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THE BINDING OF ADP TO MYOSIN*

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SUMMARY

The binding of [^{14}C]ADP to myosin (EC 3.6.1.3) was measured by equilibrium dialysis and centrifugation methods.

At low ionic strength (0.05) the ADP binding was largely independent of free Mg^{2+} concentration in the range of 1 nM–1 mM. The affinity constant at 1 mM Mg^{2+} concentration was estimated to be about 10^6 and the linear Scatchard plots yielded a maximum of 1.2–1.6 moles of ADP bound per mole of myosin. At high ionic strength (0.6 M KCl) the binding of ADP to myosin was dependent upon the presence of Mg^{2+} in a free concentration of about 1 μM. The affinity of ADP binding to myosin was reduced by inorganic pyrophosphate, actin, IDP and 2,4-dinitrophenol. The possible role of ADP in the regulation of the rate of ATP hydrolysis is discussed on the basis of the above observations.

INTRODUCTION

Information concerning the interaction of ATP, ADP or its analogue, inorganic pyrophosphate (PP_i), with myosin (EC 3.6.1.3) is of significance not only in contributing to our understanding of the mechanism of ATP hydrolysis, but also in relation to the postulated role of interacting substrates in regulating the conformation of myosin, its actin binding ability and ultimately, its contractility.

It is well established[1–4] that myosin contains a single set of sites for the binding of PP_i with an affinity constant of about 10^6. The binding of PP_i shows[3] an absolute dependence on the presence of divalent cations, Mg^{2+} or Ca^{2+}, and is markedly influenced by chemical modification of myosin[5] or by changes in the pH or temperature of the incubation medium[3]. The competitive inhibition of the pyrophosphate binding of myosin by actin implies that the interaction of actin and the substrate analogue with myosin occur at overlapping sites[3].

Based on the inverse relationship of the dependence of ATPase activity, and PP_i binding on the free Mg^{2+} concentration, we proposed a working hypothesis[3] in which the rate of ATP hydrolysis was regulated by the equilibrium of the polyphosphate chain of the substrate between the hydrolytic site and a non-hydrolytic regulatory site that is also involved in the interaction of actin with myosin.

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid; CDTA, cyclohexyldiaminetetraacetic acid; PP_i, inorganic pyrophosphate.

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A more detailed analysis of the contribution of the base moiety to the binding of nucleoside polyphosphates with myosin became possible with the recent demonstration of the existence of high affinity ADP binding sites on myosin[6]. According to YOUNG[6], myosin and heavy meromyosin contain close to three ADP binding sites per 595000 and 380000 g protein, respectively, on the basis of equilibrium dialysis and ultracentrifuge transport measurements, with affinity constants of about $5 \cdot 10^4$ in the presence of 1 mM Mg^{2+}. In a short note LUCK AND LOWEY reported[7] the maximum binding of 1.50 moles of ADP per 500000 g of myosin; the binding was shown to be dependent upon the presence of Mg^{2+} or, less effectively, Ca^{2+}, in a medium of 0.5 M KCl.

In order to correlate the characteristic requirements of ADP binding with those of the myosin–PP_i interaction described previously, a detailed examination of the temperature, pH and cation dependence of the ADP binding of myosin was carried out, supplemented by a preliminary analysis of the myosin–Mg^{2+} equilibrium. These data, to be reported below, point to the possible regulatory influence of the myosin–ADP interaction on the rate of ATP hydrolysis.

EXPERIMENTAL

Myosin obtained as described by PERRY[8], with minor modifications was fractionated with $(NH_4)_2SO_4$ according to KIELLEY AND BRADLEY[9]; the 40–50 % $(NH_4)_2SO_4$ fraction was used for all experiments except those of Fig. 4. The $(NH_4)_2SO_4$ fractionation reduced the myokinase (ATP:AMP phosphotransferase, EC 2.7.4.3) and AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) activities by 75 and 95 % respectively. Tropomyosin-free actin was prepared as described earlier[10]. Actin thus prepared was found to be essentially free of myokinase. F-Actin labeled with [8-^{14}C]ADP was prepared by allowing G-actin to exchange with [8-^{14}C]ATP[11] followed by polymerization in 0.1 M KCl. Extensive dialysis against 0.1 M KCl, 10 mM Tris–HCl (pH 7.0) was used to remove unbound nucleotides. Protein concentrations were measured by the method of LOWRY *et al.*[12] or by the biuret method calibrated against nitrogen measured by the Kjeldahl method, using a factor of 6.2 to convert mg nitrogen to mg myosin[13].

The binding of [8-^{14}C]ADP and ^{32}P-labeled PP_i to myosin was measured by equilibrium dialysis[14] or by centrifuging myosin suspensions under conditions where myosin solubility is low (low ionic strength, pH values close to neutrality). With the latter method, binding was calculated by comparing the radioactivity of the suspension with the radioactivity remaining in the supernatant following centrifugation. Radioactivity was measured on a Packard Liquid Scintillation counter according to LOFTFIELD AND EIGNER[15]. In cases where interfering substances such as DNP or PP_i (at mM concentrations) were present, the Beckman Lowbeta II Geiger counter was used applying proper correction for self-absorption. The concentration of protein remaining in the supernatant was measured by the method of LOWRY *et al.*[12], and appropriate corrections were made. In experiments with dinitrophenol, the protein in the supernatant was precipitated by adding trichloroacetic acid to a concentration of 3 %, the precipitate was washed three times with 1-ml volumes of acetone, the dried precipitate was redissolved in 0.1 M NaOH–2 % Na_2CO_3 and assayed by the method of LOWRY *et al.*[12].

