (19, 20). A peak assignment was based on the database at Biological Magnetic Resonance Data Bank (21). The mixing time was varied to measure time-dependent changes in transferred nuclear Overhauser effect (NOE) (Figure 2). After taking the spectra, all the 1D ¹H NMR spectra were processed using NMR Pipe (22), then the area under each peak was determined by integration via nmrglue software available on-line (23, 24). Equation 1 was used to fit the build-up curves and the apparent rate constant, k_{app} and the maximal intensity, I_{max} were obtained for each actin concentration added to S1.

$$I(t) = I_{max} \cdot [1 - \exp(-k_{app} \cdot t)] \quad (\text{Equation 1})$$

S1 binding assays

The extent of binding between myosin S1 and actin was measured in a similar manner described elsewhere (25, 17, 26). To obtain fluorescently labeled F-actin, F-actin was reacted with PIA according to the method described by Criddle *et al.* with minor modifications (25). Briefly, F-actin was incubated overnight in the dark with an excess amount of PIA dissolved with dimethylformamide. The reaction was quenched by addition

of DTT to 2 mM. The resulting pyrene-labeled F-actin was separated from denatured protein by centrifugation at 2000 g and then sedimented by ultracentrifugation at 140000 g. After dissolving the pellet (F-actin) with G-buffer (2 mM Tris/HCl, 0.1 mM ATP, 0.1 mM CaCl₂, and 0.01 % NaN₃) the labeled actin was dialyzed vs. a buffer containing 0.15 M NaCl, 20 mM MOPS, pH 7.0, and 0.01 % NaN₃. The buffer condition used for titration was: 0.15 M NaCl, 20 mM MOPS, pH 7.0, containing 0.2 mg/mL bovine serum albumin, 2 mM ADP, 1 mM DTT, 1 mM D-glucose, 1 U/mL hexokinase, 0.2 mM P1, P5-di(adenosine-5')pentaphosphate (Ap5A), and 1 mM EDTA. For Mg²⁺ samples, MgCl₂ was added to 6 mM. Before initiating titration with myosin S1, for no Mg²⁺ samples with ADP, hexokinase was added to the cuvette containing 1 mM MgCl₂ and all the components except EDTA and the solution was incubated at 25 °C for more than 10 minutes to facilitate the conversion of contaminating ATP to ADP, then EDTA was added to 1 mM. All the data were collected on Model 2000–4 spectrofluorometer equipped with two model 814 PMT photon-counting detectors from PhotonTechnology International (PTI) and the changes of the pyrene fluorescence (*F*) were recorded at wavelength 407 nm with excitation at 365 nm. The cell holder was maintained at 25°C throughout the experiment. In order to monitor changes in fluorescence intensity, 1.0 μM of the labeled actin was titrated with S1. Addition of myosin S1 caused intensity decrease as documented elsewhere (25, 17). Therefore, $F = F_0 - F$ was calculated as a fluorescence change where *F*₀ and *F* are the initial intensity and intensity at any given titration point, respectively. After titration of myosin-S1 into pyrene-labeled actin, the binding curves were fit with the following bi-molecular equilibrium binding equation (Equation 2) to obtain apparent dissociation constants.

$$\Delta F = \Delta F_{max} \times \frac{\{[M]_{added} + [A]_0 + K_d - \sqrt{([M]_{added} + [A]_0 + K_d)^2 - 4[A]_0[M]_{added}}\}}{2[A]_0} \quad (\text{Equation 2})$$

, where [M]_{added} and [A]₀ denote the added myosin S1 concentration and initial actin concentration, respectively, *K*_d is the apparent dissociation constant, and *F*_{max} is the maximal fluorescence change. The *K*_d and *F*_{max} values were obtained by curve fitting. (Equation 2)

ADP binding assays

The equilibrium binding between myosin S1 and ADP was determined using mantADP. The initial mantADP concentration was 1 μM and the indicated amount of myosin S1 was added to the cuvette containing mantADP. For acto-S1 samples, F-actin was added to 15 μM to ensure that the myosin heads not forming acto-S1 were negligible. The fluorescence anisotropy of mantADP was recorded at wavelength 442 nm with excitation at 366 nm. The buffer condition described above was used except that there was no added MgCl₂ in low Mg²⁺ samples. The data were collected on the same fluorometer equipped with polarizers and polarized fluorescent components, *I*_{VV}, *I*_{VH}, *I*_{HV}, and *I*_{HH} were recorded for anisotropy calculation. For example, *I*_{HV} represents horizontally polarized excitation and vertically polarized emission. Equation 3 shown below was used to calculate fluorescence anisotropy (*r*) for each titration point. The titration curves were fit with the bi-molecular equilibrium

binding equation (in this case $[A]_0$ represents the initial mantADP concentration in Equation 2) to obtain apparent dissociation constants.

