THE BINDING OF Mn²⁺ AND ADP TO MYOSIN

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The metal ion requirement of myosin-ADP binding was investigated by use of Mn^{2+}. Mn^{2+} binds to two sets of noninteracting sites on myosin which are characterized by affinity constants of 10^6 and 10^3, M^{-1} at 0.016 M KCl concentration. The maximum number of sites is 2 for the high affinity and 20–25 for the low affinity set. Binding of Mn^{2+} to the high affinity sites increases the affinity of ADP binding to myosin.

F-actin inhibits ADP binding (Kiely, B., and Martonosi, A., Biochim. Biophys. Acta 172: 158–170 [1969]), but even at F-actin concentrations much higher than that required to saturate the actin binding sites of myosin or its proteolytic fragments, significant ADP binding remained. The actin insensitive portion of ADP binding was inhibited by 10^{-4} M inorganic pyrophosphate or ATP. The results are discussed on the basis of a model in which actin and ADP bind to myosin at distinct but interacting sites.

During muscle contraction ATP dissociates actomyosin followed by hydrolysis and the formation of a series of myosin-product complexes (1). The rate of product release is enhanced by actin which interacts with the crossbridges and initiates tension development (2).

A crucial but largely unknown aspect of this scheme is the relationship between the actin and nucleotide binding sites of myosin. As 2 moles of actin (3), ADP (4), ATP (5), and PPi (6, 7) are bound per mole of myosin, each myosin head is likely to contain one actin and one nucleotide binding site. The actin and ADP binding display different metal ion requirements (4, 6) and involve different functional groups, permitting selective chemical modification of each set of sites (8–11). As actomyosin is readily dissociated by ATP (12), and F-actin decreases the affinity of ADP (4), PPi (6), and ATP (5) to myosin, the actin and the nucleotide binding sites of myosin interact with each other in a functionally significant manner.

As a first step toward the elucidation of the nature of this interaction we investigated the effect of F-actin upon the equilibrium constant of ADP binding to myosin and its fragments under conditions which may give information about some aspects of the physiological process. We also extended our previous studies (4) on the interaction of divalent metal ions with myosin by measuring the binding of Mn^{2+}. Mn^{2+} in view of its paramagnetic properties offers interesting possibilities in the study of the structure of enzyme-metal-substrate and enzyme-metal-product complexes (13).

METHODS

All techniques used in this work are described in detail in earlier publications from our laboratory (4, 6) and therefore only a brief summary is presented.

Myosin was isolated from predominantly white leg muscles of the rabbit as described earlier (4). Tryptic heavy meromyosin (HMM) was prepared by the method of Lowey and Cohen (14) and the S1 fragment according to Young et al. (15), or Margossian and Lowey (3).

Actin obtained from acetone-dried muscle powder was purified by three cycles of polymerization, centrifugation, and depolymerization (16). The unbound nucleotides were removed from F-actin solutions by treatment with Dowex-1-X2-100 resin (17) or by extensive dialysis against three changes of 0.1 M KCl-5 mM imidazole. It was ascertained that under the conditions of the experiment no significant exchange of bound nucleotide occurred (4, 18).

For measurement of Mn^{2+} binding, the myosin was freed from contaminating metal ions as described earlier (4). Mn^{2+} binding was measured by equilibrium dialysis (4), ultra-centrifuge transport (19), or EPR spectroscopy (19). ^{54}Mn isotope was used in all experiments except the EPR studies. Radioactivity measurements were performed on a Packard model 3001 spectrometer (Packard Instrument Co., Downers Groves, Ill.), or a Packard model 3320 liquid scintillation counter.

The binding of ADP to myosin was measured by equilibrium dialysis (4), ultra-centrifuge transport (19), or centrifugation of precipitated myosin at low ionic strength (4).

The binding of F-actin to myosin or its fragments was determined by the increase in $90°$ scattering upon addition of F-actin to solutions of myosin or its fragments (20), or by analyzing the concentration of unbound enzyme in the presence of increasing concentrations of actin in the analytical ultracentrifuge (3).