The possibility was considered that at low ionic strength a Donnan effect might influence the binding of ADP or PP_i to myosin. Equilibrium-dialysis experiments carried out in the presence of 10 μM [^{32}P]orthophosphate at pH 7.0, with 1 mM EDTA, at KCl concentrations as low as 0.015 M, and at pH-values from 6.0 to 9.0, revealed no Donnan effect. Trace amounts of myokinase and AMP deaminase cause some breakdown of ADP into AMP and IMP during prolonged equilibrium dialysis experiments in the presence of Mg^{2+} or Ca^{2+}. The extent of ADP decomposition was measured following separation of isotopically labeled nucleotides by descending chromatography on Whatman No. 4 paper using isobutyric acid–water–concentrated NH_4OH (66:33:1, by vol.), as solvent[16], or by high-voltage electrophoresis on Whatman 3 MM paper in 0.1 or 0.02 M sodium citrate buffer (pH 5.0) at 50 V/cm for 1–2 h, with adequate cooling[17]. The nucleotide spots were cut out and the radioactivity counted by the method of LOFTFIELD AND EIGNER[15]. The reported binding data were corrected for ADP decomposition which rarely exceeded 20 % of the total ADP in an equilibrium-dialysis experiment of 12–20 h duration at 3°.

A Perkin-Elmer model 303 atomic absorption spectrometer was used for the determination of Mg^{2+} and Ca^{2+}. Protein, when present, was removed with trichloroacetic acid (3 %). Phosphate compounds interfere with the measurement of Mg^{2+} and Ca^{2+} (ref. 18); this can be overcome by inclusion of 1 % $LaCl_3$ or by diluting the solutions. 1 mM PP_i or 0.1 mM ADP did not significantly affect the measurement of Mg^{2+} or Ca^{2+} in the range of 1 to 10 μM.

Traces of Mg^{2+} and Ca^{2+} were removed from solutions of NH_4Cl and alkali metal salts (except CsCl) by passage of 2.4 M solutions through a 2 cm × 25 cm column of Dowex chelating resin which had been thoroughly washed with 2 l each of 0.1 M HCl, water, 0.1 M KOH, water, and 1 mM EDTA, in that order. Finally, the column was washed with 8 l of water and was equilibrated with the salt to be purified. The effluent solutions of NH_4Cl and other salts had a pH near neutrality and were essentially free of Mg^{2+} and Ca^{2+}.

The following association constants[19,20] were used for the calculation of free Mg^{2+} and Ca^{2+} concentrations at pH 7.0: magnesium EDTA, $10^{5.38}$; calcium EDTA, $10^{7.28}$; calcium ethyleneglycol-bis-(β-aminoethylether)-*N*-*N*′-tetraacetate (EGTA), $10^{6.72}$; magnesium EGTA, less than 10^1; magnesium cyclohexyldiaminetetraacetate (CDTA), $10^{5.55}$; calcium CDTA, $10^{7.75}$.

The concentrations of nucleotides were determined by ultraviolet absorption spectrophotometry using Beckman Model DBG Spectrophotometer.

Materials

Only analytical grade reagents were used. ADP, AMP, adenosine, IDP, and IMP were obtained from P-L Biochemicals. ^{32}P-labeled PP_i was obtained from the New England Nuclear Corporation. [8-^{14}C]ADP and [8-^{14}C]ATP were obtained from Schwartz BioResearch. Only distilled water subsequently treated with ion-exchange resins was used in the experiments.

The homogeneity of [8-^{14}C]ADP and unlabeled ADP was tested by chromatography or electrophoresis. The only contamination was due to AMP, the amount of which was less than 1 % in the experiments represented by Figs. 1 and 2, and in all experiments was below 6 %.

RESULTS

In the presence of 0.6 M KCl, the binding of ADP to myosin is largely dependent on a free Mg^{2+} concentration in the micromolar range (Fig. 1a). The Mg^{2+} requirement for ADP binding under these conditions resembles that previously reported for PP_i[3]. Half-maximum binding of ADP occurs at a free Mg^{2+} concentration of 2–4 μM. Below 1 μM free Mg^{2+}, a residual ADP binding is observed, which appears to be independent of the concentration of free Mg^{2+} to 5 nM. Presentation of the data of Fig. 1a in the form of Scatchard plots (Fig. 1b) indicates that the affinity constant for the ADP–myosin interaction falls with decreasing concentration of free Mg^{2+}. The number of accessible binding sites, determined by extrapolation to the abscissa, shows that a maximum of 1.2–1.3 moles of ADP were bound per $5 \cdot 10^5$ g of myosin, independently of the free Mg^{2+} concentration.

Figs. 2a and 2b present the results of similar experiments carried out at low ionic strength (0.03 M KCl). Under these conditions the changes produced in the ADP binding by varying the concentration of free Mg^{2+} between 1 mM and 1 nM are small, although as shown in Fig. 2b the binding constant decreases from $1.4 \cdot 10^6$ at free Mg^{2+} concentrations of 0.1 μM or greater, to about $6 \cdot 10^5$ at the lowest Mg^{2+} concentration. In this case a maximum of 1.5–1.6 moles of ADP were bound per $5 \cdot 10^5$ g of myosin. Alkali metal halides, KCl, NaCl, LiCl, and NH_4Cl, influence the binding of ADP to myosin as shown in Fig. 3a. In the presence of 5 mM EDTA, *i.e.*, at free Mg^{2+} concentration about 10 nM, increasing concentrations of all salts tested depressed ADP binding equally, producing nearly complete inhibition at 0.8 M.

