

Effect of Adenosine Di- and Triphosphates on the Stability of Synthetic Myosin Filaments[†]

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ABSTRACT: Synthetic myosin filaments are dissociated in the presence of low concentrations (1–5 mM) of ATP, ADP, and relevant analogs. Filaments prepared under identical solvent conditions from headless myosin (rod) show similar behavior. Plots of $\log K$ vs. $\log [A]$ for the monomer \rightleftharpoons polymer equilibrium reaction of myosin and rod in the presence of the ligand (A) indicate that destabilization of the fila-

A number of workers have searched for a well-defined conformational change in myosin induced by substrate binding or as a consequence of ATP cleavage, but results to date appear to rule out any *gross* alteration in the secondary or tertiary structure. Early reports of conformational changes in myosin upon its reaction with ATP as detected by optical rotatory dispersion and ultraviolet absorption spectrophotometry have not been confirmed by more recent studies using the same techniques (Gratzer and Lowey, 1969). Moreover, in two separate investigations of hydrogen exchange of heavy meromyosin (Hartshorne and Stracher, 1965; Segal and Harrington, 1967) no detectable difference in secondary structure was observed as a result of its interaction with ATP. Differential velocity sedimentation studies of myosin in the presence of substrate or of the inactive competitive inhibitor, pyrophosphate, have also failed to detect changes in the hydrodynamic behavior of the molecule (Gratzer and Lowey, 1969; Godfrey and Harrington, 1970a). However Seidel and Gergely (1971) have recently reported a shift in the electron spin resonance spectrum of spin-labeled myosin during the early stages (1–5 min) of ATP hydrolysis which they interpret as resulting from a local conformational change which persists into the steady-state phase of the reaction.

In the studies cited above, ionic conditions were such that myosin exists in a dispersed monomer \rightleftharpoons dimer state (Godfrey and Harrington, 1970b; Herbert and Carlson, 1971). It seems possible that any alteration in structure in individual myosin molecules during interaction with substrate, requires that these molecules be in their associated state in the filament and that some type of cooperative interaction is required to generate the conformational changes responsible for cross-bridge movement and tension development. Although one could suppose that the expected conformational change requires coupling of the cross-bridge (subfragment I) structure with actin, a recent preliminary report suggests that movement of cross bridges occurs on excitation at regions of the thick filament not overlapped by actin (Haselgrove, 1970).¹

In the work to be presented below we have investigated the

ment results from the binding of one to two ligand molecules per monomeric protein unit. Sedimentation studies of the intact filament during the steady-state-cleavage reaction of ATP show no significant changes in gross hydrodynamic properties. Results suggest that binding of a small number of nucleoside phosphate molecules to specific sites within the filament core may be relevant to contraction.

sedimentation and binding behavior of synthetic myosin filaments in the presence of ATP and ADP in an attempt to detect conformational changes on binding of these substrates which might be reflected in the hydrodynamic or association properties of the macrostructure. As we will demonstrate, the gross hydrodynamic properties of the filament remain unchanged during the steady-state hydrolytic reaction. However our studies do reveal the presence of one to two binding sites in the rod segment of myosin which exert a profound effect on the stability of the filaments at low substrate concentration.

Materials and Methods

Glass-distilled water was used throughout; inorganic salts and reagents were analytical grade. The adenosine triphosphate (P-L Biochemicals) was freed of Ca^{2+} by passage over an ion-exchange resin, either Chelex-100 (Bio-Rad Laboratories) or Dowex 50 (Baker's reagent) as described by Seidel and Gergely (1963).

Myosin and Myosin Polymer Preparations. Rabbit myosin was prepared as described previously (Godfrey and Harrington, 1970a). Solutions of myosin filaments were prepared as described by Josephs and Harrington (1966) except that the final dialysis step in 0.5 M KCl (prior to dialysis at low ionic strength) was carried out at the pH desired for the specific filament preparation (either pH 8.3 or 7.0). Veronal or Tris-HCl was used as a buffer for the pH 8.3 filament system and bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane, synthesized according to Lewis (1966), was used for all filament preparations at pH 7.0. This buffer has a pK of about 6.8 at 10°.

Myosin rod was prepared employing soluble myosin and insoluble papain (Kominz *et al.*, 1965; Lowey *et al.*, 1969). The procedure followed in the present study has been fully described elsewhere (Harrington and Burke, 1972).

Tryptic light meromyosin² preparations followed the procedure of Mihalyi and Harrington (1959) with no further purification steps. The myosin to trypsin ratio used was 127 to 1 (mg/mg) and digestion times of 4, 8, and 15 min were used to prepare LMM of differing lengths.

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¹ As reported by H. E. Huxley.

² Abbreviation used is: LMM, light meromyosin; PNP, imidodiphosphate.

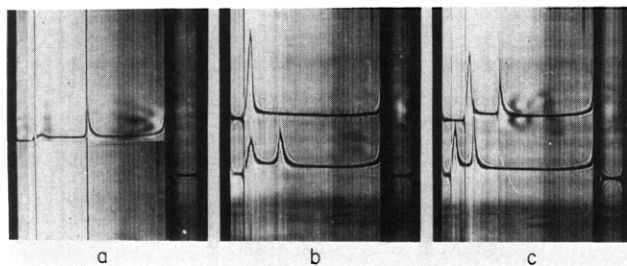


FIGURE 1: Velocity sedimentation patterns of synthetic myosin filament in the presence of ATP and ADP. (a) Myosin filament in the absence of added ligand. Solvent is 0.10 M KCl–0.05 M Tris (pH 8.3). Protein concentration 0.35%; temperature = 6°; 24,000 rpm. Time of centrifugation, 64 min; bar angle 70°. (b) Myosin filament in the presence of ligand. Upper: wedge cell, solvent as in (a) plus 5×10^{-3} M ATP– 10^{-5} M Ca^{2+} ; lower: regular cell, solvent as in (a) plus 5×10^{-3} M ADP– 5×10^{-3} M PO_4^{2-} – 10^{-5} M Ca^{2+} ; protein concentration 0.35%; temperature 7°; 24,000 rpm. Time of centrifugation, 48 min; bar angle, 60°. (c) Myosin filament in the presence of ligand. Aliquots of solutions used in (b) after standing overnight at 4°. Temperature 7°; 24,000 rpm. Time of centrifugation, 32 min; bar angle 60°.