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}, \text{ where } G = \frac{I_{HV}}{I_{HH}}. \quad (\text{Equation 3})$$

The apparent K_d values for both S1 binding and ADP binding were obtained from three titration experiments.

Near UV CD

The tertiary structure of myosin S1 or acto-S1 were examined by near UV CD. All the spectra were collected on a JASCO 810 spectrometer. Protein solutions were prepared in 150 mM NaCl, 20 mM MOPS, 1 mM EDTA and 0.01 % NaN_3 , pH 7.0. For Mg^{2+} samples, MgCl_2 was added to 6 mM and for ADP samples ADP was added up to 150 μM . For S1 only samples, 10.9 μM S1 was added to the buffer. For acto-S1 samples, 5.4 μM of each protein was mixed. The number of scans was six for 10.9 μM S1 and 20 for 5.4 μM S1 concentration samples. After acquisition of the spectra, the spectral contributions of the buffer and ADP were subtracted. Since the noise around 260 nm was due to ADP absorbance (the applied high tension voltage was also monitored during acquisition to maintain below 800V), more rigorous smoothing was applied around 260 nm.

RESULTS AND DISCUSSION

Mg^{2+} inhibits myosin ATPase activity

As shown in Figure 1A, the catalytic activity of striated muscle myosin S1 was inhibited by increasing amount of free Mg^{2+} concentration above 1 mM in the presence of filamentous actin (F-actin) with 1.25 mM ATP, whereas in the absence of F-actin the inhibitory effect on the myosin ATPase activity was not observed above 1 mM free Mg^{2+} . Since the ionic strength was kept constant in these activity assays, it is not likely that the reduced activity under higher free Mg^{2+} solely resulted from weakened interaction between actin and myosin S1 due to high ionic strength. Further, only Mg^{2+} uncomplexed with ATP appeared to exert the inhibitory effect on the actin-activated ATPase activity, because Mg^{2+} in the form of MgATP acted as an activator rather than an inhibitor (Figure 1B), which is consistent with the results obtained by Bagshaw and Trentham (27). Therefore, the activation phase observed at the lower free Mg^{2+} concentration (below ~ 1 mM free Mg^{2+}) reported in Figure 1A is likely to be a result of driving the equilibrium into more MgATP formation available to myosin. Even in the presence of 100 μM Ca^{2+} , the inhibitory effect of Mg^{2+} on actin-activated myosin ATPase was clearly observed (Figure S2). Thus, we conclude Mg^{2+} is the inhibitor of ATPase in question rather than a random phenomenon caused by a metal ion bound to the system. Since the inhibitory effect exerted by free Mg^{2+} was much more prominent in the actin-activated myosin ATPase activity than that of myosin S1 alone, we think that the product release step, which actin facilitates, is affected by high free Mg^{2+} concentration. It has been demonstrated that both in non-muscle myosins and muscle myosins the ADP release is inhibited by Mg^{2+} (14, 9, 13, 10). Thus it is tempting to speculate that Mg^{2+} affects ADP release rate for striated muscle myosin II in the presence of

F-actin. Furthermore, the inhibitory effect of Mg^{2+} on actin-activated myosin ATPase was observed in the presence of other thin filament components, both with Tm or with Tm and Tn complex bound with Ca^{2+} (Figure 1C). Therefore, the inhibitory effect of Mg^{2+} is relevant to physiologic myosin ATPase in the striated muscle.