Previously reported techniques (4) were used for the measurement of protein concentration and ATPase activity.

MATERIALS

[8-^{14}C]-ADP, ^{54}Mn, and ^{45}Ca were obtained from New England Nuclear, Boston, Mass. All other reagents were of analytical grade.

RESULTS AND DISCUSSION

The Binding of Mn^{2+} to Myosin

At low (Fig. 1) and at high ionic strength (Fig. 2) the binding of Mn^{2+} to myosin occurs at two sets of noninteracting sites with widely different affinities. The maximum number of high affinity binding sites (n_1) at both ionic strengths is about two, in reasonable agreement with the number of high affinity sites reported previously for Mg^{2+} (4), ADP (4), ATP (5), and PPi (6, 7) binding to myosin. The affinity constant of Mn^{2+} binding to these sites is 1.5×10^6 M^{-1} at low ionic strength (Fig. 1), and $0.8 - 3.6 \times 10^5$ M^{-1} at high ionic strength. There is evidence that the high affinity sites are involved in the binding of ADP to myosin (see below). The number of low affinity sites is less well defined but it is likely to be greater than 20–25, with affinity constants of 2×10^3 M^{-1} at low and

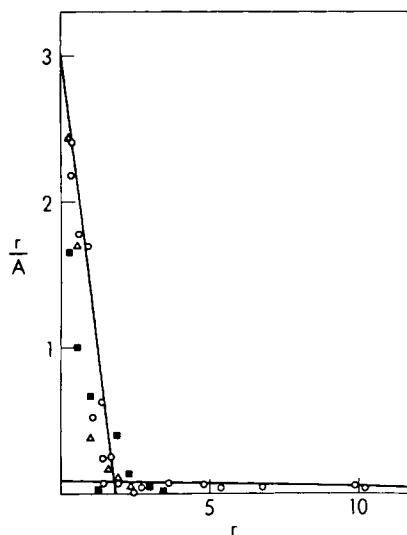Fig. 1. Mn^{++} binding to myosin at low ionic
strength. The binding of $^{54}Mn^{2+}$ was measured
in 0.05 M Tris-HCl buffer and 0.016 M KCl, pH
7.4, at 2–5°C by the centrifuge transport method.
○—○ no addition; ■—■ 1.1 × 10^{-4} M ADP;
△—△ 1.1 × 10^{-4} M inorganic pyrophosphate
(from ref. 30).

0.5×10^3 M^{-1} at high ionic strength. Similar low affinity sites were also observed with
other divalent metal ions. The functional significance (if any) of the low affinity sites
is unknown.

Effect of Mn^{2+} Concentration Upon the Binding of ADP to Myosin

The binding of ADP to myosin in media of high ionic strength requires Mg (4),
while at a KCl concentration of 0.05 M significant ADP binding occurs even in the presence
of 5 mM EDTA (4). The data of Fig. 3 show a similar relationship between Mn^{2+} concen-
tration and ADP binding. At low ionic strength the binding of ADP was not influenced
significantly by varying the Mn^{2+} concentration, while at high ionic strength there was a
sharp increase in the stability of ADP binding at Mn^{2+} concentrations close to 10^{-5} M,
which presumably reflects the saturation of high affinity Mn^{2+} binding sites. The increase
in the stability of ADP binding by Mn^{2+} is accompanied by inhibition of ATPase activity
(21). Mn^{2+} under similar conditions also inhibits the rate of dissociation of myosin-ADP
complex (22).