Concentration Determinations. Concentrations of myosin and myosin polymer solutions were determined spectrophotometrically. Filament systems were dissociated to monomeric myosin by gravimetric dilution to 0.5 M KCl. A PMQ II spectrophotometer was employed for these measurements and an extinction coefficient, $\epsilon_{280}^{1\%}$, of $5.50\%^{-1} \text{ cm}^{-1}$ was assumed (Godfrey and Harrington, 1970a). Concentrations of rod were determined using either the extinction $\epsilon_{278}^{1\%} = 2.00\%^{-1} \text{ cm}^{-1}$ or the Rayleigh interference optical system using a value of 39 fringes for a 1% solution (Harrington and Burke, 1972). Concentrations of LMM were determined by the Rayleigh interference optical system assuming 40 fringes equivalent to 1%.

The concentrations of adenosine triphosphate, adenosine diphosphate, and AMP-PNP were determined spectrophotometrically using $\epsilon_{260}^{\text{max}} = 15.4 \times 10^3$ (Morele and Bock, 1954) for all three samples. We are indebted to Dr. Ralph G. Yount for his kind gift of the ATP analog, adenylylimidodiphosphate (AMP-PNP).

Centrifugation Experiments. The Spinco Model E analytical ultracentrifuge was used at temperatures of 5°; photographic plates were analyzed using a Nikon Model 6C shadowgraph microcomparator.

In all cases double-sector Kel F cells, either 12 or 30 mm in path length, were employed. We used the Rayleigh interference optical system for determination of the myosin monomer concentration in the presence of polymer according to the procedure described by Josephs and Harrington (1966). The total concentration of protein was determined spectrophotometrically as described above and the polymer concentration calculated by difference. In most filament preparations an initial centrifuge run was performed and the above methods used to determine the monomer and polymer concentration in 30-mm path-length double-sector cells prior to initiating binding experiments.

Spectrophotometric Experiments. During spectrophotometric measurements of binding affinity the temperature was maintained at 7–11° by circulating water from a thermostatted bath through the jacket of the cuvet compartment of a Zeiss PMQ II spectrophotometer. Optical density readings were made at 310 nm. The linearity of the turbidity reading at 310 nm vs. protein concentration was checked for every filament

preparation before initiating a binding study. The turbidity of the myosin–filament equilibrium system obeyed Beer's Law over the protein concentration range 0.08–0.40%. Solutions of ligands were always made up to the ionic strength and the pH of the filament solvent system; filament concentrations ranged from 0.25 to 0.30%. Protein and solvent were each weighed into a cuvette and an initial optical density reading determined. The material to be added was then delivered from a Carlsberg pipet and mixed as quickly as possible with a plastic rod. In the case of addition of magnesium ion to the adenosine diphosphate or pyrophosphate–filament systems, additives were mixed prior to addition to the protein. In the experiments with adenosine triphosphate and in the case of the pH 7.0 filaments the optical density changes were monitored by a Bausch and Lomb VOM 10 linear recorder.

We were unable to generate a homogeneous system of LMM filaments under a variety of ionic conditions including those used in the preparation of myosin and rod filament systems. To determine the concentration range of ligand required for dissociation of the LMM filament system the clearing of the insoluble LMM upon addition of ADP or ATP (to the precipitated sample in a pH-Stat cell) was followed. The reaction was monitored by an Ole Dich recorder; the pH-Stat cell was maintained at 10° with a Haake bath.

Results

Effect of ATP and ADP on Filament Stability. Earlier work has shown that synthetic myosin filaments, prepared by lowering the ionic strength of 0.5 M KCl solutions of myosin by dialysis or dilution, have a close topological similarity to the native thick filaments of muscle (Huxley, 1963; Kaminer and Bell, 1966a,b; Josephs and Harrington, 1966). Filaments generated by dialysis near pH 8 are shorter ($L = 6300 \text{ \AA}$) than native thick filaments ($L \cong 16,000 \text{ \AA}$), but they have numerous irregular projections, a bare central region and diameter of 100–150 Å characteristic of the native structures. Length vs. number histograms derived from electron micrographs of these particles show a relatively sharp size distribution. In solution a single, well-defined polymer species is observed ($s_{20,w}^0 = 150 \text{ S}$) which has been shown to be in rapidly reversible equilibrium with monomeric myosin (Josephs and Harrington, 1968). A typical sedimentation velocity pattern of the equilibrium system prepared at pH 8.3 ($\mu = 0.15$) is presented in Figure 1a. It consists of two sedimenting boundaries: a hypersharp polymer peak with $s_{20,w} = 35 \text{ S}$ and a slower sedimenting "monomer" peak with $s_{20,w} = 6 \text{ S}$. When ATP ($5 \times 10^{-3} \text{ M}$) and Ca ($1 \times 10^{-5} \text{ M}$) is added to the equilibrium system and the resulting solution examined immediately in the ultracentrifuge, schlieren patterns reveal complete transformation of the hypersharp polymer peak into the slower sedimenting peak with sedimentation coefficient characteristic of monomer (Figure 1b, upper). ADP at an equivalent concentration also shifts the monomer \rightleftharpoons polymer equilibrium toward monomer (Figure 1b, lower) but the transformation is incomplete. At higher concentrations of ADP ($9 \times 10^{-3} \text{ M}$), however, a pattern similar to Figure 1b (upper) is observed.

