Equilibrium Binding of Adenosine Diphosphate to Myosin*

Susan Lowey and Seta Mahakian Luck

ABSTRACT: Equilibrium dialysis has been used to determine the number of adenosine diphosphate binding sites in the myosin molecule and in its tryptic fragment, heavy meromyosin. From spectrophotometric measurements of the free adenosine diphosphate concentration in 0.5 M KCl, 0.1 M Tris-HCl, and 1 mM $MgCl_2$ (pH 7.7) at 259 mμ, we found 1.8 ± 0.2 sites per 5×10^6 g of myosin, with an average intrinsic association constant of 1.4×10^5 M^{-1}. Slightly lower values were obtained for heavy meromyosin. Replacement of Mg by Ca or EDTA significantly reduced the ADP binding. It was shown that the presence of trace contaminants of myokinase and 5′-adenylic acid deaminase can lead to erroneously high values for the number of binding sites. To minimize such errors, it was necessary to purify myosin both by cellulose phosphate chromatography and ammonium sulfate fractionation.

Electron microscopy, in conjunction with hydrodynamic studies on myosin and its subfragments, has shown that myosin consists of two globular subunits attached to a rodlike tail (Slayter and Lowey, 1967; Lowey *et al.*, 1969). Such a structure raises the question of the functional significance of two globular entities. Does each globule possess sites to which ATP and actin can bind, and if so, are these two subunits equivalent?

It has been known for some time that when the globules are separated from the rod by proteolytic enzymes, both of the globular fragments are able to complex with actin. This property has, in fact, been used to isolate the globular fragments from the digestion mixture (Mueller, 1965; Jones and Perry, 1966). More recently, Young (1967a) demonstrated by ultracentrifugal transport experiments that 1 mole of the globular subfragment binds to 1 mole of F-actin monomer. He also showed by equilibrium dialysis, as well as by transport experiments, that 1 mole of heavy meromyosin subfragment 1 binds 1 mole of ADP (Young, 1967b). Measurements of ATP binding to the whole myosin molecule by gel filtration chromatography provided additional support for the concept of two ATPase sites (Schliselfeld and Bárány, 1968).

Although these results would seem to show unequivocally that each globule in myosin has one actin binding site and one nucleotide binding site, other data are at variance with these conclusions: A maximum value of *three* sites was obtained by transport studies on the binding of ADP to heavy meromyosin (Young, 1967b), whereas a minimum value of *1* mole ATP or ADP bound per mole of heavy meromyosin was indicated by the difference spectra at 280–290 mμ (Morita and Yagi, 1966; Morita, 1967; Sekiya and Tonomura, 1967). Earlier investigators of the binding of ATP to myosin also reported about one ATP binding site in the myosin molecule (Nanninga and Mommaerts, 1960). Low binding ratios can perhaps be accounted for by myosin's well-known instability and tendency to lose ATPase activity on ageing. This may explain the range in reported pyrophosphate binding sites from 1.4 (Kiely and Martonosi, 1968) to 1.8 per myosin molecule (Nauss *et al.*, 1969). A previous study by some of these authors gave only one pyrophosphate binding site in myosin (Gergely *et al.*, 1959), but this low value has now been attributed to the use of a phosphate buffering system in the earlier work (Nauss *et al.*, 1969). Experiments suggesting more than two ATPase sites are harder to reconcile with our structure of myosin, particularly in view of the finding that myosin is composed of primarily two chemically similar subunits (Weeds and Hartley, 1967).

The aim of the present work is twofold: (1) to show that trace contaminants of 5′-adenylic acid deaminase and myokinase may give spuriously high values for the binding of ADP to myosin when the nucleotide concentration is determined spectrophotometrically; and (2) to demonstrate that close to 2 moles of ADP are bound to purified myosin and heavy meromyosin when artifacts from contaminating enzymes are eliminated and inactivation of myosin is avoided.

A brief description of this study has been presented previously (Luck and Lowey, 1968).