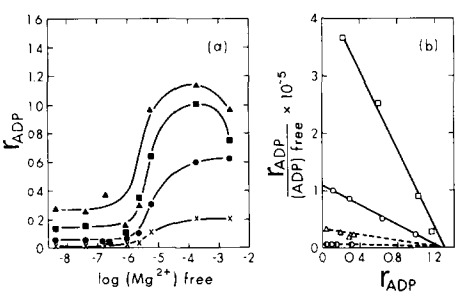


Fig. 1. a. The magnesium dependence of ADP binding at high ionic strength. 2.5-ml vol. of myosin solution containing 7.0 mg/ml protein were dialyzed against 5.0 ml medium for 16 h at 3°. The medium contained 0.6 M KCl, 0.052 M Tris–HCl (pH 7.0), and 10 mM Tris–EDTA, $MgCl_2$ was added in concentrations from zero to 12 mM, and ADP was present in the following amounts per 7.5 ml: 15 nmoles (×—×); 50 nmoles (●—●); 150 nmoles (■—■); 0.5 μmole (▲—▲). Immediately following separation of the dialysis systems, 0.5-ml aliquots of inner and outer solutions were treated with 0.1 ml 25% $HClO_4$, centrifuged, and 0.025 ml of the supernatant was subjected to paper chromatography. Spots corresponding to AMP, IMP, and ADP were cut out and counted in liquid scintillation counter. On average, 89.5% of the applied counts were recovered. ADP breakdown ranged from 10 to 38%, in different experiments, increasing with increasing Mg^{2+}. Approximately equal amounts of IMP and AMP were present as breakdown products. The apparent free ADP concentration was corrected for this breakdown. The Mg^{2+} and Ca^{2+} contamination of the combined reagents measured by atomic absorption spectroscopy was 12 and 13.5 μM, respectively. The extra Mg^{2+} was taken into account in calculating the free Mg^{2+}; most of the Ca^{2+} was present as calcium–EDTA complex. r is defined as the number of moles of ADP bound per $5 \cdot 10^5$ g of myosin, while n represents the maximum number of binding sites. b. Scatchard plots of ADP binding at various levels of free Mg^{2+} (high ionic strength). The data are drawn from the experiments shown in a. Concentrations of calculated free Mg^{2+} were as follows: 205 μM (□—□); 6.3 μM (○—○); 2.73 μM (△—△); 4.2 nM (◐—◐).

In the presence of 1 mM $MgCl_2$, the same salts have little effect on the ADP binding even at the highest concentrations. PP_i did not bind measurably to myosin in the presence of 5 mM EDTA, even at 0.04 M KCl concentration. In the presence of 1 mM $MgCl_2$, the binding of PP_i was hardly influenced by variations in the concentration of KCl.

Under conditions similar to those used above, in the presence of 0.035 M KCl and 0.84 mM EGTA, 1.52–2 moles of Mg^{2+} are bound per $5 \cdot 10^5$ g of myosin (Fig. 4).

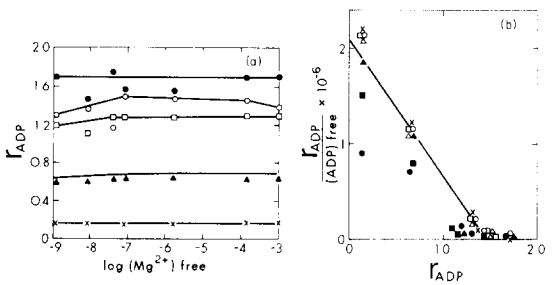


Fig. 2. a. Effect of free Mg^{2+} concentration on ADP binding at low ionic strength. 4-ml suspensions of myosin containing 3.25 mg protein per ml in 0.03 M KCl, 5 mM EDTA, and $MgCl_2$ concentrations from zero to 6 mM were prepared, and adjusted to pH 7.0 at 0–3°. The following amounts of [^{14}C]ADP were included per 4 ml: 4 nmoles (×—×); 20 nmoles (▲—▲); 60 nmoles (□—□); 120 nmoles (○—○); 200 nmoles (●—●). The suspensions were allowed to equilibrate at 3° for 15 min with occasional shaking, and were centrifuged at 8000 × *g* for 20 min. Binding was calculated on the basis of radioactivity removed from the supernatant. 0.03–0.07 mg protein per ml remained in the supernatant. 13 μM Mg^{2+} and 15.5 μM Ca^{2+} were present as contaminants in the system. The contaminating Mg^{2+} was included in the calculation of free Mg^{2+} concentration. b. Scatchard plots of ADP binding at various levels of free Mg^{2+} (low ionic strength). The data are drawn from the experiments shown in a. Free Mg^{2+} concentrations were as follows: 1.15 nM (●—●); 9.93 nM (■—■); 42 nM (▲—▲); 85 nM (△—△); 2.1 μM (□—□); 145 μM (○—○); 1 mM (×—×). Except at the lowest Mg^{2+} concentrations the binding data can be represented by a single line.