Under the solvent conditions and temperature of these experiments the half-time of ATP hydrolysis was approximately 2 hr. When aliquots of the solutions examined in Figure 1b were allowed to stand overnight (at 4°), sedimentation velocity patterns of the ATP system (Figure 1c) showed partial regeneration of the filament species, but no time-dependent alterations in the ADP system. The pH of the solutions

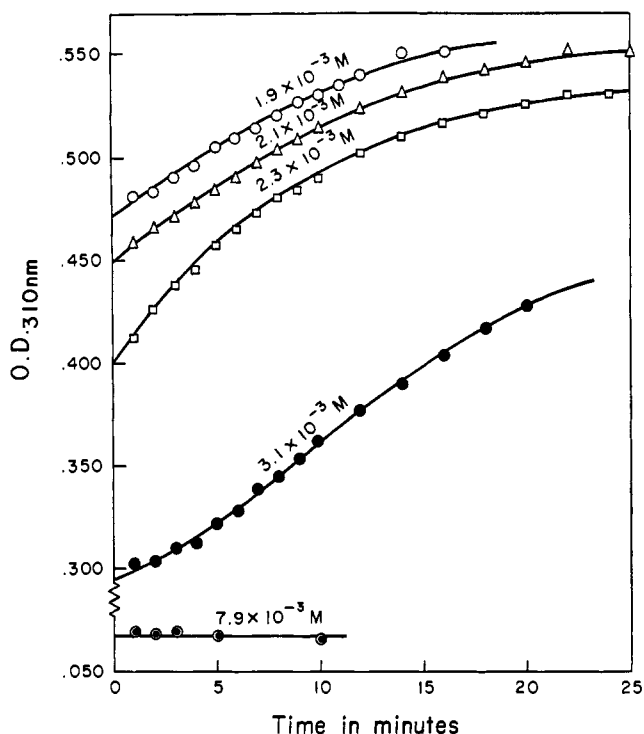


FIGURE 2: Time-dependent turbidity changes in a myosin filament system following addition of ATP. Individual curves are labeled with initial concentration of ATP. Initial $OD_{310} = 0.580$. Protein concentration 0.29%; solvent is 0.1 M KCl–0.05 M Tris (pH 8.3); temperature 10° . ATP solutions adjusted to pH 8.3 before addition.

was measured immediately after addition of the nucleoside phosphates and again after 24-hr incubation at 4° and was found to be invariant (within ± 0.03 pH unit) in the buffer system employed indicating that the changes observed were not related to alterations in the hydrogen ion concentration resulting from ATP cleavage. Sedimentation coefficients of the regenerated filament following completion of the ATP splitting reaction were indistinguishable from the untreated synthetic filaments at the same protein concentration.

In the presence of Mg^{2+} , ATP-dependent filament dissociation is depressed (see below) and it was therefore possible to investigate the sedimentation behavior of the hypersharp polymer peak under steady-state hydrolytic conditions. The sedimentation coefficient of polymer was measured at several protein concentrations, over the range 0.17–0.39%, in the presence of 7×10^{-3} M Mg^{2+} and 7×10^{-3} M ATP. In these experiments the monomer concentration remained invariant at $\sim 0.06\%$ and the area of the polymer peak (determined at low speed) increased with increasing protein concentration consistent with the behavior expected for a monomer–polymer system in rapid reversible equilibrium. The variation in sedimentation rate of the faster peak was found to obey the equation: $1/s = 0.0073 + 0.075c$; $s_{20,w}^0 = 140$ S where c is the concentration of polymer in grams per deciliter. The sedimentation *vs.* concentration dependence of the filament in the absence of $MgATP$ was determined in earlier work (Joseph and Harrington, 1968) giving: $1/s = 0.0066 + 0.075c$; $s_{20,w}^0 = 150$ S. Within the accuracy of the present measurements the gross hydrodynamic properties of the filament remain unchanged during the steady-state hydrolysis of ATP.

In the absence of Mg^{2+} , ATP cleavage was too rapid at lower concentrations of substrate to be followed by ultracentrifugation but the reaction could be monitored spectro-

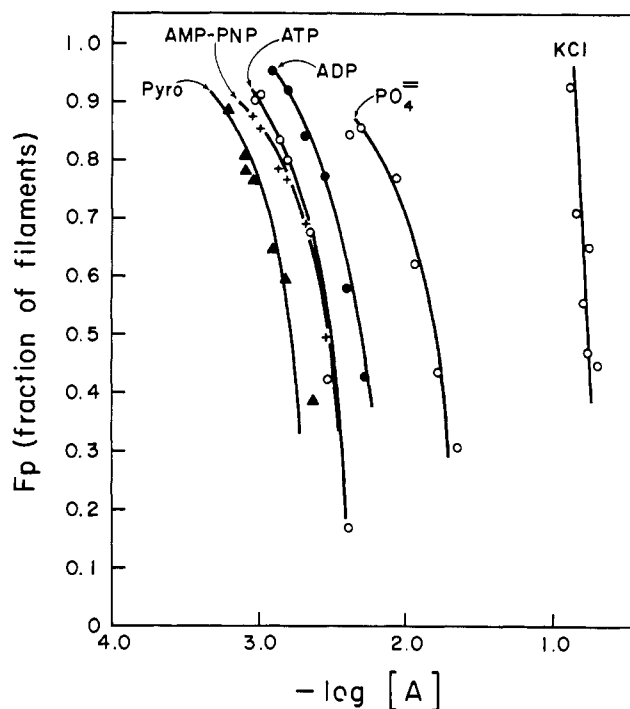


FIGURE 3: Fraction of filament *vs.* logarithm of ligand concentration. Solvent is 0.135 M KCl–0.002 M Veronal (pH 8.3). KCl data taken from Josephs and Harrington (1968); temperature 10° . See text for experimental procedure.