Materials and Methods

Myosin was prepared from rabbit muscle as previously described (Holtzer and Lowey, 1959). To remove trace quantities of 5′-adenylic acid deaminase, the myosin was passed through a freshly prepared 4 × 60 cm cellulose phosphate (Sigma) column equilibrated and eluted with 0.5 M KCl–0.04 M Tris-HCl (pH 7.8) essentially as described by Harris and Suelter (1967). The myosin is not retained under these conditions and passes through the column as a single peak with some trailing. The recovered protein (about 75% of that applied) was concentrated by the addition of an equal volume of neutral, saturated $(NH_4)_2SO_4$ (1 mM in EDTA) to 50% saturation. The precipitated myosin was resuspended in 1 M KCl and dialyzed against 0.5 M KCl, 1 mM $MgCl_2$, and 0.1 M Tris-HCl (pH 7.7). In some experiments (see text), 0.5 M NaCl was substituted for KCl, or 1 mM $CaCl_2$ or 2 mM EDTA replaced $MgCl_2$. In one experiment, the myosin was not purified with phosphocellulose

* From The Children's Cancer Research Foundation and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts. *Received February 20, 1969.* These studies were supported in part by Research Grants AM-04762 and AM-10630 from the National Institutes of Arthritis and Metabolic Diseases, and FR-05526 from the Division of Research Facilities and Resources, National Institutes of Health.

but only fractionated with $(NH_4)_2SO_4$ to 50% saturation. This procedure removes most of the contaminating myokinase, which remains in the supernatant (Kress *et al.*, 1966). Myosin prepared by these methods continued to migrate as a single peak in the analytical ultracentrifuge.

Heavy meromyosin was prepared by the method of Lowey and Cohen (1962). The myosin was digested for 10 min, after having been freed from 5′-adenylic acid deaminase and myokinase as described above. The heavy meromyosin was either dialyzed against 0.5 M KCl–1 mM $MgCl_2$–0.1 M Tris-HCl (pH 7.7) or 0.05 M KCl was substituted when a lower ionic strength was desired.

Protein concentrations were determined by the micro-Kjeldahl procedure using a nitrogen factor of 6.2.

The ATPase activity of the proteins was assayed at 25° and pH 7.9, as described by Perry (1960). The incubation mixture contained 0.25 M KCl, 2.5 mM ATP, 5 mM $CaCl_2$, 50 mM Tris-HCl, and 0.1 mg of protein in a total volume of 2 ml. The reaction was initiated by the addition of ATP and terminated by adding 1 ml of 15% trichloroacetic acid. Inorganic phosphate was measured by the Fiske and Subbarow method.

Na_2ADP and NaAMP were purchased from Sigma. The purity of the nucleotides was ascertained by ascending paper chromatography in isobutyric acid–NH_4OH–H_2O (66:1:33, v/v) at pH 3.7 as described in the Pabst Laboratories Circular. By this method Sigma ADP showed 2–3% AMP within a week after receipt. A sample of ADP from Nutritional Biochemicals Corp., which was several months old, gave 12% AMP by this criterion. All nucleotides were stored over silica in a desiccator at −15°. However, even under optimal storage conditions ADP can decompose to AMP at the rate of 1%/month.

Difference spectra of the proteins in the presence of nucleotides were obtained on a Cary Model 15 recording spectrophotometer using matched pairs of 1-cm quartz cylindrical cells in tandem. One of the two cells of the sample pair contained about 0.1% protein plus 1×10^{-4} M nucleotide while the other cell contained dialysate. The reference pair had one cell filled with protein and the other with nucleotide at the same concentrations as those present in the sample pair. After zeroing the cells at 340 mμ, the spectral difference was recorded from 340 to 240 mμ. The pairs of cells were matched to within 0.01 optical density unit within this range. Solutions were prepared with sufficient precision to ensure differences of less than 0.01 optical density unit between duplicates.

Dialysis cells were constructed out of Lucite with a 3-ml central well, according to the specifications of commercially available cells from Techni-Laboratory Instruments, Calif. The cells were boiled successively in 1% $NaHCO_3$, 0.05 M HCl, 0.05 M EDTA, and 50% ethanol to remove all ultraviolet-absorbing material. The dialysis membranes were cut from viscose tubing and exposed to the same treatment as the cells. When the assembled cells were filled with water and shaken for 48 hr, no ultraviolet-absorbing material was released.