Interestingly, N-ethylmaleimide (NEM), an SH group reagent which inhibits ATPase
activity and at higher concentrations the binding of ADP to myosin (23, 24), left the
Mn^{2+} binding unaffected up to an NEM/myosin mole ratio of 1,000. This clearly indicates
that the demonstrated SH group involvement in product (23, 24) and substrate binding
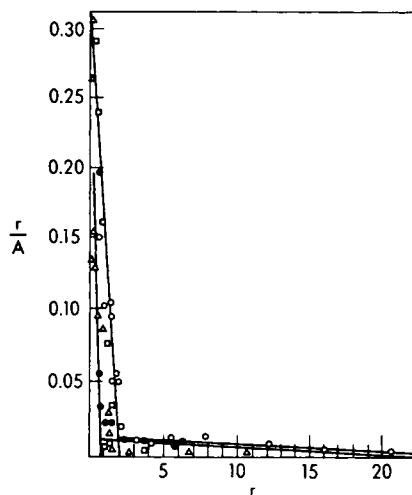(25) is not related to the binding of the stabilizing metal ion.

Fig. 2. The binding of Mn^{2+} to myosin in 0.6
M KCl. The binding was measured by EPR
(○, ●) or by equilibrium dialysis (△, ❑) methods.
For EPR measurements myosin (14.9–15.7 mg
protein per ml) was dissolved in solutions con-
taining 0.6 M KCl and $2 \times 10^{-5} - 6 \times 10^{-3}$ M
$MnCl_2$, pH 7.4 (○). In some experiments (●) myosin
was inactivated by exposure to 37°C for 1 hr in
0.6 M KCl prior to the EPR measurements.

    The equilibrium dialysis studies were performed
using $^{54}MnCl_2$ at 2–5°C in the presence of 0.6
M KCl and 0.05 M Tris-HCl, pH 8.0 (❑), or pH
7.4 (△) (from ref. 30).

Competition Between Mn^{2+} and Other Divalent Metal Ions

Mg^{2+} is bound to two high affinity sites on myosin with affinity constants greater
than 2×10^6 (4), and several lines of evidence indicate that Mg bound at these sites has an
important physiological role in energy transduction during muscle contraction (2).
Although Mg^{2+} competitively inhibits the binding of Mn^{2+} to myosin at low free Mn^{2+}
concentrations, where primarily the high affinity sites are saturated, the dissociation con-
stant of Mg^{2+} calculated from this competition is unexpectedly high, close to 1 mM
(Fig. 4). Ca^{2+} and other divalent metals, which on the basis of ATPase activity measure-
ments (21) bind less tightly to myosin than Mg^{2+}, were surprisingly effective com-
petitors for the Mn^{2+} binding (Fig. 5).

    It is possible that under the conditions of the experiments, in spite of the rather
low free Mn^{2+} concentrations, most of the Mn^{2+} binding occurred at the low affinity
sites. Alternatively, if Mg^{2+} and Mn^{2+} are bound at the same site, the configuration of
bound Mn^{2+} is such as to make competition by Mg^{2+} and Ca^{2+} less effective.
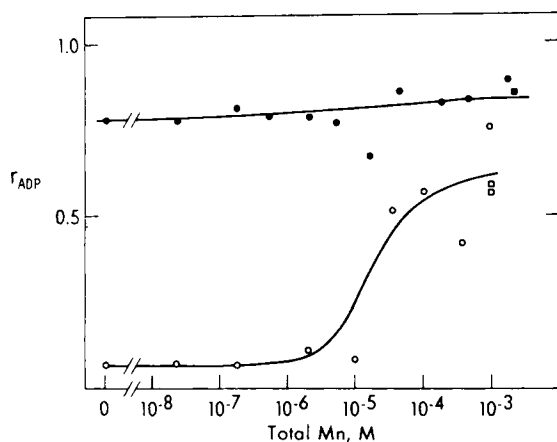
Fig. 3. The effect of Mn^{++} upon the binding of ADP to myosin. The binding of ADP was measured at low ionic strength by the centrifugation method in a medium of 0.05 M KCl, 0.009 M Tris, pH 7.0, and 4.4×10^{-6} M ADP (●). In control experiments Mn was replaced by 2.2×10^{-3} M $MgCl_2$ (■).