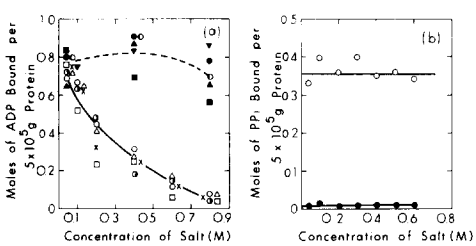
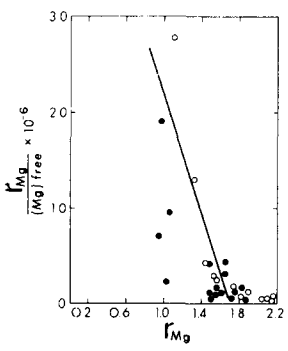


Fig. 3. a. Effect of increasing salt concentration on ADP binding. Myosin was largely freed of metal ions by two washings in 0.06 M Tris–HCl (pH 7.0). 2.5-ml vol. of myosin suspension or solution, at a salt concentration indicated on the abscissa, were dialyzed for 18 h at 3° against an outer volume of 5 ml containing the same salt concentration. The medium also contained 0.03 M Tris–HCl (pH 7.0), 75 nmoles of [^{14}C]ADP per 7.5 ml, and either 5 mM EDTA (solid line) or 1 mM $MgCl_2$ (broken line). The different salts used are indicated as follows: ●---●, ○—○, KCl; ■---■, □—□, LiCl; ▼---▼, ×—×, CsCl; ▲---▲, △—△, NH_4Cl; ◐---◐, ◑—◑, NaCl. The maximum free concentrations of contaminating Mg^{2+} and Ca^{2+} were 42 nM and 0.63 nM respectively, in the presence of 5 mM EDTA. b. Effect of increasing KCl concentration on the binding of PP_i to myosin. 2.5-ml vol. of myosin suspension or solution, containing 4.0 mg protein per ml and KCl in concentrations indicated on the abscissa, were dialyzed for 18 h at 3°, against 5 ml of a solution containing the same concentration of KCl. The medium also contained 26 mM Tris–HCl (pH 7.0), 15 nmoles of ^{32}P-labeled PP_i per 7.5 ml, and either 1 mM $MgCl_2$ (○—○) or 5 mM EDTA (●—●). Contaminating Mg^{2+} and Ca^{2+} levels correspond to free concentrations of approximately 3 nM for Mg^{2+} and less than 0.1 nM for Ca^{2+}.

The binding constant is too high to be measured accurately by the method used but its value appears to be at least $2 \cdot 10^6$, although it may be much higher than this.

When Ca^{2+} (1 mM) replaces Mg^{2+} as the principal divalent cation in the system, the binding constant for ADP is reduced by about one order of magnitude, either in the presence of 0.6 M KCl or at low ionic strength (Fig. 5). In the presence of 0.6 M KCl, a binding constant of $4 \cdot 10^5$ was found in the presence of Mg^{2+}, and a constant of $3 \cdot 10^4$ was found in the presence of Ca^{2+}. At 0.03 M KCl, a binding-constant of $1.4 \cdot 10^6$ was found in the presence of Mg^{2+}, and at 0.015 M KCl in the presence of Ca^{2+}, the constant was about $2.5 \cdot 10^5$.

In order to clarify the relationship between the binding sites involved in the



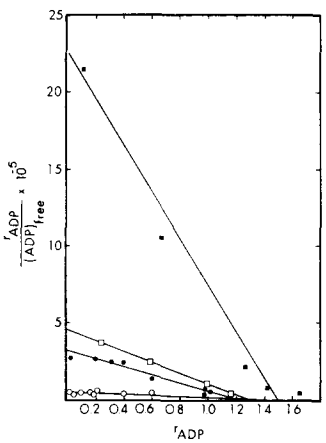


Fig. 4. Mg^{2+} binding to myosin. Myosin was suspended in 0.04 M KCl, 1 mM potassium EGTA (pH 7.0) and precipitated by centrifugation at 8000 × *g* for 10 min. The washing was repeated three times to minimize the divalent cation content of the protein suspension. 6-ml vol. of a suspension which contained about 6 mg myosin per ml, 0.035 M KCl, 840 μM potassium EGTA with $MgCl_2$ added to a concentration of 0–75 μM were prepared in thoroughly cleaned glass test tubes. After centrifugation at 1640 × *g* for 10 min at about 3°, 3 ml of the supernatant were withdrawn and assayed for Mg^{2+} and Ca^{2+} by atomic absorption spectrometry. The protein was resuspended in the remaining fluid and precipitated by addition of 0.3 ml of 30% trichloroacetic acid. After centrifugation the supernatant was assayed for Mg^{2+} and Ca^{2+} using an appropriate trichloroacetic acid blank. The concentrations of Mg^{2+} and Ca^{2+} in the first supernatant were taken as free; the amounts of these ions released by trichloroacetic acid were taken as bound. The protein content of each tube was estimated by dissolving the trichloroacetic acid precipitate in alkali and assaying the solution by the biuret method. In this experiment, the concentration of Ca^{2+} in the first supernatant was 6.2 ± 2.4 μM and 0.38 ± 0.20 mole of Ca^{2+} was bound per $5 \cdot 10^5$ g myosin. 6.2 μM Ca^{2+} in the presence of 840 μM EGTA under these conditions gives approx. 1.4 nM free Ca^{2+}; less than 1% of the Mg^{2+} is bound by EGTA at any of the levels of Mg^{2+} in these experiments. The open and closed circles correspond to two similar experiments.