Mg^{2+} modulates myosin S1- ADP interaction

There have been several reports demonstrating that the ADP release is inhibited by Mg^{2+} both in non-muscle and muscle myosins (7, 8, 9, 13, 10). In order to detect the possibility of an altered interaction between myosin S1 and ADP caused by F-actin and Mg^{2+} , we employed WaterLOGSY NMR experiment which utilizes through space dipolar couplings between proton nuclei. The inverted magnetization of bound water transfers to the protein of interest and then finally relayed to a bound ligand. Hence, if the ligands bind to the protein the resulting signals are also “inverted”. In the WaterLOGSY experiment, the protons in water are selectively excited by a shaped pulse (19, 20) applied in such a manner that the dipolar couplings transfers modulated by the protein (i.e. bound to protein) result in the appearance of positive peaks. The WaterLOGSY signals for unbound small molecules are opposite in sign from that of large molecules due to the difference in their correlation times (28). The correlation time of isolated S1 (myosin head) was estimated to be 10 μ s (29), which is much slower than the correlation time enough for the change in the sign of NOE signals. One of the ADP proton resonances appeared to overlap or to be located in the vicinity of the water resonance (4.7 ppm) judging from the spectrum taken in D_2O (Figure S3). For this reason, it is expected that there are intra-molecular NOEs as well as those from the bulk water. Nonetheless, appearance of positive peaks in WaterLOGSY spectra is a clear indication of protein-small molecule interactions. The assignment of peaks was carried out according to the database at Biological Magnetic Resonance Data Bank (21). Peaks 1 and 2 were assigned to the protons in the methanediyl group connecting the pyrophosphate and ribose groups. In this study they were quantified as one species (Figures 2A and 2B), since they were not separable at high Mg^{2+} concentration (Figure S4). Peaks 3 and 5 were assigned to the protons in the ribose moiety and peaks 7 and 8 were assigned to those in the adenine moiety. Peak 6 belongs to the amine protons in the adenine moiety, which is exchangeable with the bulk water (Figure 2). Therefore peak 6 employs a different mechanism when it appears in WaterLOGSY spectra, thus always giving a positive peak. Although there were two more proton resonances visible (the proton of peak 4 and the proton aforementioned) when measured in D_2O (Figure S3), they were not quantified in the WaterLOGSY experiments due to the fact that they appeared close to or at the water resonance (4.7 ppm) and that their appearance was severely quenched by the water suppression pulse placed before acquisition. Without myosin S1 the ADP peaks appeared in the negative sign while peak 6 appeared in the positive sign (Figure S5). Those peaks which were sensitive to addition of myosin S1 did not change its sign by addition of F-actin in the absence of myosin (Figure S6). Upon addition of myosin S1, most of the peaks started to point toward the positive sign or at least several of them increased indicating the interaction between myosin S1 and ADP. Interestingly as our data show here (Figure 3), addition of F-actin to the myosin S1 – ADP mixture in both presence and absence of Mg^{2+} caused significant intensity increase presumably caused by actin-myosin interaction. We speculate this observation was due to the weakened S1 – ADP interaction (30) and/or increased

dissociation rate from S1 (31) upon F-actin binding because the actomyosin formation may have alleviated too tight ADP binding and/or too slow dissociation of ADP from myosin S1 (this appears to be counterintuitive, however, too tight binding and/or too slow dissociation actually cause signal decrease). As a result of actin-myosin interaction, more ADP molecules that have come in contact with myosin S1 are released into solution with a trace left on them detected as an NOE increase in a “free” or “unbound” ADP molecule. Because of different longitudinal relaxation times, T_1 , for each proton within ADP (Table S1), the degree of the signal increase upon addition of F-actin was necessary to be quantified such that the T_1 bias was minimized when different protons were compared. There were not significant differences in the measured T_1 relaxation times between with Mg^{2+} and without Mg^{2+} (Table S1). Rather than taking a single time point which may entail such a bias where one proton magnetization is almost saturated while another is not (32), we assumed that after an infinite amount of time past from application of the shaped pulse the NOE signals detected for all the protons reach their attainable maxima for each titration step (in our case, titration with F-actin). After fitting the exponential equation to the time-dependent NOE-build up curve for each proton (Figure 2C), the estimated maximal intensities, I_{max} , were plotted versus actin concentrations in order to demonstrate actin dependency in intensity increase (Figures 3A–3E). A significant attenuation was observed in the maximal intensities in the presence of Mg^{2+} compared with in the absence of Mg^{2+} (Figure 3). The curves of actin dependent increase in the maximal intensity, I_{max} , appeared to correspond well to myosin S1 – actin interaction (Figure 3) whose binding constants were determined independently of these NMR experiments and will be discussed later (Figure 5).

Also the apparent rate constants of NOE build-up obtained by the curve fitting were plotted versus actin concentrations (Figures 3F–3J). In the presence of Mg^{2+} , the apparent rate constants for all the peaks measured here except peak 8 (the proton which is in the adenine moiety) appeared not to exceed $\sim 3 \text{ s}^{-1}$ (Figures 3F–3J). When the build-up rate constants were below 2 s^{-1} such as peaks 3 and 5 (both in the ribose moiety) at lower actin concentrations, they increased gradually as the actin concentration increased. In the case of peak 3, there was an apparent difference with and without Mg^{2+} regardless of actin concentration. In peaks 1 and 2, there was virtually no enhancement in the apparent rate constant in the presence of Mg^{2+} .