photometrically. As a result of the large molecular size of the synthetic filament, the turbidity of the equilibrium system can be related directly to the concentration of this species in solution (see Materials and Methods). Figure 2 shows time dependent changes in OD_{310} of the filament system after addition of ATP. It will be seen that the concentration of filament immediately following ATP addition falls to a level (<1 min) dependent on the concentration of the substrate, then gradually increases with time during the hydrolytic reaction. The rate of change of turbidity approximated the rate of ATP hydrolysis indicating that the filament was regenerated concomitant with the depletion of ATP. In these studies the amount of filament regenerated was also dependent on the initial concentration of ATP. With increasing concentration of ATP the elevation in concentration of the reaction products, ADP and inorganic phosphate, acts to lower the concentration of filament in equilibrium with monomer. It is conceivable that this process results from a change in the conformational state of the myosin molecules in the filament structure on binding nucleoside phosphate. Such a profound change in stability may also result from an increasing electrostatic repulsive force within the core of the filament resulting from binding of the highly charged ligands. It was therefore of interest to investigate the stoichiometry of binding of these substrates as well as other ATP analogs.

Stoichiometry of Substrate Binding at pH 8.3. Figure 3 presents plots of the apparent weight fraction of filament, derived from spectrophotometric measurements, of the myosin–polymer equilibrium (pH 8.3), *vs.* the logarithm of substrate concentration. Varying amounts of the substrates previously adjusted to pH 8.3 were added to solutions of the myosin polymer equilibrium in 0.135 M KCl and the absorption recorded at $\lambda = 310$ nm. Data for the time-dependent ATP transition was obtained by extrapolating OD_{310} *vs.* time plots,

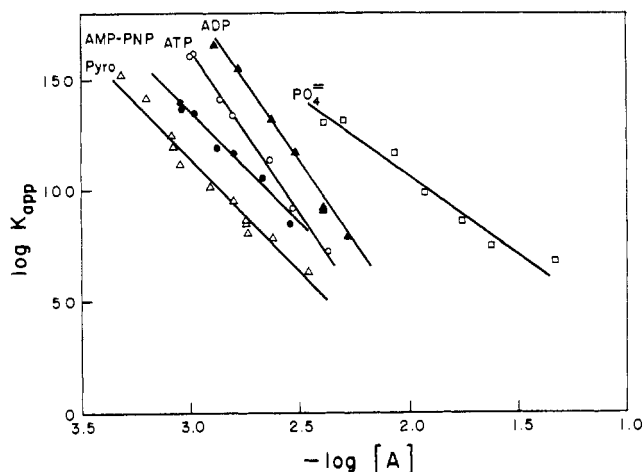
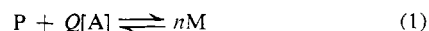


FIGURE 4: Plot of logarithm of apparent equilibrium constant *vs.* logarithm of ligand concentration for monomer \rightleftharpoons polymer equilibrium of myosin. Solvent is 0.135 M KCl–0.002 M Veronal (pH 8.3); temperature 10°.

similar to Figure 2, to zero time. A number of features of these plots will be noted. First, the filaments are dissociated by the nucleoside phosphate substrates in the millimolar concentration range. The effect of the substrates on the stability of the filaments parallels the binding constants of these substrates to myosin which have been reported by other workers, with the dissociation effectiveness of the substrates increasing in the order $\text{PO}_4^{2-} < \text{ADP} < \text{ATP} < \text{PP}_i$. All of the plots exhibit the sharp profiles characteristic of cooperative phase transitions. In the case of ATP, ADP, and PP_i the sharpness of the transition below $F_p < 0.2$ – 0.3 precluded measurements in this range, further emphasizing the cooperative nature of the dissociation. The ATP analog, AMP-PNP, dissociates the filament at a concentration and with F_p *vs.* $\log [A]$ profile indistinguishable from ATP. Yount *et al.* (1971) have recently demonstrated that AMP-PNP binds to the ATP binding site of myosin, but without hydrolysis of the PNP linkage. Thus these experiments are in agreement with the sedimentation studies in showing that the steady-state hydrolytic reaction has no apparent effect on the monomer–polymer equilibrium. From the work of Taylor and his colleagues the steady-state rate should be established within 1–2 sec under the enzyme and substrate conditions of the present study (Lymn and Taylor, 1970; Taylor *et al.*, 1970).

The data of Figure 3 have been analyzed according to the procedure used previously (Josephs and Harrington, 1968) for the myosin–polymer equilibrium system to ascertain whether the behavior observed involves stoichiometric reaction of the protein component with the substrate. We assume that the myosin association reaction can be expressed as



where n is the number of myosin monomers, P is the polymer (filament), and Q the number of moles of substrate participating in the equilibrium reaction per mole of polymer. Then

$$K = \frac{[\text{C}_m]^n}{[\text{C}_p][\text{A}]^Q} = K_{\text{app}} \frac{1}{[\text{A}]^Q} \quad (2)$$

According to this formulation the monomer protein species includes the bound ions which are released on polymerization.