At high ionic strength the ADP binding was measured by equilibrium dialysis in a medium of 0.6 M KCl, 0.03 M Tris, pH 8.0, 3.3×10^{-6} M ADP. In some experiments (□) $MnCl_2$ was replaced by 1 mM $MgCl_2$ (from ref. 30).
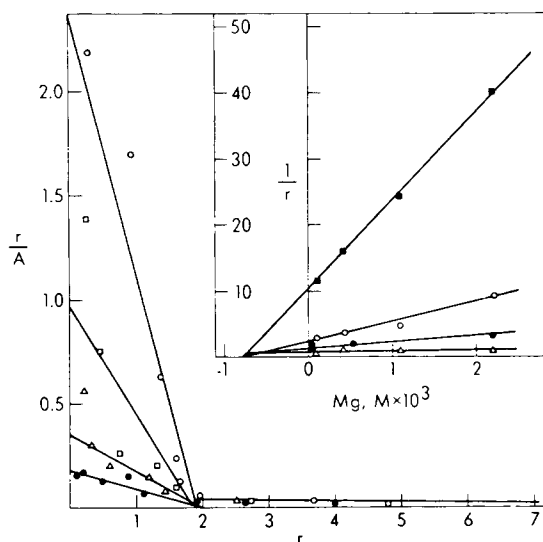

Fig. 4. Competition between Mg^{++} and Mn^{++}. The Scatchard plots represent data obtained at low ionic strength. ○---○, no Mg; ●——●, 1.1×10^{-3} M $MgCl_2$; △——△, 2.2×10^{-4} M $MgCl_2$; □——□, 4.4×10^{-5} M $MgCl_2$.

The Dixon plots (inset) show similar data at the following total $MnCl_2$ concentrations: ■——■, 4.4×10^{-7} M; ○——○, 2.2×10^{-6} M; ●——●, 5.5×10^{-6} M; △——△, 3.3×10^{-5} M (from ref. 30).
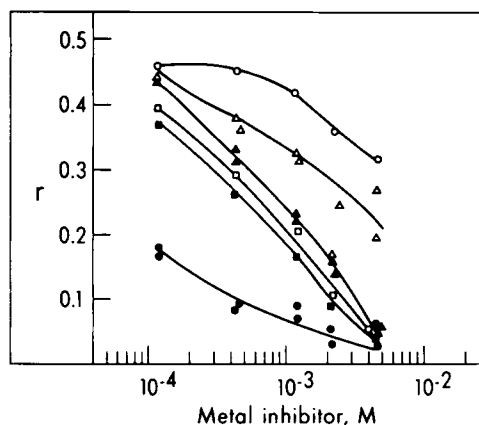
Fig. 5. The effect of divalent metal ions upon the binding of Mn^{++}. The medium contained 0.05 M Tris, pH 7.4, 0.05 M KCl, 2.2×10^{-6} M $^{54}MnCl_2$, 2.12 mg myosin/ml, and the various divalent metal ions in the concentrations indicated. ●——●, $CaCl_2$; ■——■, $CoCl_2$; □----□, $MgCl_2$; ▲——▲, $ZnCl_2$, △——△, $SrCl_2$; ○----○, $NiCl_2$ (from ref. 30).

The Effect of F-Actin Upon the Binding of ADP to Myosin and Its Fragments

Kiely and Martonosi (4) observed that F-actin inhibits the binding of ADP to myosin but even at a relatively high F-actin to myosin ratio, which was sufficient to saturate the myosin with actin, significant ADP binding remained.

This observation was further developed recently in collaboration with Dr. M. Beinfeld. In agreement with previous findings, addition of F-actin to myosin, HMM, or S1 fragments at 0.015–0.05 M KCl concentration inhibited ADP binding (Fig. 6). The inhibition reached a maximum at an actin to enzyme ratio which provided saturation of the actin binding sites and further increase in F-actin concentration caused little or no change in ADP binding. Similar observations were made at 0.3 M (not shown) and at 0.6 M KCl concentration (Fig. 7). The saturation of the actin binding sites of myosin was reached at an actin to myosin mole ratio of 2. The corresponding mole ratios for HMM and S1 were close to 2 and 1, respectively, as determined by ultracentrifuge transport.