On the contrary, in the absence of Mg^{2+} , all the peaks examined, including peaks 1 and 2, exhibited substantial increases in the apparent rate constant. This could be attributed to several factors including differences in protein-ligand structures (33), kinetics (33), and hydration status (19). Furthermore, the ADP proton resonances showed trends different from each other even if one compares with and without Mg^{2+} taking the T_1 times into account (Table S1), which is an example of indirect evidence that suggests existence of distinct binding modes with and without Mg^{2+} . For example, the rate constants of peaks 3 and 5 both in the presence and absence of Mg^{2+} gradually increased. On the contrary, in peaks 1 and 2 as mentioned above there was no increase observed in the presence of Mg^{2+} whereas there was some increase in the rate constant in the absence of Mg^{2+} (since peaks 1 and 2 have the lowest T_1 , quick saturation of magnetization by this mechanism alone is quite unlikely and there was no significant difference between the T_1 times in the presence and absence of Mg^{2+}). Although it is difficult to pinpoint as to what exactly causes this

difference, this set of results indicates that there exist distinct binding modes with and without Mg^{2+} .

In order to provide further support for our hypothesis that Mg^{2+} modulates myosin S1-ADP interaction, we took another independent approach with measurements of the equilibrium binding between mantADP and myosin S1. In the absence of F-actin, myosin S1 with Mg^{2+} bound mantADP about five times tighter than that of no Mg^{2+} . The apparent dissociation constants, K_d with and without Mg^{2+} were 0.13 μM and 0.65 μM , respectively (Figure 4A). This trend is consistent with the literature (9) and partially explains the observations of the WaterLOGSY experiments described above. In the presence of F-actin, the affinity toward mantADP drastically diminished to the extent where it was almost impossible to determine an accurate binding constant especially in the absence of Mg^{2+} by this method (Figure 4B). In spite of the difficulty, if one imposes reasonable assumptions on the results in the presence of F-actin (Figure 4B), approximate dissociation constants may be deduced. In the first round of fitting, the data set for Mg^{2+} was fit to estimate both the maximal anisotropy and apparent K_d values: the maximal anisotropy was 0.44 which is very close to the theoretical value, i.e., 0.4, and the apparent K_d value was 9.8 μM ($R^2 = 0.86$). The obtained maximal anisotropy of 0.44 should be interpreted as the best estimate obtained from the curve fit rather than the actual maximum. Hence, in the second round of fitting, the maximal anisotropy was substituted with the theoretical maximal anisotropy of 0.4. When the maximal anisotropy was fixed at 0.4, the apparent K_d value with Mg^{2+} was 8.0 μM with $R^2 = 0.86$, which is similar to the results obtained in the first round of fitting, suggesting 0.4 as a reasonable maximal anisotropy value. The maximal anisotropy value close to 0.4 can be explained in terms of more restricted mobility of the bound dye due to addition of F-actin. The theoretical maximum anisotropy value of 0.4 is derived by assuming that fluorescent molecules are equally distributed in all directions and that these fluorescent dipoles need not be precisely aligned with the vertically polarized excitation light (34). Therefore, even in the absence of Mg^{2+} the maximal anisotropy is expected to be close to 0.4 in the presence of F-actin. If the fluorescence anisotropy without Mg^{2+} increased up to 0.4, when saturating amount of myosin S1-ADP was added to F-actin, then the approximate dissociation constant without Mg^{2+} would be 20.4 μM with $R^2 = 0.71$. Under these assumptions, it was suggested that there was a moderate difference, about two fold, in the apparent K_d values. These K_d values differ by one order of magnitude from the one reported elsewhere (31). This could be partly attributed to the fact that in the current experiment a fluorescently-labeled ADP analog was used instead of ADP. Nonetheless, our current results have shown at least qualitatively that Mg^{2+} moderately modulated the binding between myosin S1 and ADP even in the presence of F-actin.

Our WaterLOGSY experiments showed that the maximum of actin dependent increase (actin dependent response) for each proton examined was attenuated in the presence of Mg^{2+} compared with that in the absence of Mg^{2+} while the curves of maximal intensity, I_{max} , increase corresponded well to those of myosin S1 – actin binding both in the presence and absence of Mg^{2+} (Figures 3A–3E). The latter set of results may confirm that myosin S1 – ADP interaction is weakened as actin concentration increases since the free ADP molecules which are the ones detected by this technique. Judging from the attenuation in intensity caused by Mg^{2+} , the affinity of myosin S1 – ADP interaction is altered, because the I_{max}