We have taken the molecular weight of the filament to be 50×10^6 (Josephs and Harrington, 1966), and the molecular weight of the myosin monomer to be 4.6×10^5 (Godfrey and Harrington, 1970b; Herbert and Carlson, 1971; Gershman *et al.*, 1969). Values of Q/n , the number of moles of substrate bound to the monomeric species, have been derived from the slopes of linear plots of $\log K_{\text{app}}$ *vs.* $\log [A]$ (Figure 4) and are given in Table I. Results indicate that the binding of one to two molecules of substrate per myosin molecule are sufficient to dissociate the filament at pH 8.3. Our estimate of the apparent binding number assumes the filament to be in rapid, reversible equilibrium with monomer. This is likely to be an oversimplified view in the light of recent studies demonstrating the presence of a monomer–dimer equilibrium in high-salt media. Preliminary evidence J. S. Barton and W. F. Harrington, unpublished) indicate that this equilibrium is shifted toward increased formation of dimer on lowering the salt concentration to the threshold level for filament formation (0.2 M KCl, pH 8.0). Consequently the slower sedimenting peak in Figure 1a may well be a monomer–dimer reaction boundary. If the low molecular weight species in solution were entirely dimer, the binding number reported here would represent the number of ligand molecules associated with this molecular unit.

A separate study of the effect of ADP on the monomer filament system was carried out using the ultracentrifuge to compare to the binding results obtained spectrophotometrically. Solutions of the filament system containing various concentrations of ADP were centrifuged at low speed (24,000 rpm) to resolve the monomer and polymer peaks and the concentration of monomer was determined from the fringe count using the Rayleigh optical system. A plot of $\log K_{\text{app}}$ *vs.* $\log [\text{ADP}]$ was linear providing a binding number (1.3) identical with that obtained from the spectrophotometric studies. It will be noted that in these experiments we are measuring the binding of substrate in a concentration range ($\sim 10^{-3}$ M) which would normally not be accessible to the conventional equilibrium dialysis technique, since the bound ligand represents an extremely small fraction of the concentration of that species in solution. The sensitivity of the binding measurement is increased manifold through the effect of the bound ligand on the filament stability.

The profiles of Figure 3 have also been analyzed for cooperative binding according to the well-known Hill equation (see, for example, Van Holde, 1971).

$$\bar{v} = \frac{K[\text{A}]^\beta}{1 + K[\text{A}]^\beta} \quad (3)$$

where \bar{v} is the average number of ligand, A, bound per macromolecule, K , the association constant and β , the Hill coefficient. We assume that the fraction of specific binding sites occupied by substrate and responsible for dissociation is the fraction of protein in solution in the “monomeric” state; then it follows that

$$\frac{\theta}{1 - \theta} = \frac{[\text{sites occupied}]}{[\text{sites vacant}]} = \frac{[\text{C}_m]}{[\text{C}_p]} \quad (4)$$

$$\frac{\theta}{1 - \theta} = K[\text{A}]^\beta \quad (5)$$

Plots of $\log [\theta/(1 - \theta)]$ *vs.* $\log [A]$ were linear over the range of substrate concentrations which were measured yielding

TABLE I: Summary of Filament-Ligand Interaction Parameters.

Filament Monomer	pH	Solvent	Ligand	Binding No. (Q/n)	Hill Coef (β)	Midpoint of Transition ($-\log [A]$)
Myosin	8.3	0.135 M KCl	ATP	1.3	2.3	2.5
Myosin	8.3	0.002 M Veronal				
Myosin	8.3	0.1 M KCl	ATP	1.7	2.3	2.5
Myosin ^a	8.3	0.05 M Tris				
Myosin	8.3	0.135 M KCl	ADP	1.3	2.6	2.3
Myosin	8.3	0.002 M Veronal				
Myosin	8.3	0.135 M KCl	ADP	1.3	2.3	2.3
Myosin	8.3	0.002 M Veronal				
Myosin	8.3	0.135 M KCl	AMP-PNP	0.93	1.7	2.5
Myosin	8.3	0.002 M Veronal				
Myosin	8.3	0.135 M KCl	Pyrophosphate	0.96	1.8	2.8
Myosin	8.3	0.002 M Veronal				
Myosin	8.3	0.135 M KCl	Phosphate	0.67	1.4	1.8
Myosin	8.3	0.002 M Veronal				
Rod	8.3	0.17 M KCl	ATP	1.0	1.9	2.9
Rod	8.3	0.002 M Veronal				
Rod	8.3	0.17 M KCl	ADP	0.70	1.8	2.6
Rod	8.3	0.002 M Veronal				
Myosin	7.0	0.1 M KCl	ATP	~ 2.6	5.9	2.0
Myosin	7.0	0.05 M Bis-Tris ^c				
Myosin	7.0	0.1 M KCl	ADP	~ 2.6	5.9	1.8
Myosin	7.0	0.05 M Bis-Tris				
Myosin ^b	8.3	0.002 M Veronal	KCl	9.1	4.5	0.75

^a Ultracentrifuge data. ^b Data from Josephs and Harrington (1968). Plotted assuming monomer molecular weight of 4.6×10^5 .

^c Bis-tris = bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

the Hill coefficient presented in column 6 of Table I. All of the interacting systems show a high degree of binding cooperativity ($\beta \gtrsim 2$), with the exception of inorganic phosphate ($\beta = 1.4$), in keeping with the sharpness of the F_P vs. $\log [A]$ profiles near the midpoint of the two-state transition. A highly cooperative filament \rightleftharpoons monomer transition also occurs in the presence of KCl alone (see Figure 2 and Table I) as was shown in earlier work (Josephs and Harrington, 1968). This transition, however, takes place over a concentration range of KCl about 50-fold higher ($pK_a = 0.75$) than that observed in the presence of ATP and requires the binding of about 9 moles of KCl/monomer unit.