The actin-insensitive portion of ADP binding is not attributable to dissociation of actomyosin, since ADP up to 100 μM concentration had little effect upon the steady-state rate of actin-activated ATP hydrolysis, the light scattering of actomyosin or actomeromyosin solutions (Table I), or upon the concentration of actomeromyosin or acto-S1 complexes measured by ultracentrifuge transport (Table I). 10^{-4} M inorganic pyrophosphate or ATP inhibited the actin-insensitive ADP binding.

These data clearly establish simultaneous binding of F-actin and ADP to myosin, leading to the formation of actomyosin-ADP complex. The inhibitory effect of F-actin
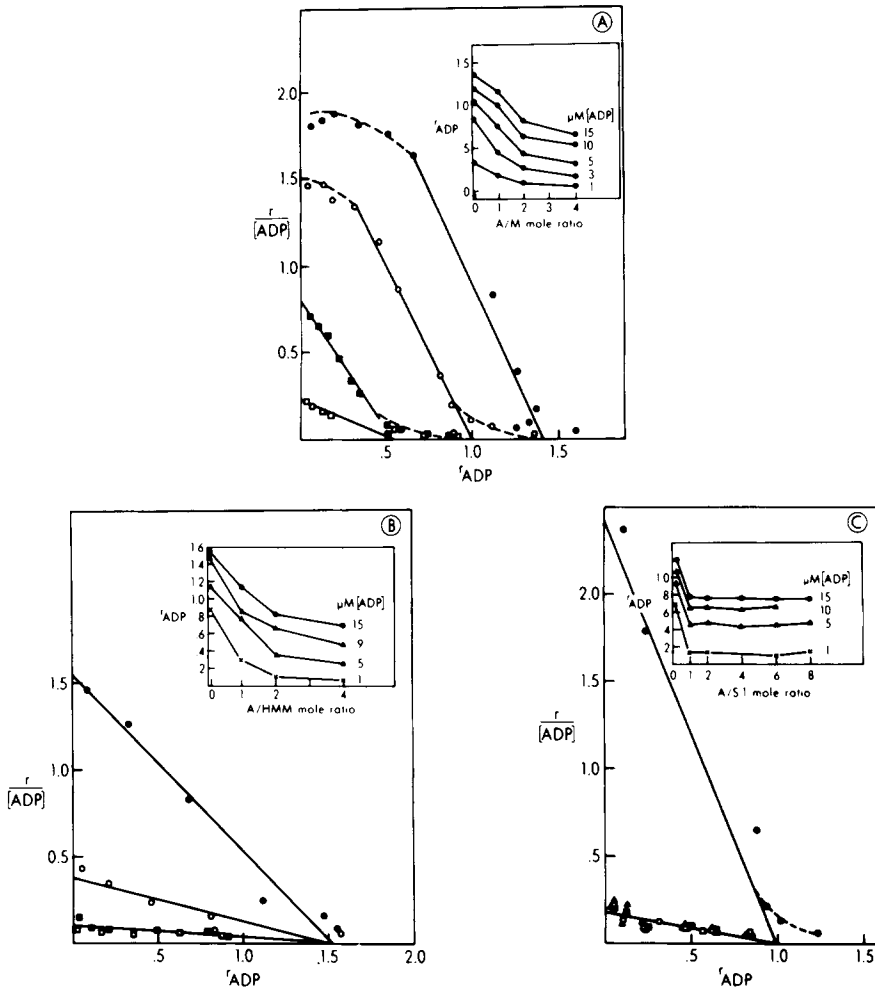
Fig. 6. The effect of F-actin upon the binding of ADP to myosin and its fragments at low ionic strength. A. The binding of ^{14}C ADP to myosin was measured by the precipitation technique (4), in a medium of 0.015 M KCl, 1.3 mM imidazole, and 0.6 mM $MgCl_2$. ●——●, myosin alone; ○——○ actomysin at actin to myosin (A/M) ratio of 1.0, ■——■ actomyosin, A/M ratio 2; □——□ actomyosin, A/M ratio 4.0. Free ADP concentration is expressed in μM throughout. The insert figures represent the number of moles of ADP bound per mole of myosin at the indicated free ADP concentrations as a function of A/M ratio. B. H