values also reflect the population of the ADP molecules that are released into the solution after having bound to myosin. Thus it is safe to say that the affinity of myosin towards ADP is strengthened by Mg^{2+} , which was suggested by the ADP binding assays in this study and those of others (9, 13). Our results of the equilibrium binding between mantADP and myosin S1 revealed that in the absence of F-actin myosin S1 in high Mg^{2+} concentration bound mantADP about five times tighter than that in low Mg^{2+} concentration. A similar trend is found in the literature describing myosin V (9). In the presence of F-actin, however, our results only suggested because of the technical difficulties the possibility that there may be a slight enhancement, i.e. up to two- or three-fold, in the affinity towards ADP in the presence of Mg^{2+} . Swenson *et al.* claims that the interaction between skeletal muscle myosin II and ADP is relatively insensitive to Mg^{2+} and there was a moderate enhancement in the affinity by Mg^{2+} (13). Utilizing a tryptophan mutant of *Dictyostelium* myosin II, namely Trp – 501 mutation, the same authors demonstrated that there was ~ 1.6 – fold difference in ADP affinity with and without Mg^{2+} , in which they speculate that its behavior in ADP binding is somewhat similar to skeletal myosin II (13). Therefore, our observations seem to be consistent with their results in terms of ADP binding.

Mg^{2+} also alters myosin S1- actin interaction

Next, we further investigated contributing factors for the Mg^{2+} inhibition of myosin ATPase. The aforementioned mechanism in which Mg^{2+} inhibits ADP-release may be explained in part by weakened myosin S1 – actin interaction in addition to slowed ADP dissociation rate alone, when F-actin was included in the system. Therefore, the binding equilibrium between myosin S1 and F-actin was measured both in the presence of either low or high Mg^{2+} to test if Mg^{2+} can alter the interaction between the two proteins. The pyrene-labeled F-actin was titrated with myosin S1 in the presence or absence of ADP (Figure 5). When ADP was absent (rigor state), tighter binding affinity toward F-actin with high Mg^{2+} concentration was observed compared with that of low Mg^{2+} concentration (data not shown). However, in the absence of ADP, the binding curve with Mg^{2+} did not fit well to the equation because the S1- actin interaction was too tight. When 2 mM ADP was present, without Mg^{2+} the apparent K_d was 0.15 μ M, while with Mg^{2+} it was 0.86 μ M (Figure 5), which indicates that Mg^{2+} significantly weakens the interaction between myosin S1 and actin in the presence of ADP resulting in more than a 5 – fold difference in average apparent K_d (Table 1). Thus, Mg^{2+} actively contributes to alter the myosin S1 – actin interaction in the presence of ADP.

Actin binding in turn is considered to facilitate ADP release from myosin. Therefore, the weakened actin-binding in the presence of Mg^{2+} would be likely to cause slower ADP release. However, this hypothesis has not been proven in striated muscle myosin II (13).

Distinct tertiary structures of myosin S1 and acto-S1 are brought on by Mg^{2+} and ADP binding

The earlier studies employing CD spectroscopy reported structural changes in myosin S1 caused by Mg^{2+} or ADP (35, 36, 37). However, to our knowledge a high resolution structure of acto-S1 has not been available up to now, and it is of our interest to capture structural changes in acto-S1 associated with ligand binding. Therefore, we carried out near UV CD

measurements on acto-S1 in addition to myosin S1 alone in this study to examine their tertiary structures.

Upon addition of Mg^{2+} to the S1 alone, only slight changes in ellipticity throughout the region examined without the nucleotide (Figure 6A) were found. In the absence of nucleotides, the affinity of Mg^{2+} toward the NBP is presumably weak. In fact, when there were no nucleotides, the crystal structure of myosin S1 prepared with papain digestion lacked a bound Mg^{2+} in the NBP and Mg^{2+} was found only in the regulatory light chain (38). Since, unlike the myosin S1 prepared with papain digestion, myosin S1 prepared with chymotryptic digestion is completely devoid of the regulatory light chain (39), Mg^{2+} - binding to the myosin light chains is thought to be negligible. This may explain the relatively small change in myosin S1 alone (rigor) in the near UV CD.

The near UV CD spectra of acto-S1 in the absence of ADP showed a Mg^{2+} - dependent difference indicative of the presence of distinct tertiary structures, which is clearly distinguishable from either myosin S1 alone or F-actin alone (Figures 6A and 6C). The heavy chain of rabbit skeletal muscle myosin S1 alone has seven tryptophan and 33 tyrosine residues. Since actin monomer adds four more tryptophan and 16 more tyrosine residues to the myosin S1 alone sample, the system is certainly more complicated. Because of minimal spectral contributions from Mg^{2+} -binding to the regulatory light chain, the observed change in acto-S1 without ADP in the near UV CD is more likely to have arisen from Mg^{2+} - binding to the actin filament. However, at least, it is safe to conclude that either some of the tyrosine or tryptophan residues or both in acto-S1 underwent a structural rearrangement that results in the spectral difference by addition of Mg^{2+} .