The possibility that dissociation of the filament in the presence of the highly charged anions is simply an ionic strength rather than an ion-specific effect has been considered. If this were so, plots of $\log K$ vs. μ or F_P vs. μ for all ions would be expected to fall along a common curve. Such plots show, however, a monotonic decrease in filament concentration with increasing ionic strength for each ion species, but the slopes as well as the effective range in μ observed for the various families of curves are markedly different. Complete filament dissociation is observed at an ionic strength near 0.176 M in the presence of pyrophosphate but values of $\mu \sim 0.23$ –0.24 M are required for KCl alone or the KCl–Na₂HPO₄ solvent system. Moreover, it will be noted that the F_P vs. $\log [A]$ profile obtained in the presence of pyrophosphate is displaced significantly with respect to the profile observed with

AMP-PNP or ATP (see Figure 3). Since these systems have identical ionic strengths, the results argue against a simple ionic strength effect. This conclusion is also supported by the behavior of the filament system in the presence of ATP (Figure 1b,c). Cleavage of ATP results in an increase in the ionic strength of the medium, yet this process leads to partial regeneration of the filament.

Effect of Added Magnesium. At low Mg concentration ($\sim 10^{-4}$ M) no effect was observed on the F_P vs. $\log [A]$ profile in the presence of ADP or PP_i. At concentrations of $Mg^{2+} = \sim 10^{-3}$ M, the F_P vs. $\log [A]$ profile for PP_i was shifted toward higher substrate concentrations (~ 0.35 log unit), but the estimated binding number remained unchanged.

Stoichiometry of Substrate Binding at pH 7. The binding number, Hill coefficient and midpoint of the filament-monomer transition in the presence of ATP and ADP have also been determined (see Table I) at pH 7. Synthetic myosin filaments prepared by dialysis of 0.5 M KCl myosin preparations vs. 0.1 M KCl at this pH show a rather broad size distribution (Huxley, 1963; Kaminer and Bell, 1966a; Josephs and Harrington, 1966) with lengths varying between 0.3 and 2 μ and the dissociation parameters, which have been determined by turbidity measurement, are, therefore, not as convincing as those reported for the pH 8.3 system. Dissociation of the filaments requires higher nucleoside phosphate concentrations than the equilibrium system formed at pH 8.3 with mid point of the transition near a ligand concentration

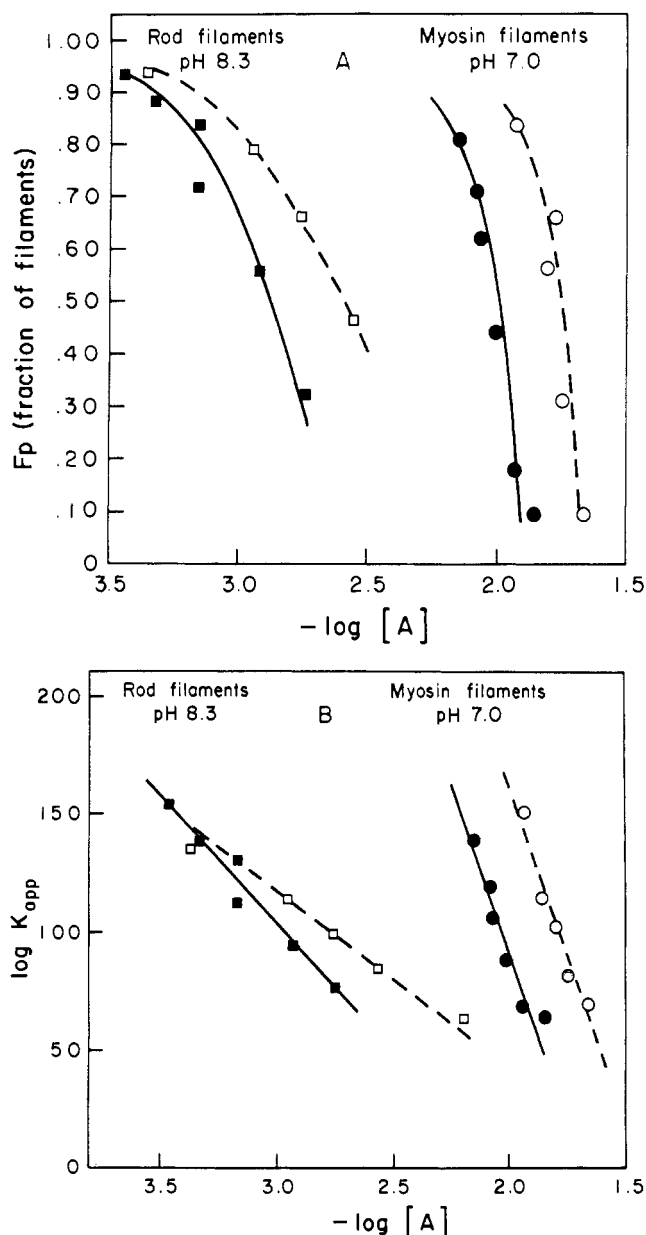


FIGURE 5: (A) Fraction of filaments *vs.* logarithm of ligand concentration. Solvent for rod filaments is 0.17 M KCl–0.002 M Veronal (pH 8.3). (■) ATP, (□) ADP. Solvent for myosin filaments is 0.135 M KCl–0.05 M Tris-bis (pH 7.0); (●) ATP; (○) ADP. (B) Plot of logarithm of apparent equilibrium constant *vs.* logarithm of ligand concentration for monomer \rightleftharpoons polymer equilibrium. Solvents and symbols as in part A; temperature 10°.

of 10^{-2} M. At pH 7 the apparent binding number is elevated ($Q/n \cong 2.5$) and the Hill coefficient indicates substantially increased cooperativity in ligand binding.