In a typical experiment, five cells were set up with varying concentrations of ADP in one half and protein dialysate in the other half; a second set of five had exactly the same concentrations of nucleotide on one side and a constant amount of protein (usually about 10 mg in 1 ml) on the other. Solutions were delivered into the cells through fine polyethylene tubing from an all-glass AGLA syringe (Burroughs Wellcome Co.) attached to a micrometer. A stock solution of 1×10^{-3} M ADP, together with appropriate amounts of dialysate, was delivered into the cells to give final concentrations of approximately $3–10 \times 10^{-5}$ M ADP in a total volume of 1 ml. Equilibrium was reached after gently shaking the cells for 24 hr at 4°. After equilibration the nucleotide solutions were withdrawn and read in 2-mm cells at 259 mμ in a Zeiss spectrophotometer. From the ADP concentration in the absence of protein and the corresponding concentration with protein present, the amount of bound nucleotide could be calculated (preliminary experiments showed that no binding took place to the membrane or the cell). The molar extinction coefficient for ADP was taken as 15.4×10^3 cm^{-1} at 259 mμ. The molar extinction coefficient for IMP was taken as 12.1×10^3 cm^{-1} at 248.5 mμ.

The data were calculated and plotted according to the equation: $\bar{\nu}/(ADP) = (n - \bar{\nu})k_a$, where $\bar{\nu}$ is the average number of moles of ADP bound per mole of protein, (ADP), is the free nucleotide concentration at equilibrium, k_a is the apparent intrinsic association constant, and n is the apparent number of equivalent binding sites on each protein molecule (Scatchard, 1949; Klotz, 1953). As $\bar{\nu}/(ADP) \rightarrow 0$, $\bar{\nu}$ approaches n. The molecular weight of myosin was taken as 500,000 and that of heavy meromyosin as 350,000 (Holtzer *et al.*, 1962).

Results

When the free nucleotide concentration is determined spectrophotometrically, as it was in this study, it is extremely important that there be no change in the absorption of the nucleotide solution other than that due to binding. We found that our usual method of purifying rabbit myosin was not adequate to remove the trace quantities of AMP deaminase and myokinase which interfere with the quantitative measurement of ADP concentration. Myokinase catalyzes the formation of AMP and ATP from 2 moles of ADP, while AMP deaminase catalyzes the transformation of AMP to IMP. Since inosine nucleotides absorb at a lower wavelength than adenine nucleotides, the optical density at 259 mμ is diminished.

The first two figures illustrate the effect of these enzymic impurities on the difference spectra of myosins with varying degrees of purity. If no chemical reaction occurred in the sample cell containing myosin plus nucleotide, the spectrum would not deviate by more than about 0.01 optical density unit from a straight line, such deviations being due to imperfections in the cells and volumetric errors. The large difference spectrum for myosin prepared by repeated precipitations at low ionic strength (Figure 1A) can be accounted for by assuming a complete conversion of ADP into IMP. If a solution of 10^{-4} M IMP is measured against 10^{-4} M ADP, a difference spectrum identical with that of Figure 1A is obtained. If the myosin is then further purified by precipitation with ammonium sulfate, most of the myokinase activity appears to be removed but deaminase activity remains (Figure 1B). The difference spectrum obtained with ADP is mainly due to AMP already present in the stock ADP solution. Of course, the experiment in Figure 1B exaggerates the effect of AMP contamination; unlike the ADP solution used for that spectrum, the ADP used in the equilibrium dialysis experiments usually contained no more than 2–3% AMP. However, we wish to emphasize that misleading data can be obtained unless one removes both the myokinase activity and the deaminase activity