As reported earlier (35), the near UV CD spectrum of myosin S1 alone shifted downward in the 265 – 292 nm region when ADP was added (Figure 6B). Since both tyrosine and tryptophan residues contribute to the spectrum in the 275 – 285 nm region, it was concluded that the environment of these residues was strongly affected by ADP. Moreover, it was found that addition of Mg^{2+} caused a positive shift in the CD spectra of myosin S1 in the presence of ADP, which is consistent with the findings made by Muhlrud who utilized a trinitrophenylated S1 (36). It appears difficult to point out which residues are more affected by addition of Mg^{2+} in the presence of ADP. However, our results presented here are sufficient to conclude that there is a structural change in myosin S1 by addition of Mg^{2+} in the presence of ADP.

Even in the presence of ADP, a spectral difference with and without Mg^{2+} in acto-S1 was observed in the 265 – 292 nm region as without the nucleotide (see the difference spectrum in Figure 6D). However, the difference in the region was distinct from that of myosin S1 alone: a larger positive shift in the 265 – 272 nm region which could be attributed to changes in phenylalanine and/or tyrosine residues in acto-S1 or to some extent ADP itself. These changes in phenylalanine and/or tyrosine residues may be confined either in myosin S1 or actin or may happen to both of the components. Recent studies on F-actin showed that Mg^{2+} ion induces conformational changes which cause stiffness in the actin filament (40, 41, 42). Nonetheless, the difference in the spectrum of F-actin between the presence and absence of Mg^{2+} was distinct from that of acto-S1, which indicates that in some way the observed

change involved myosin. Since the observed effect of Mg^{2+} on each sample in near UV CD differed (i.e. S1 with and without ADP and acto-S1 with and without ADP), the results suggest that F-actin binding and ADP/ Mg^{2+} binding to myosin S1 have a synergic effect on the structure of the actin and myosin S1 assembly.

The apparent rates of NOE build-up in WaterLOGSY were larger in the absence of Mg^{2+} than those in the presence of Mg^{2+} except peak 8 (Figures 3F – 3J). Furthermore, each ADP proton resonance showed somewhat different trends from each other in terms of presence or absence of Mg^{2+} even if one takes the T1 times into account. These results indicate that there must exist distinct binding modes with and without Mg^{2+} although further investigation is necessary to determine exactly what caused the observed differences. Muhlrad concluded from observation of changes in the CD spectra of a trinitrophenylated S1 that the tertiary structure of myosin was sensitive to Mg^{2+} when pyrophosphate was added to the myosin (36). The structure of scallop striated muscle myosin II crystalized with MgADP contained several water molecules in the NPB (the water molecules were omitted in Figure 7C), two of them were bound to the P-loop and Switch I residues, which was speculated to be similar to the interaction with the γ - phosphate of ATP (43). The bound water molecules were in close vicinity of the bound Mg^{2+} .

The crystal was not co-crystalized with actin and we also do not know how these residues of myosin II interact with ADP in the absence of Mg^{2+} . Therefore, we could only speculate regarding the detailed structure of the NBP either in the presence of F-actin or in the absence of Mg^{2+} and how F-actin and Mg^{2+} influence each other. Perhaps interaction with F-actin could further widen the difference relevant to myosin ATPase with and without Mg^{2+} or alter the nature of difference, which is partly supported by the fact that Mg^{2+} - inhibition of myosin ATPase was observed only in acto-S1 sample not in S1 alone sample. Our near UV CD results also suggested that there exist distinct modes of binding of ADP to myosin, in which a change in orientation of aromatic residues or in the structure itself due to Mg^{2+} - binding that affected on the 265 – 292 nm region when myosin S1 was complexed with the nucleotide. Addition of F-actin intensified in the 265 – 272 nm region in the difference spectrum with and without Mg^{2+} in the presence of ADP (Figure 6D). At least, it suggests that these originate from underlining structural differences in how myosin S1 when complexed with F-actin, binds ADP depending on whether Mg^{2+} is present or not. This is further strengthened by the fact that the WaterLOGSY experiments indirectly indicated that Mg^{2+} exerts a further change in the NBP structure in the presence of F-actin, because the protons close to the pyrophosphate moiety of ADP behaved differently from the other protons when F-actin was added in the presence of Mg^{2+} . Therefore, these observations point toward the conclusion that actin binding and ADP/ Mg^{2+} binding to myosin S1 have a synergic effect on the structure of the actin and myosin S1 assembly.