Fig. 5. The effect of Ca^{2+} and Mg^{2+} on the binding of ADP, at high and at low ionic strength. ■—■, ADP binding to myosin in the presence of 1 mM free Mg^{2+}, 0.03 M KCl (pH 7.0) under conditions described in Fig. 2b. □—□, ADP binding to myosin in the presence of 205 μM free Mg^{2+}, 0.6 M KCl (pH 7.0) under conditions described in Fig. 1b. ●—●, ADP binding was measured by the centrifugation method described in the legend to Fig. 2a. Prior to centrifugation the suspensions contained 4.4 mg myosin per ml, 0.015 M KCl, 1 mM $CaCl_2$, and 2 to 100 nmoles of [^{14}C]ADP per ml. The pH was 7.0 and the temperature approx. 3°. ○—○, ADP binding was measured by dialysis of 2-ml vol. of myosin solution (5.5 mg/ml) against an outer volume of 4 ml for 14 h at 3°. The medium contained 0.6 M KCl, 1 mM $CaCl_2$, 0.05 M Tris–HCl (pH 7.0) and 6 to 24 nmoles of [^{14}C]ADP per 6 ml. The free ADP concentration was corrected for breakdown (10–15%) by a method similar to that described for Fig. 1a, except that paper electrophoresis was used to separate the nucleotides.

interaction of myosin with PP_i, actin, and nucleoside phosphates, the effects of PP_i, IDP and actin on the ADP binding were measured. PP_i at low concentrations effectively inhibits the binding of ADP to myosin in the presence of 0.6 M KCl and 1 mM $MgCl_2$ (Fig. 6). At low ionic strength (0.015 M KCl, 2.5 mM CDTA) much higher concentrations of PP_i are required to inhibit ADP binding, as expected from the low affinity of PP_i for myosin under these conditions (see also Fig. 3b). Either in the presence of 0.6 M KCl with 1 mM $MgCl_2$, or in the presence of 0.015 M KCl with 5 mM EDTA, IDP competes weakly with ADP (Fig. 6). IMP, and adenosine, under conditions similar to those described in the legend to Fig. 8, had no effect on ADP binding, even

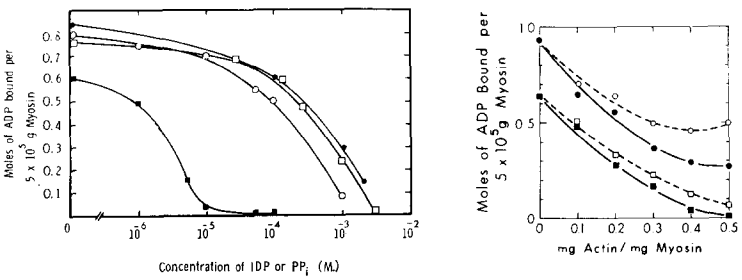


Fig. 6. PP_i and IDP as inhibitors of ADP binding. ■—■, 2.5-ml vol. of myosin solution, 4.8 mg per ml, were dialyzed against outer volumes of 5 ml for 38 h. The medium contained 0.6 M KCl, 0.053 M Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM EGTA, 50 nmoles [^{14}C]ADP per 7.5 ml, and increasing concentrations of PP_i as indicated on the abscissa. □—□, ADP binding was measured by centrifugation of suspensions containing 5.0 mg myosin per ml, 0.015 M KCl, 2.5 mM CDTA, and increasing concentrations of PP_i as indicated on the abscissa. The pH was 7.0 and the temperature 3°. The levels of contaminating Mg^{2+} and Ca^{2+} in the system at the highest concentration of PP_i were 8 and 10.7 μM respectively, corresponding to a free Mg^{2+} concentration of 9 nM and a free Ca^{2+} concentration of 0.08 nM. ●—●, 2.5-ml vol. of myosin (5.5 mg/ml) were dialyzed against an outer volume of 5 ml for 18 h at 3°. The medium contained 0.6 M KCl, 1 mM $MgCl_2$, 0.054 M Tris–HCl at pH 7.0, 50 nmoles [^{14}C]ADP per 7.5 ml, and increasing concentrations of IDP as indicated on the abscissa. ○—○, 2-ml suspensions containing 5.1 mg myosin per ml, 0.015 M KCl, 10 μM [^{14}C]ADP, 5 mM EDTA and increasing amounts of IDP, at pH 7.0 and 3°, were centrifuged as described in the legend to Fig. 2a. Maximum levels of contaminating Mg^{2+} and Ca^{2+} were 20.8 and 43.8 μM respectively, corresponding to 1.8 nM free Mg^{2+} and 0.46 nM free Ca^{2+}.

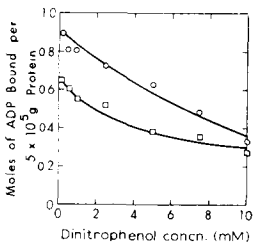
Fig. 7. Inhibition of ADP binding by actin (equilibrium dialysis). Expt. A: The dialysis bag contained 2.5 ml myosin solution at a concentration of 5 mg/ml, with increasing amounts of F-actin as indicated on the abscissa. The medium contained 0.6 M KCl, 1 mM $MgCl_2$, and 0.04 M Tris–HCl at pH 7.0, with 75 nmoles of [^{14}C]ADP per 7.5 ml. Dialysis was carried out at 3° for 20 h. Electrophoresis of the outer fluid after dialysis indicated that 10–13 % of the ADP had been broken down to AMP and IMP. The apparent binding of ADP must be corrected for the exchange of actin-bound ADP. Correction was made on the basis of two types of control experiments. One control set of tubes contained [^{14}C]ADP-labeled F-actin, with 75 nmoles of unlabeled ADP in the medium. The amount of label released from actin under these conditions results partly from the exchange of the actin-bound ADP with the ADP of the medium and partly from the release of the bound nucleotide. By measuring the maximum nucleotide release in a second set of control tubes which contained [^{14}C]ADP-labeled F-actin but without ADP added to the medium, the corrected value of net exchange was calculated. On the average 24 % of the actin nucleotide was released with, and 18 % without ADP in the medium, giving a corrected value of 6 % for the exchange. The ADP exchange when subtracted from the total ADP bound (○---○) yields the corrected value for ADP binding (●—●). Expt. B: Dialysis sacs containing 2.5-ml mixtures of myosin and actin in 0.6 M KCl (pH 7.0), were prepared as in Expt. A, and were dialyzed against 0.1 M KCl. Equilibrium dialysis was then carried out for 24 h at 3° against an outer volume of 5 ml. The medium contained 0.1 M KCl, 1 mM Tris–EDTA, and 14 mM Tris–HCl (pH 7.0). 8–13 % of the ADP was broken down to AMP and IMP. Values for the apparent (10.5 %) and corrected (3 %) exchange of actin-bound ADP were obtained as above. □---□, the ADP binding uncorrected for exchange; ■—■, the corrected binding. In the presence of EDTA, the maximum levels of contaminating Mg^{2+} and Ca^{2+} were 5.9 and 7.1 μM respectively, corresponding to 25 nM free Mg^{2+} and 0.16 nM free Ca^{2+}.