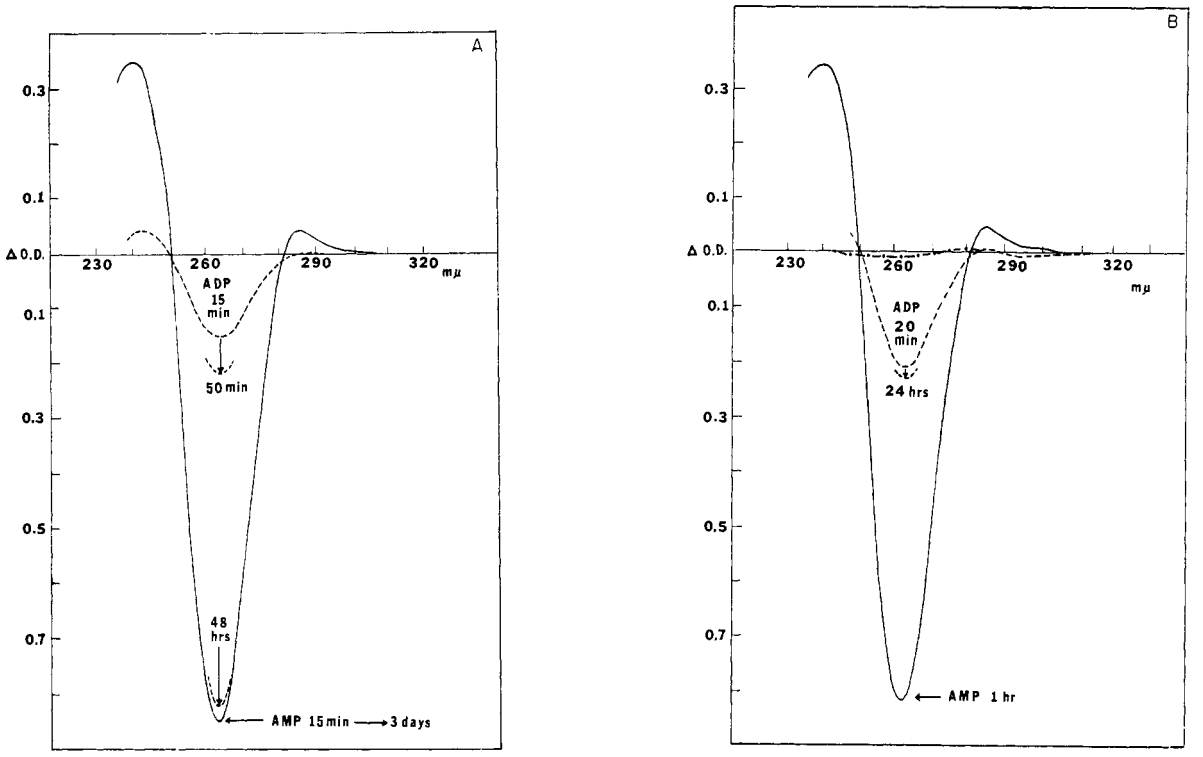

FIGURE 1: Difference spectra for myosin in ADP and AMP. The concentration of the myosin was approximately 1 mg/ml and the nucleotide concentration was 1×10^{-4} M. The solvent was 0.5 M KCl, 0.1 M Tris-HCl, and 1 mM $MgCl_2$ (pH 7.7). (A) Three-times-precipitated myosin in AMP (—). The difference spectrum was maximal 15 min after mixing and remained unchanged for 3 days. The final spectrum in ADP was identical with the previous one, but attained at a much slower rate (---). (B) The myosin used in these spectra had an additional fractionation step with 50% saturated $(NH_4)_2SO_4$. The myosin spectrum in AMP was identical with that in (A) but reached more slowly (—). The myosin spectrum in ADP did not change appreciably with time; the solution of ADP contained about 25% AMP (---). Myosin treated by cellulose phosphate chromatography and $(NH_4)_2SO_4$; the difference spectrum in ADP remained unchanged after 4 days (·····).