Effect of ATP and ADP on Filaments Prepared from the Rod Segment of Myosin. Information presented in the previous sections suggests that occupation of one to two binding sites on the myosin molecule by nucleoside phosphate is sufficient to dissociate the filament at pH 8.3. Since the concentration range of ligand required for dissociation is well above that necessary to saturate the ATP binding site in subfragment I ($K \cong 10^6$), it was of interest to investigate the stability of filaments prepared from “headless” myosin under comparable experimental conditions. Rod segments obtained by papain digestion of myosin were dialyzed against a solvent consisting

of 0.17 M KCl–0.002 M Veronal (pH 8.3). The resulting slightly turbid solution showed sedimentation patterns (at low rotor velocity ($<15,000$ rpm)) similar to those seen in the monomer–polymer equilibrium system of myosin, including a slowly sedimenting “monomer” boundary and a single hypersharp polymer boundary. The rod filament, like that prepared from native myosin, is dissociated at high hydrostatic pressure and a rapid increase in the rotor velocity during a sedimentation experiment results in splitting of the hypersharp polymer boundary leading to the appearance of a slower sedimenting differential monomer boundary on the centripetal side of the polymer peak. Electron micrographs of negatively stained rod filaments reveal particles of a rather sharp size distribution with mean length near 4850 Å and diameter 220 ± 50 Å. The surfaces of these particles are smooth and devoid of the corrugated appearance characteristic of myosin thick filaments (M. Burke and W. F. Harrington, unpublished data; see Harrington, 1972).

We found that addition of ATP or ADP dissociates the rod filaments near the effective concentration range observed for the synthetic myosin filaments (see Figure 5). Plots of $\log K_{app}$ *vs.* $\log [A]$ according to eq 1 and assuming 108 monomeric units in the polymer yield binding numbers of 1.0 and 0.7 for ATP and ADP, respectively. These values are insensitive to the actual number of monomeric units in the polymer as long as the number is large. The apparent binding number is unchanged at values of n up to 500.

Attempts were also made to prepare a filament system from LMM under ionic conditions similar to those used for the myosin and rod systems. Overnight dialysis of LMM *vs.* 0.135 M KCl (pH 8.3) invariably resulted in the formation of insoluble aggregates. On addition of ATP or ADP the turbid, two-phase system cleared and the OD_{310} value dropped into the range characteristic of monomer. The concentration of the nucleoside phosphates required to bring the solution to the clearing threshold was approximately the same ($4\text{--}5 \times 10^{-3}$ M) as that required to dissociate the myosin and rod filaments. No difference in “clearing behavior” was seen among LMM systems which had been prepared by tryptic digestion for 4, 8, and 15 min (at a myosin:trypsin ratio of 127:1, w/w) although the per cent protein precipitating out of solution during the dialysis step was 68, 59, and 14%, respectively. It seems likely from these results that the binding sites responsible for filament dissociation are located in the LMM segment of the rod.

Discussion

It is now well established from a number of earlier studies that each of the two globular subfragment I segments of myosin has a single nucleoside phosphate binding site with association constant, $K_A \cong 10^5$ for ATP and ADP (Young, 1967; Schliselfeld and Barany, 1968; Lowey and Luck, 1969; Murphy and Morales, 1970; Eisenberg and Moos, 1970) and $K_A \cong 10^6$ for pyrophosphate (Nauss *et al.*, 1969). Assuming that the two ATP binding sites are independent and identical in the associated state of myosin and with affinity constant the same as that in the monomeric state, they should be saturated at ligand concentrations well below that required for filament dissociation. Thus our results point to the existence of a separate set of binding sites for the nucleoside phosphates in myosin which play a crucial role in destabilizing the filament. The finding that filaments prepared from “headless” myosin undergo dissociation in the presence of ATP and ADP over the same range of ligand concentration as that found for

the myosin filament system reinforces this conclusion and indicates that the sites are located along the rod segment. Moreover, according to the experiments of Lymn and Taylor (1970) virtually all of the subfragment I binding sites should exist in the myosin·ADP·P_i complex within a few seconds after mixing the protein with ATP. Thus we might expect the F_P vs. log [ATP] profile to lie close to the ADP curve. The virtual coincidence of the AMP-PNP and ATP profiles argues that the binding sites responsible for filament dissociation are distinct from the active sites located on subfragment I.

Our results are in accord with the proposals of several other research groups (Tonomura and Morita, 1959; Levy and Ryan, 1966; Chaplain, 1966; Kominz, 1966; Bowen and Evans, 1968; Brahms and Brezner, 1961), who have interpreted their work in terms of multiple classes of ATP binding sites on myosin and actomyosin. The report of Bowen and Evans (1968) is particularly relevant since they measured the binding stoichiometry of ATP to myosin in low salt (0.05 M KCl) and in the millimolar concentration range of the ligand. A rapid filtration technique was employed to separate bound and unbound nucleotides and protein in the presence of an ATP-generating system. Their findings were interpreted as indicating two classes of sites with binding numbers $n_1 = 2.3$ and $n_2 = 14$ moles/460,000 g of myosin and with association constants $K_1 = 5.5 \times 10^3$ and $K_2 = 5.5 \times 10^2$. The value cited above for the association constant of the first class ($K_1 = 5.5 \times 10^3$) is about two orders of magnitude lower than that reported for monomeric myosin in high-salt and subfragment I in low-salt solvent systems and the significance of this finding is therefore open to question. The association constant for the weak binding site is comparable to that found in the present study (Table I, column 7). Brahms and Brezner (1961) have reported that myosin has at least 44 binding sites for pyrophosphate based on the electrophoretic mobility of the protein in 0.025 M PP_i. Within this second class of binding sites, it appears likely that one to two ligand molecules per myosin monomer is sufficient to dissociate the filament. We believe that dissociation results from binding at a specific site(s) and is probably the result of a local electrostatic charge repulsion, since the binding stoichiometry required for dissociation is not related directly to the charge on the ligand. At pH 8.3 the apparent binding numbers are 1.7 (ATP), 1.3 (ADP), 0.9 (PP_i), and 0.7 (PO₄²⁻), whereas the net charge on these substrates are -4, -3, -3, and -2, respectively. Thus nonspecific binding and a smeared-out electrostatic charge interaction between surfaces of neighboring molecules within the filament is not consistent with the effectiveness of various ligands in dissociating the filament based on their binding stoichiometry and charge number.