meromyosin. The ^{14}C ADP binding was measured by ultracentrifuge transport in a medium of 0.05 M KCl, 50 mM Tris, pH 8.0, and 1 mM $MgCl_2$. ●——●, HMM alone, ○––○ actin and HMM at an A/HMM ratio of 1.0; ■——■ actomeromyosin, A/HMM ratio of 2; □——□ actomeromyosin, A/HMM ratio of 4; △——△ actomeromyosin A/HMM ratio of 5; △——△ actomeromyosin A/HMM ratio of 6. Free ADP concentration is expressed in μM. The inset figures illustrate the effect of actin upon the binding of ADP to HMM at the indicated free ADP concentrations. C. Subfragment I. The conditions were essentially identical to those under B. ●——●, S1 alone; ○——○ actin and S1 at an A/S ratio of 1; ■——■, A/S-1 ratio of 2; □——□, A/S-1 ratio of 4; △ A/S-1 ratio of 6; △ A/S-1 ratio of 8. Free ADP concentration is expressed as μM. The inset figures represent the number of moles of ADP bound per mole of S1 at the indicated free ADP concentrations as a function of actin/S1 ratio.
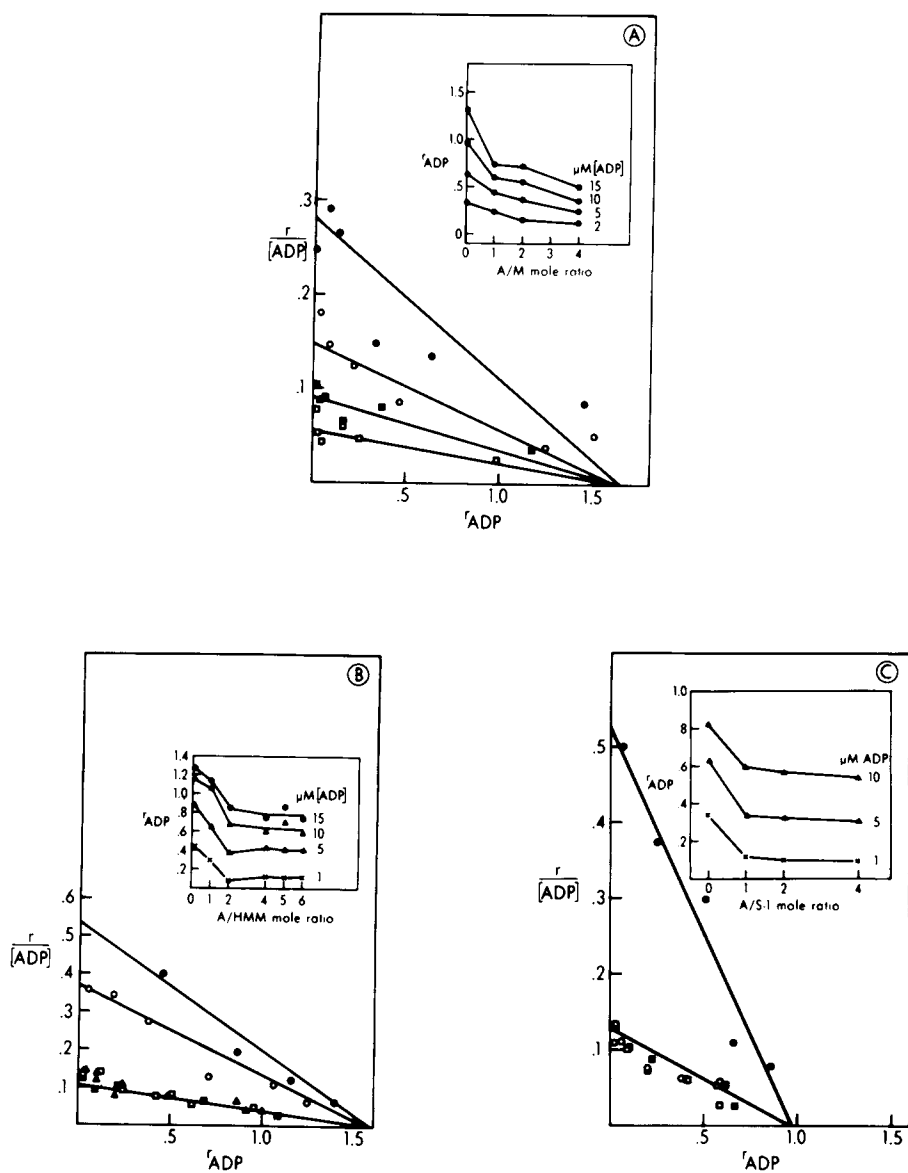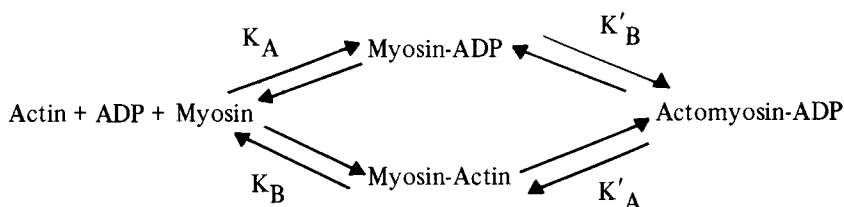
Fig. 7. The effect of F-actin upon the binding of ADP to myosin (A), HMM (B), and subfragment 1 (C) at high ionic strength. The binding was measured by ultracentrifuge transport in a medium of 0.6 M KCl 50 mM Tris, pH 8.0, and 1 mM MgCl₂. For other details see legend to Fig. 6.