at a concentration of 2 mM; under the same conditions, 2 mM AMP, half of which was converted into IMP during the experiment, produced only 14.5 % reduction in the amount of bound ADP.

F-Actin can also displace ADP from myosin, the effect approaching a maximum at an actin/myosin weight ratio of 1:3 (Fig. 7). The dependence of the inhibition of ADP binding on actin concentration is similar in media containing either 0.6 M KCl and 1 mM $MgCl_2$ or 0.1 M KCl and 1 mM EDTA, in spite of the differences in the physical properties of actomyosin at high and low ionic strengths.

The incomplete inhibition of ADP binding produced by actin in the presence of 0.6 M KCl and 1 mM $MgCl_2$ may reflect a true equilibrium between actin, myosin, and ADP, or may be due to a slight underestimation of the exchange of actin-bound ADP under these conditions.

2,4-Dinitrophenol, a well known activator of myosin ATPase inhibits the binding of ADP to myosin. The concentration dependence of this effect is shown in Fig. 8.



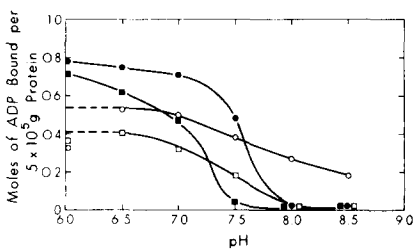


Fig. 8. Inhibition of ADP binding by dinitrophenol. 4-ml myosin suspension containing 4.5 mg myosin per ml, 0.015 M KCl, 5 mM EDTA, 10 μM [^{14}C]ADP, and dinitrophenol as indicated on the abscissa was adjusted to pH 7.0 and ADP binding was measured at either 3° (○—○) or 25° (□—□) as described for Fig. 2a. Protein remaining in the supernatant was determined following removal of dinitrophenol, as described in EXPERIMENTAL.

Fig. 9. Effect of pH, temperature, and dinitrophenol on ADP binding (2.5 mM CDTA, low ionic strength). 4-ml suspensions of myosin (5.6 mg/ml) containing 0.015 M KCl, 2.5 mM CDTA, 10 μM ADP and 5 mM buffer (sodium succinate at pH 6 and 6.5, Tris–HCl from pH 7 to 8.5) adjusted to the pH indicated on the abscissa. The ADP binding was measured as described in Fig. 8. ■—■, 3°, 5 mM dinitrophenol; ●—●, 3°, dinitrophenol omitted; □—□, 25°, 5 mM dinitrophenol; ○—○, 25°, dinitrophenol omitted.

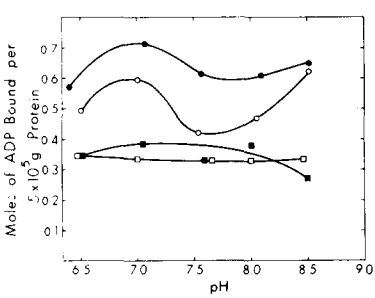


Fig. 10. Effect of pH, temperature, and dinitrophenol on ADP binding (1 mM $MgCl_2$, high ionic strength). The medium contained 0.6 M KCl, 33 mM buffer (sodium succinate at pH 6.5, Tris–HCl from pH 7 to 8.5), 1 mM $MgCl_2$, and 60 nmoles of [^{14}C]ADP per 6 ml. Dialysis was for 12 h at 3° or for 8 h at 25°. Control experiments showed that these times permitted equilibration. Further details were as follows: ○—○, 3°, 5 mM dinitrophenol; ●—●, 3°, dinitrophenol omitted; □—□, 25°, 5 mM dinitrophenol; ■—■, 25°, dinitrophenol omitted. The pH values plotted on the abscissa were measured in the external fluids after dialysis.

10 mM dinitrophenol reduces the binding of ADP by about 60 % in a solution of 0.015 M KCl, 5 mM EDTA (pH 7.0) at a temperature of 3 or 25°.

The ADP binding to myosin at low ionic strength (0.015 M KCl) in the presence of 5 mM EDTA, is markedly pH dependent (Fig. 9). The binding of ADP falls off above pH 7.0, the effect being more marked at 3° than at 25°. 5 mM dinitrophenol causes a decrease in the amount of ADP bound at either temperature and at any pH where binding occurs. The maximum effect of dinitrophenol occurs at pH 7.5 and 3°. In 0.6 M KCl, with 1 mM $MgCl_2$, the binding of ADP to myosin is nearly independent of pH in the range 6.5–8.5, at 3° or at 25° (Fig. 10). Under these conditions, 5 mM dinitrophenol has little or no effect on the ADP binding at 25° and only a small effect is observed at 3°.