Although high resolution structures of striated muscle myosin II in the presence of F-actin for these conditions studied above, i.e., ADP and Mg^{2+} are not available yet, MgADP.BeFx bound myosin V (PDB code: 1W7J) may give an idea on how the nucleotide and Mg^{2+} ion fit into the active site in the strongly MgADP bound state, which results in opening of the actin binding cleft. With Mg^{2+} (PDB code: 1W7J), in general, the surrounding residues around ADP are brought close together while those around ADP are sparse without Mg^{2+} .

(PDB code: 1W7I). In particular, K169 in the P-loop is not in contact with β -phosphate of ADP which weakens the interaction between ADP and myosin V in the crystal structure (PDB code: 1W7I). Although Y439 in myosin V is replaced with alanine at the corresponding position in muscle myosin II denoted as A462, T183 (in P-loop), S241 (in Switch I) and D460 (in Switch II) are all present in muscle myosin II (Figure 7). Several residues in the switch II including the aspartic residue at the corresponding site of myosin V (D437 in myosin V) are thought to play an important role in the closed conformation of NPB in the presence of actin (44). In the case of striated muscle myosin, the phosphate release rate is considered to be strongly affected by actin binding. Therefore, the alteration of ADP and actin binding to muscle myosin II by Mg^{2+} may not have as much impact on ATPase rate as that on the phosphate release step. Nonetheless, our biochemical studies reported here suggest that there exist changes in actomyosin when associated with ADP and Mg^{2+} (a proposed scheme is shown in Figure 8), which may exert some effect on ATPase cycle of muscle myosin II.

Our current study describes Mg^{2+} -modulation of striated muscle myosin II both in the presence and absence of F-actin, using several different methods. In addition to Ca^{2+} -regulation of striated muscle, we address importance of the Mg^{2+} -modulation in myosin ATPase. We demonstrated that excess free Mg^{2+} but not the complexed form, i.e. $MgATP$, inhibits actin-activated muscle myosin II ATPase activity and that high Mg^{2+} alters the actin – myosin interaction in the presence of ADP as well as myosin – ADP interaction. Further, the tertiary structure of myosin S1 alone or acto-S1 in the presence and absence of the ligands was investigated, which may shed light on the reasons why excess Mg^{2+} affects the ADP – myosin and actin – myosin interactions. In conclusion, our results demonstrate that free Mg^{2+} alters the interaction between actin and myosin in the ADP bound state that may affect ATPase in the striated muscle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Mg^{2+} inhibited striated muscle myosin ATPase in the presence of F-actin.
- Both Mg^{2+} and F-actin had modulatory effects on striated muscle myosin S1–ADP interaction.
- Mg^{2+} also modulated myosin S1–actin interaction.
- Both in the presence and absence of ADP, there was a Mg^{2+} -dependent difference in the tertiary structure of actomyosin.

Figure 1. Myosin S1 ATPase activity inhibition by Mg^{2+} . (A) Free Mg^{2+} effect on actin-activated myosin S1 ATPase rate (black circle) and myosin S1 ATPase rate (gray square). (B) Increasing amount of $MgATP$ does not inhibit actin-activated myosin S1 ATPase activity. (C) Free Mg^{2+} effect on myosin ATPase rate in the presence of different thin filaments: actin (black circle and same data presented in panel A), actin with Tm (gray square), and actin with Tm and Tn in the presence of saturating Ca^{2+} (light gray triangle). Each data point represents the average value from multiple experiments ($n = 2 \sim 4$) and error bars represent the standard deviation.

Figure 2. Resonance assignment of ADP and NOE build-ups in WaterLOGSY.

(A) Resonance assignment of ADP was carried out using the database (see methods). Each proton assigned is numbered in parentheses according to the ordering in the NMR spectra in panel B. (B) An NOE build-up rate in WaterLOGSY spectra for each peak was estimated by changing the mixing-time (spectra at $\tau_m = 1.20$ s, 0.48 s and 0.32 s are shown). The resonance peaks appearing between 2 and 4 ppm which correspond to MOPS protons are pointing in the negative direction because MOPS does not interact with myosin S1. (C) An example of NOE build-up curve was shown for peak 5 in the presence of acto-S1 in the presence (gray square) and absence (black circle) of Mg²⁺. The intensity of peak 5 relative to peak 6 at $\tau_m = 2.0$ s was plotted v.s. the NOE mixing time.

Figure 3. Actin concentration-dependent changes in NOE intensity and apparent transfer rate in the presence and absence of Mg²⁺.