TABLE I: Representative Data on the Binding of ADP to Myosin.[a]

ADP Total $OD_{259}^{1\,cm}$	(ADP) Total (M × 10^5)	$\Delta OD_{259}^{1\,cm}$	(ADP) Bound (M × 10^5)	(ADP) Free (M × 10^5)	$\bar{\nu}$	$\bar{\nu}$/(ADP) × 10^{-5}
0.455	2.95	0.175	1.14	1.81	1.04	0.57
0.585	3.80	0.190	1.23	2.57	1.12	0.44
0.735	4.77	0.220	1.43	3.34	1.30	0.39
1.010	6.56	0.250	1.62	4.94	1.47	0.30

[a] Myosin concentration = 1.11%, solvent = 0.5 M NaCl–0.1 M Tris-HCl (pH 7.7)–1 mM $MgCl_2$.

associated with myosin. If myosin is purified by cellulose phosphate chromatography (Harris and Suelter, 1967) and ammonium sulfate fractionation, as described in the Methods section, essentially no difference spectrum is obtained in the presence of ADP or AMP over a period of several days (Figure 1B). Binding experiments were performed only on myosin preparations which displayed negligible difference spectra.

Some representative binding data are given in Table I. Least-mean-squares plots of similar data for myosin and heavy meromyosin under various solvent conditions are shown in Figure 2. Table II summarizes our results for the association constant and number of binding sites: we find an average value of 1.85 ± 0.2 sites per 5×10^6 g mole^{-1} of myosin with an average association constant of 1.4×10^5 M^{-1}.

Occasional binding values greater than 2 (see Table II) may be due to the incomplete removal of contaminants despite the precautions taken, whereas values of 1.4 may be ascribed to an inactivation of the active site leading to a loss of binding capacity. There appeared to be some correlation between n and the ATPase activity of myosin and heavy meromyosin; myosin preparations with a high value of n usually had an ATPase activity between 0.5 and 0.6 μmole of P_i per mg per min, whereas a decrease in activity usually meant a correspondingly lower value in the number of sites. This argument is particularly applicable to heavy meromyosin which, having undergone proteolytic degradation, is all the more likely to lose binding capacity. Although these explanations no doubt account for some of the scatter in the data, one must

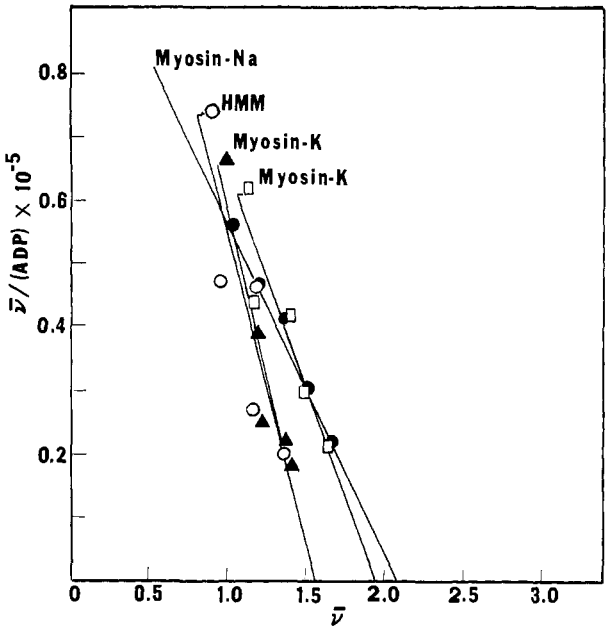

FIGURE 2: Experimental data for the equilibrium binding of ADP to myosin and heavy meromyosin. (○) 1.3% heavy meromyosin in 0.05 M KCl, 0.1 M Tris-HCl, and 1 mM $MgCl_2$ (pH 7.7); (▲) 1.46% myosin in same solvent as above but 0.5 M KCl; (□) 1.35% myosin in same solvent; and (●) 1.11% myosin with 0.5 M NaCl substituted for KCl.

TABLE II: Summary of Association Constants and Binding Sites for Myosin and Heavy Meromyosin.

Prepn Concn (%)	$k_a \times 10^{-5}$	n	Solvent[a]
		Myosin	
1.46	1.1	1.54	
1.35	0.43	1.91	
1.36	...	...	2 mM EDTA
1.30	0.054	2.17	1 mM $CaCl_2$
1.30	0.95	1.29	
1.13	7.0	1.44	
1.13	1.0	1.42	
1.11	0.54	2.02	0.5 M NaCl
1.00	0.49	2.16	0.5 M NaCl
0.95	0.43	2.58	
0.95	0.70	1.94	
		Heavy Meromyosin	
1.30	1.0	1.53	0.05 M KCl
1.30	1.4	1.39	

[a] Solvent = 0.1 M Tris (pH 7.7), 1 mM $MgCl_2$, and 0.5 M KCl unless otherwise specified.

also realize that there are considerable technical difficulties in measuring the binding of only two nucleotides to a molecule as large as myosin, especially when the binding is relatively weak.