The effect of dinitrophenol was reversed when dinitrophenol was removed by washing myosin three times in 0.06 M KCl at pH 7.0.

DISCUSSION

The requirements for the binding of ADP to myosin differ in important aspects from those previously established for the binding of PP_i.

At an ionic strength of 0.05 the binding of ADP to myosin was influenced only slightly by changes in the free Mg^{2+} concentration between 1 nM and 1 mM. The apparent independence of the ADP binding from added divalent cations at low ionic strength sharply contrasts the absolute divalent cation requirement of the PP_i interaction[3]. This difference between the two substrate analogues probably reflects the stabilizing influence of the adenine ring interaction on the binding of polyphosphates to myosin.

The apparent independence of myosin ADP binding from divalent cations is surprising in view of the universal cation dependence of enzymes acting on nucleoside polyphosphate substrate. The association constant for Mg^{2+} binding to myosin is high (Fig. 4) and it is possible that a further stabilization of the bound Mg^{2+} in a myosin–Mg^{2+}–ADP complex would protect the Mg^{2+} against removal by EDTA. Such stabilization was observed in systems containing actin[3] or PP_i (unpublished observations). The slight decrease in the affinity of ADP for myosin as evidenced by the decreasing slope of the Scatchard plot at very low Mg^{2+} concentrations might result from reduction of the bound divalent cation concentration.

It is unlikely that at low ionic strength, in the presence of 5 mM EDTA, monovalent cations participate in the ADP binding. Should this be the case, it would be expected that the affinity of ADP binding would depend on the nature of the monovalent cation present in the system. In fact, the amounts of ADP bound in the presence of varying concentrations of KCl, NaCl, LiCl, NH_4Cl, and CsCl were closely similar. Furthermore, the binding of ADP was markedly reduced in the presence of 5 mM EDTA by increasing the monovalent cation concentration from 0.04 to 0.8 M.

On the basis of these considerations, it is assumed that in the presence of 5 mM EDTA, at low ionic strength the binding of ADP occurs with the mediation of divalent cations. The decreased ADP binding with increasing monovalent cation concentration might represent competition between monovalent and divalent cations at the binding site.

With 5 mM EDTA, the binding of PP_i to myosin is strongly inhibited irrespective

of salt concentration. The inhibition of ADP binding by 1 mM PP_i in the presence of a chelating agent indicates that binding of PP_i still occurs although with severely reduced affinity.

In 0.6 M solutions of alkali-metal halides the binding of ADP to myosin is dependent upon divalent cations in a manner analogous to that reported for the binding of PP_i (ref. 3). Mg^{2+} is considerably more effective than Ca^{2+} in promoting the binding of ADP (Fig. 5; see also ref. 7). Scatchard plots of ADP binding at different levels of free Mg^{2+} concentration in a medium containing 0.6 M KCl clearly indicate that the apparent affinity of ADP binding increases as a function of the free Mg^{2+} concentration while the maximum number of ADP binding sites remains constant, 1.2–1.3 moles of ADP being bound per 500000 g of myosin. The linearity of Scatchard plots at free ADP concentrations ranging from approx. 0.1 to 50 μM suggests the existence of a single set of ADP binding sites. Since the maximum number of binding sites obtained by extrapolation in similar experiments using fresh myosin is about 1.6, in agreement with the findings of LUCK AND LOWEY[7], we assume the existence of two identical ADP binding sites per mole of myosin. The lower values of maximum binding described in Figs. 1 and 5 were obtained on myosin after storage in 50% glycerol at $-15°$.

The affinity constant of ADP decreases somewhat with increasing concentration of KCl even in the presence of 1 mM Mg^{2+}, reaching a value of about $4 \cdot 10^5$ at 0.6 M KCl concentration. This value is close to the dissociation constant of $1.4 \cdot 10^{-5}$, determined from the dependence of ADP-induced difference spectra of heavy meromyosin on ADP concentration[21].

The binding of ADP is effectively inhibited by PP_i .Nearly complete inhibition of ADP binding was also observed in the presence of stoichiometric concentrations of actin. An accurate assessment of the degree of inhibition at high actin concentrations is difficult due to the uncertainties connected with the measurement of the exchange of actin-bound ADP. These observations, taken in conjunction with the earlier reported inhibition of PP_i binding by actin[3] and by ADP[2,5] and with the known dissociating effect of PP_i (ref. 22) and ADP[2] on actomyosin suggest that the binding sites for actin, ADP, and PP_i overlap, and that ADP, under these experimental conditions, is not bound to a site uninfluenced by actin or PP_i.

While PP_i in concentrations equal to that of the ADP produces substantial inhibition of ADP binding in the presence of 1 mM $MgCl_2$, IDP under the same conditions was ineffective. This surprising finding implies that the interaction of the polyphosphate chain with myosin is actually weakened by the inosine moiety. The competition between IDP and ADP is not influenced significantly by the presence of a chelating agent. It is noteworthy, that the relative effectiveness of PP_i and IDP in inhibiting the ADP binding to myosin under different conditions closely parallels their relative effectiveness in producing viscosity decrease in actomyosin solutions[22].