(A-E) Estimated maximal intensity in the presence (filled square) and absence (filled circle) of Mg²⁺ was expressed relative to peak 6 at $\tau_m = 2.0$ s and plotted against actin concentration for each assigned peak. (F-J) Apparent NOE build-up rate in the presence (filled square) and absence (filled circle) of Mg²⁺ was plotted against actin concentration for each peak. Because the NOE build-up curves for peak 8 in the presence of Mg²⁺ appeared to be saturated early on, its apparent build-up rates were not obtained and only apparent rates in the absence of Mg²⁺ are shown in J. Also the NOE build-up curves for peak 7 in the

presence of Mg^{2+} at low actin concentration were severely contaminated by free ADP resonance whose sign was negative and the data were not suitable for fitting the same exponential curve. (**A, F**) peaks 1 and 2, (**B, G**) peak 3, (**C, H**) peak 5, (**D, I**) peak 7, and (**E, J**) peak 8.

Figure 4. Myosin S1 – mantADP binding in the presence and absence of Mg²⁺.

(A) A fluorescently-labeled ADP analog, mantADP, was titrated with myosin S1 in the presence (filled square) and absence (filled circle) of Mg²⁺ in the absence of F-actin. A representative set of titration data points is shown for each case. The average apparent equilibrium dissociation constants with and without Mg²⁺ concentrations were 0.13 ± 0.03 μM and 0.65 ± 0.07 μM, respectively. (B) The same analog was titrated with myosin S1 in the presence (filled square) and absence (filled circle) of Mg²⁺ in the presence of F-actin. In panel B, data points from multiple experiments conducted at different myosin S1 concentrations were shown rather than a representative set (see text).

Figure 5. Myosin S1 – actin binding in the presence and absence of Mg²⁺.

Fluorescently-labeled actin was titrated with myosin S1 in the absence (A) and presence (B) of Mg²⁺ in the presence of ADP. A representative set of titration data points is shown for each of (A) and (B). The best fit to each of the experimental data is shown as a solid line. The initial fluorescence intensity before titration was regarded as 100 % and an intensity change caused by myosin S1 was expressed relative to the initial intensity. The average apparent K_d values in the absence and presence were $0.15 \pm 0.04 \mu\text{M}$ and $0.86 \pm 0.43 \mu\text{M}$, respectively (see Table 1).

Figure 6. Near UV CD spectra of myosin or actomyosin.

(A) S1 only (solid line) and acto-S1 (dashed line) in the absence (black) and presence (gray) of Mg^{2+} in the absence of ADP. (B) The difference spectra between without Mg^{2+} and with Mg^{2+} in the absence of ADP are shown for myosin S1 only (gray) and acto-S1 (black). (C) S1 only (solid line) and actin-S1 (dashed line) in the absence (black) and presence (gray) of Mg^{2+} in the presence of ADP. ADP was added to 150 μM . (D) The difference spectra between without Mg^{2+} and with Mg^{2+} in the presence of ADP are shown for myosin S1 only (gray) and acto-S1 (black). In order to see the effect of Mg^{2+} on the tertiary structure, the difference spectra were obtained by subtracting spectrum without Mg^{2+} from that with Mg^{2+} . The dotted line in (B) and (D) represents the difference spectrum between with and without Mg^{2+} for actin only sample in the absence of ADP for comparison. Ellipticities shown here were corrected for concentration.

Figure 7. Magnified view of the nucleotide binding pocket of myosin.

Myosin II from scallop striated muscle (PDB: 1B7T). The corresponding residues in myosin II to the key residues in Mg²⁺-binding in Myosin V, i.e., T170, S218, D437, and Y439 around ADP (shown in red) are labeled (switch I: yellow, switch II: orange, and P-loop: magenta). Residues located within 3 Å from either Mg²⁺ or ADP are connected via yellow dotted lines.

Figure 8. Schematic representation of proposed changes in myosin S1 – ADP interaction due to the presence and absence of Mg^{2+} and actin.

This figure shows four different states of myosin bound to ADP (top left: actin-S1 with Mg^{2+} ; top right: actin-S1 without Mg^{2+} ; bottom right: S1 without Mg^{2+} ; bottom left: S1 with Mg^{2+}). It is suggested that myosin interacts weakly with ADP in the absence of Mg^{2+} and in the presence of actin. Therefore, actin-S1 without Mg^{2+} (top right) is depicted to illustrate that the NBP adopts a more open conformation than in the other states.

Scheme I.

A is actin and M is myosin.

Table 1.

Apparent dissociation constants for S1-ADP binding to pyrene-labeled filamentous actin.

	No Mg^{2+}	Mg^{2+}
Apparent K_d (μM)	0.15 ± 0.04	0.86 ± 0.43
F_{max} (%)	50.98 ± 3.03	33.20 ± 5.84
R^2	0.98 ± 0.01	0.98 ± 0.00