It is well known that both the chelating agent, EDTA, and calcium ions activate the ATPase activity of myosin (Gergely, 1966). It was therefore of interest to see how these reagents would affect the binding of ADP to myosin. We find that EDTA completely suppresses the binding and calcium markedly reduces it (Table II). The substitution of sodium for potassium ions, or lowering the ionic strength of the medium, did not seem to affect the binding (Table II). Other investigators (Nauss *et al.*, 1969; Young, 1967b) have found a similar insensitivity to ionic strength in the binding of ligands to myosin.

Discussion

The purity of myosin becomes an important consideration in experiments dealing with the binding of nucleotides to the active sites of the molecule. Myosin prepared by repeated precipitations at low ionic strength and ammonium sulfate fractionation still contains the contaminating enzyme, 5′-adenylic acid deaminase, and perhaps traces of myokinase not entirely removed by the ammonium sulfate step. We have shown that the presence of these enzymes interferes with measurements of the binding of adenine nucleotides to myosin, and is probably responsible for those values greater than two obtained for the number of nucleotide binding sites in myosin (Young, 1967b). Additional purification of myosin by cellulose phosphate chromatography (Harris and Suelter, 1967) removes the adenylic acid deaminase activity and minimizes the slow conversion of adenine into inosine nucleotides. Equilibrium dialysis measurements of the binding of ADP to myosin and heavy meromyosin purified by these procedures have given an average value of two binding sites per mole of protein. This result agrees well with recent determinations of approximately two pyrophosphate sites (Nauss *et al.*, 1969) and two ATP binding sites (Schliselfeld and Bárány, 1968) in myosin. The value of the equilibrium constant is approximately an order of magnitude smaller than that found for ATP and pyrophosphate binding by these authors. The relatively weak binding is consistent with the fact that ADP is a less effective inhibitor of myosin ATPase than pyrophosphate and does not dissociate the actomyosin complex.

The present study avoids the uncertainties associated with measurements of ATP binding; ATP is slowly hydrolyzed by myosin under even the most inhibitory conditions. By using the competitive inhibitor, ADP, we have circumvented this difficulty and still measured the binding of an adenosine nucleotide. Although many techniques, including spectrophotometric and kinetic analyses, have been used in the past to determine the number of binding sites in myosin, equilibrium dialysis is the only method which provides a value under conditions of true thermodynamic equilibrium.

The finding of two nucleotide sites in myosin is compatible with the known structure of the molecule. Each of the two globular entities visualized in the electron microscope (Slayter and Lowey, 1967) contains one site for the nucleotide and one site for interactions with actin (Young, 1967a). The interdependence of these sites is evidenced by the effect of actin on the enzymic activity of myosin: In the presence of Mg ions, ATP is tightly bound to heavy meromyosin (Szent-Györgyi, 1968) and little hydrolysis takes place. The addition of actin reverses this inhibition and activates the Mg–ATPase of heavy meromyosin (Leadbeater and Perry, 1963). Accompanying this activation by actin is the release of the bound ATP from

heavy meromyosin (Szent-Györgyi, 1968). Similar effects of actin on bound ADP (Szent-Györgyi, 1968) and pyrophosphate (Nauss *et al.*, 1969) have been observed. Conversely, when ATP or pyrophosphate is added to acto-heavy meromyosin at low ionic strength or actomyosin at higher ionic strength, the polymer dissociates into its constituents (Eisenberg and Moos, 1968; Gergely, 1956). Therefore, despite the physical separation of the actin and the nucleotide binding sites on each globular subunit of myosin (Bárány, 1959), interactions at one site influence the reactivity of the other site.