2,4-Dinitrophenol, the well-known activator of the ATPase activity of myosin and mitochondria, inhibits the binding of ADP to myosin. Although the inhibition is moderate, it is reproducibly observed at both $3°$ and $25°$, and its magnitude is moderately influenced by the pH. In the presence of 5 mM EDTA the affinity of ADP for myosin decreases with increasing pH in the range of 6.5–8.0. The effect of pH is more pronounced at $3°$ than at $25°$. At low temperature, especially in the presence of 5 mM dinitrophenol, the ADP binding is virtually abolished at pH 8.0, when measured at a

free ADP concentration of 10 μM or less. In the presence of 1 M $MgCl_2$, the ADP binding is nearly independent of pH in the range of 6.5–8.5.

BLUM AND FELAUER[23] proposed many years ago that the rate of ATP hydrolysis is limited by the release of ADP from the active site, and that activators of myosin ATPase effect rate acceleration by promoting the dissociation of the myosin–ADP complex. In this context, it is of interest that conditions which permit a high rate of ATP hydrolysis markedly diminish the affinity of ADP for myosin. Such correlations were established in the following instances. (1) The well-known activation of myosin ATPase by EDTA requires KCl or NH_4Cl. The increase of EDTA-activated ATPase with increasing concentration of these monovalent cations closely mirrors the decrease in the affinity of ADP for myosin. The effect of increasing KCl concentration on the ADP binding cannot be the only contribution of K^+ or NH_4^+ to the hydrolytic process, since Na^+ also inhibits ADP binding in the presence of EDTA without activating myosin ATPase[24]. (2) Replacement of Mg^{2+} with Ca^{2+} as the principal divalent cation activates myosin ATPase 20 to 50 fold, while it reduces the affinity constant of ADP for myosin by about one order of magnitude, either at low ionic strength or in the presence of 0.6 M KCl. (3) Well-known activators of myosin ATPase (actin, dinitrophenol, PP_i) inhibit ADP binding. (4) The weak interaction of IDP with myosin in the presence of 1 mM $MgCl_2$, as evidenced by the data of Fig. 6, can be correlated with the high rate of ITP hydrolysis under similar conditions.

It is unlikely that in all these instances the observed activation of ATPase activity would result simply from the cancellation of product inhibition by ADP. It is probable, however, that underlying the observed change in the affinity constant of myosin for ADP is a marked increase in the rate constant for the dissociation of the myosin–ADP complex.

The inverse relationship in the Mg^{2+} requirement of ATPase activity and PP_i binding, and the consistently observed inhibition of PP_i binding by agents which activated myosin ATPase led us to the formulation of a working hypothesis in which the rate of ATPase activity was defined by the equilibrium of the triphosphate chain of ATP between the hydrolytic site and a non-hydrolytic regulatory area that required Mg^{2+} for interaction with polyphosphates[3]. Treating PP_i as an analogue of the triphosphate chain of ATP we assumed that under conditions of high ATPase activity the binding of the triphosphate chain to the regulatory site was weak, resulting in a shift of equilibrium in favor of the hydrolytic site. This hypothesis provided a simple explanation for the activation of myosin ATPase by EDTA, Ca^{2+}, or actin. The activation of ATP hydrolysis by PP_i or by high concentrations of ATP was explained by assuming that the activator polyphosphate itself was bound to the regulatory site.

There is independent evidence for the existence of a high affinity nucleotide binding site on myosin or its fragments where bound nucleotides might exert a regulatory effect on the rate of ATP hydrolysis. BURTON AND LOWENSTEIN[25] discovered that incubation of heavy meromyosin with ATP or ADP at low ionic strength in the presence of Mg^{2+} leads to the formation of a stable complex which contains 1–1.5 moles of nucleoside polyphosphate per mole of heavy meromyosin[26]. The nucleotide is not released from the complex by Sephadex chromatography or by treatment with anion-exchange resins, indicating a tight interaction. ATP while bound to the protein is protected from the ATPase activity of heavy meromyosin. The presence of actin or Ca^{2+} interferes with the formation of the heavy meromyosin–ATP complex. These

observations clearly suggest the existence of a high-affinity, non-hydrolytic site which binds nucleotide strongly in the presence of Mg^{2+} and whose affinity is reduced when actin or Ca^{2+} is present. These are the properties of the non-hydrolytic regulatory site outlined in our previous report.

The inverse relationship of the affinity of ADP binding to the rate of ATP hydrolysis resembles the relationship found with PP_i and can be interpreted in terms of the regulatory site hypothesis, considering ADP like PP_i as an analogue of ATP. However, in order to fit into this hypothesis, ADP should have relatively greater affinity than PP_i for the hydrolytic site of myosin, since activation of myosin ATPase by ADP was not observed, in fact ADP generally acts as an inhibitor of ATP hydrolysis[27].

IMAMURA, TADA AND TONOMURA reported[28] that the time-course of liberation of free ADP from a myosin–ATP system in the presence of 5 mM $MgCl_2$ did not show a rapid initial burst when measured as the rate of oxidation of NADH in the presence of pyruvate kinase and lactate dehydrogenase. Rapid initial release of both ADP and orthophosphate was found, however if the reaction was stopped with the addition of $HClO_4$ prior to the assay. These observations lead to the inevitable conclusion that during the initial rapid ATP hydrolysis about 1 mole of ADP is bound to a mole of myosin with no further change in ADP binding during the steady phase of ATPase activity. The ADP bound to myosin during the rapid initial phase might contribute to the establishment of the slower steady rate since the initial burst of P_i liberation by heavy meromyosin is inhibited by ADP[29]. This suggestion is consistent with the finding that activators of myosin ATPase inhibit the binding of ADP. In these terms, activation may be viewed as a prolongation of the initial rapid phase of hydrolysis. It is unclear whether the bound ADP acts in competition with the substrate or reduces the ATPase activity by interaction with some regulatory site.

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