Our results should not be taken as indicating dissociation of the native thick filament under physiological conditions. At pH 7.4 and in the ionic environment of resting or active muscle, the concentration of ATP (2 mM) would be too low to effect destabilization. Moreover, the negative charge on ATP and ADP is effectively reduced through formation of the Mg (nucleoside) complex. Although we have not detected any significant change in the gross hydrodynamic behavior of the intact synthetic myosin filament on binding or cleavage of ATP which could be correlated with a conformational change in the myosin molecule, it should be emphasized that under the conditions employed in the steady-state sedimentation studies (high Mg²⁺, high ATP) the filament is in a state analogous to that of resting muscle. As a result of the slow decay time of the myosin·ADP·P_i complex virtually all of the subfragment I segments are in the complex form and the seg-

ments involved in any transient conformational change as a result of the ATP-cleavage reaction would likely remain undetected. It remains to be determined if the properties of the intact filament are altered within 5–200 msec, i.e., the time period associated with the ATP-cleavage reaction, following addition of ATP.

Our results do point to the possibility that the binding of a small number of nucleoside phosphate molecules to specific sites within the filament core may play an important role in the functional behavior of this structure. The remarkable sensitivity of the filament to relatively low concentrations of these ligands—concentrations in the range characteristic of living muscle—suggests that the binding may be pertinent to contraction.

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Salt Effects on Histone IV Conformation†

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ABSTRACT: The addition of salts to water solutions of histone IV induces conformational changes. These changes have been followed by observing the circular dichroism of the peptide bond, and the fluorescence anisotropy of the tyrosine emission. Both fast and slow conformational changes occur. At low enough salt concentrations there is only a fast change, in

which the altered molecules have 17–22 residues of α helix. Critical concentrations of salt exist, however, specific for each anion, above which the fast change is followed by a much slower one. In the slow change there are 20–30 β -sheet residues per altered molecule. At sufficiently high salt concentrations, the amount of slow change diminishes with added salt.

Many workers have noted that the addition of salts to aqueous solutions of histones results in an increase in secondary structure (Bradbury *et al.*, 1965, 1967; Jirgensons and Hnilica, 1965; Tuan and Bonner, 1969; Boublik *et al.*, 1970; Shih and Fasman, 1971; Li *et al.*, 1971, 1972). It has also been long known, and commented upon frequently, that under a variety of conditions histones will aggregate (Davison and Shooter, 1956; Cruft *et al.*, 1957, 1958; Mauritzen and Stedman, 1959; Phillips, 1965, 1967; Johns, 1968, 1971; Fambrough and Bonner, 1968; Edwards and Shooter, 1969; Boublik *et al.*, 1970; Diggle and Peacocke, 1971). Aggregation may, of course, be considered a special type of structural alteration.

Insight into the nature of some of the salt-induced changes in histone IV conformation was obtained by the nuclear magnetic resonance studies of Boublik *et al.* (1970), who found that the addition of NaCl to histone IV induced line broadening in the proton magnetic resonances of certain amino acid residues, particularly the hydrophobic ones which predominate in the C-terminal half of the histone IV molecule (DeLange *et al.*, 1969; Ogawa *et al.*, 1969).

Our laboratory recently reported (Li *et al.*, 1972) that the addition of sodium phosphate to histone IV solutions produces a series of conformational changes in the protein. The overall process appears complex, but we showed how it could be decomposed into a set of more elementary steps which could be at least partially understood.

At sufficiently low phosphate concentrations, a rapid conformational change occurs. At higher concentrations, a fast conformational change occurs initially, but this is followed by

a considerably slower change. In the fast step approximately 17 residues, out of the 102, go into α -helical conformation. In the slow conformational change, 20–30 residues go into β sheet. The number that go into β sheet is not a monotonic function of salt, but, as salt is added, rises to a maximum and then declines. β -sheet formation is intermolecular. Consequently the slow step also involves the formation of higher order associations.

The circular dichroism (CD) change in the fast step is independent or nearly independent of histone concentration. In contrast, however, the intrinsic fluorescence anisotropy increases with increasing histone IV concentration. By assuming that this concentration dependence indicated an equilibrium between two different states of the system we showed that the data could be fit by a dimer equilibrium equation.

In the present paper we report further work on salt-induced conformational changes of histone IV. We have studied the comparative effects of a set of salts, *viz.*, sodium phosphate, sodium sulfate, sodium fluoride, sodium perchlorate, sodium chloride, and magnesium chloride.

Materials and Methods

Calf thymus histone IV was prepared by the method of Ogawa *et al.* (1969). Our samples were electrophoretically homogeneous, and the amino acid composition agreed with the published sequence (Ogawa *et al.*, 1969; DeLange *et al.*, 1969). Histone IV concentrations were determined spectrophotometrically using $\epsilon_{280} = 4.7 \times 10^2 \text{ cm}^{-1}/\text{mole of residue per l.}$ (Ohlenbush *et al.*, 1967; Shih and Bonner, 1970). H_0 will here denote the total histone concentration in moles of protein per liter.¹

Salt stock solutions were added to histone IV in $5.0 \times$

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¹ We note that, in two previous papers from this laboratory (Li *et al.*, 1971, 1972), we measured histone concentrations in moles of residue per liter. Histone IV has 102 residues/molecule.