TABLE I.   The Binding of Actin to S_1, HMM, and Myosin in the Presence of ADP

	Light scattering		Ultracentrifuge	
	Acto HMM (A/HMM = 2.0)	Actomyosin A/M = 2.0	Acto-S_1 (A/S_1 = 0.8)	Acto HMM (A/HMM = 1.3)
ADP concentration mM	% HMM bound	% Myosin bound	% S_1 bound	% HMM bound
0	86	100	80	40
0.1	76	88	82.5	46.6

The interaction of actin with S_1 and with HMM were measured in the Model E analytical ultracentrifuge in a medium of 0.6 M KCl, 50 mM Tris, pH 8.0, and 1 mM $MgCl_2$. Light-scattering studies on acto-HMM and actomyosin were performed in a medium of 0.6 M KCl, 44 mM Tris HCl, pH 8.0, and 1.5 mM $MgCl_2$. It is assumed that the differential scattering attributable to actomyosin formation is proportional to actomyosin concentration. The actin-to-enzyme ratios are given in parentheses.

upon the ADP binding may be explained by negative interaction between the actin and the nucleotide binding sites, according to the following scheme:

$$\text{Actin + ADP + Myosin} \begin{array}{c} \xrightarrow{K_A} \text{Myosin-ADP} \xrightarrow{K'_B} \\ \xrightarrow{K_B} \text{Myosin-Actin} \xrightarrow{K'_A} \end{array} \text{Actomyosin-ADP}$$

K_A, K_B, K'_A, and K'_B denote affinity constants. Our data would require that binding of F-actin to myosin lower the affinity of ADP binding by about one order of magnitude ($K_A > K'A$); the affinity of F-actin is not influenced detectably by ADP (20).

Alternative models based upon differences in the intrinsic affinity and actin sensitivity of the ADP binding sites located on the two heads of a myosin molecule are also possible. Although such models are supported by earlier indications of binding site heterogeneity (26—28), they require special assumptions to fit the data and therefore, at least at this moment, appear less plausible.

Simultaneous binding of actin and ADP to myosin was also inferred from experiments on glycerinated muscle fibers by Marston and Tregear (29).

The physiological significance of actin-insensitive ADP binding in living muscle is not known, as the bound ADP of actomyosin may be readily displaced by ATP.

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