Mg seems to play an important role in all these interactions: The replacement of Mg by Ca or EDTA suppresses the binding of the polyphosphates while at the same time increasing the ATPase activity of myosin (Kiely and Martonosi, 1968). A plausible explanation for the activating effect of EDTA on the enzymic activity of myosin is its ability to chelate trace amounts of Mg which would otherwise inhibit the ATPase activity (Offer, 1964). Whether the divalent cations bind to a distinct site on myosin, or whether they exert their influence by forming a stereochemically specific complex with the polyphosphate portion of the nucleotide is not known.

The presence of a second subunit does not seem to be an essential requirement for either the ATPase activity or the actin activation of myosin. The isolated proteolytic fragment, heavy meromyosin subfragment 1, displays a high ATPase activity in the presence of Ca and EDTA and, like heavy meromyosin, is activated by high concentrations of actin (Eisenberg *et al.*, 1968; Lowey *et al.*, 1969). These experiments do not, however, exclude the possibility of interactions between the subunits. Young's (1967a) work has suggested that only one of the two subunits of heavy meromyosin may be able to bind to F-actin at any given time. Whether this inhibition results from steric hindrance between actin and heavy meromyosin, or whether a more complicated sequence of events is occurring remains to be elucidated.

References

Bárány, M. (1959), Symposium on Sulfur and Proteins, New York, N. Y., Academic, p 317.

Eisenberg, E., and Moos, C. (1968), *Biochemistry 7*, 1486.

Eisenberg, E., Zobel, C. R., and Moos, C. (1968), *Biochemistry 7*, 3186.

Gergely, J. (1956), *J. Biol. Chem. 220*, 917.

Gergely, J. (1966), *Ann. Rev. Biochem. 35*, 691.

Gergely, J., Martonosi, A., and Gouvea, M. (1959), Symposium on Sulfur in Proteins, New York, N. Y., Academic, p 297.

Harris, M., and Suelter, C. H. (1967), *Biochim. Biophys. Acta 133*, 393.

Holtzer, A., and Lowey, S. (1959), *J. Amer. Chem. Soc. 81*, 1370.

Holtzer, A., Lowey, S., and Schuster, T. (1962), The Molecular Basis of Neoplasia, Austin, University of Texas Press, p 259.

Jones, J. M., and Perry, S. V. (1966), *Biochem. J. 100*, 120.

Kiely, B., and Martonosi, A. (1968), *J. Biol. Chem. 243*, 2273.

Klotz, I. M. (1953), *Proteins 1*, 727.

Kress, L. F., Bono, V. H., Jr., and Noda, L. (1966), *J. Biol. Chem. 241*, 2293.

Leadbeater, L., and Perry, S. V. (1963), *Biochem. J. 87*, 233.

Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4*, 293.

Lowey, S., Slayter, H. S., Weeds, A. G., and Baker, H. (1969), *J. Mol. Biol. 42*, 1.

Luck, S. M., and Lowey, S. (1968), *Federation Proc. 27*, 519.

Morita, F. (1967), *J. Biol. Chem. 242*, 4501.

Morita, F., and Yagi, K. (1966), *Biochem. Biophys. Res. Commun. 22*, 297.

Mueller, H. (1965), *J. Biol. Chem. 240*, 3816.

Nanninga, L. B., and Mommaerts, W. F. H. M. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1155.

Nauss, K. M., Kitagawa, S., and Gergely, J. (1969), *J. Biol. Chem. 244*, 755.

Offer, G. W. (1964), *Biochim. Biophys. Acta 89*, 566.

Perry, S. V. (1960), *Biochem. J. 74*, 94.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Schliselfeld, L. H., and Bárány, M. (1968), *Biochemistry 9*, 3206.

Sekiya, K., and Tonomura, Y. (1967), *J. Biochem. (Tokyo) 61*, 787.

Slayter, H. S., and Lowey, S. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1611.

Szent-Györgyi, A. G. (1968), Aspects of Cell Motility, London, Cambridge University, p 17.

Weeds, A. G., and Hartley, B. S. (1967), *J. Mol. Biol. 24*, 307.

Young, D. M. (1967a), *Proc. Natl. Acad. Sci. U. S. 58*, 2393.

Young, D. M. (1967b), *J. Biol. Chem. 242*, 2790.