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Formation of a ternary complex: Actin, 5′-adenylyl imidodiphosphate, and the subfragments of myosin

(myosin subfragment 1/heavy meromyosin/association constants/turbidity)

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ABSTRACT **The formation of the ternary complex composed of actin, 5′-adenylyl imidodiphosphate [AMP-P(NH)P], and myosin subfragment 1 (S-1) was studied using the analytical ultracentrifuge with UV optics, which enabled the direct determination of the extent of dissociation of actin·S-1 (acto·S-1) by AMP-P(NH)P. In contrast to the reaction with ATP, at saturating levels of AMP-P(NH)P (1.5 mM), extensive formation of the ternary acto·S-1·AMP-P(NH)P complex occurs at 22°. With 40 μM actin present, AMP-P(NH)P causes almost no dissociation of the acto·S-1 complex at 0.04 M ionic strength, while even at 0.22 M ionic strength one-third of the S-1 remains associated with actin and AMP-P(NH)P in a ternary complex. A detailed study of the binding of S-1·AMP-P(NH)P to actin using the Scatchard plot analysis shows that, at saturation, 1 mol of S-1·AMP-P(NH)P binds per mol of actin monomer. There appears to be no cooperativity occurring as the S-1·AMP-P(NH)P binds along the actin filament, with the possible exception of a slight positive cooperativity when most of the sites on the actin filament are saturated. The turbidity of the ternary complex is identical to the turbidity of acto·S-1 alone. Preliminary experiments with the two-headed subfragment of myosin, heavy meromyosin (HMM), show that the binding of HMM·[AMP-P(NH)P]$_2$ to actin is only about twice as strong as the binding of S-1·AMP-P(NH)P to actin, indicating that the second head contributes very little to the free energy of binding.**

The key event in muscle concentration is the interaction of myosin with actin and ATP. Numerous studies have been performed on this interaction, both *in vivo* and *in vitro*. In particular, *in vitro* studies using the soluble proteolytic fragments of myosin, heavy meromysin (HMM) and subfragment 1 (S-1), have suggested that the myosin head dissociates from and then reassociates with actin each time an ATP molecule is hydrolyzed (1, 2). However, during steady-state hydrolysis of ATP by actomyosin several intermediates occur in sequence, which makes it very difficult to study a specific part of the ATPase cycle, for example, the dissociation of actomyosin by ATP during each cycle of ATP hydrolysis. For this reason, the use of ATP analogs both *in vivo* and *in vitro* has been of great interest. One of the most widely used of these analogs is 5′-adenylyl imidodiphosphate [AMP-P(NH)P], which binds with strong affinity to the ATP site of myosin, but cannot be hydrolyzed (3).

An important question concerning AMP-P(NH)P is how closely it can duplicate the effects of ATP, particularly the marked ability of ATP to dissociate actomyosin. Several physiological studies have suggested that AMP-P(NH)P at least partially relaxes skinned muscle fibers (4, 5), while other more recent physiological studies employing stiffness as a measure of binding suggest that many or most of the myosin cross-bridges remain attached to actin in the presence of AMP-P(NH)P (6, 7). In x-ray diffraction studies, the pattern produced by AMP-P(NH)P in rabbit skeletal (5, 8) and insect flight (6, 9) fibers does not resemble either the rigor or the relaxed state, suggesting that many of the cross-bridges may still be attached in the presence of AMP-P(NH)P, but are somehow altered in configuration. In contrast, several biochemical studies with isolated actin and myosin (or S-1) have shown that at high salt (3) or low temperature (10) very little ternary complex occurs.

To interpret these varied results it is first necessary to systematically investigate the ability of AMP-P(NH)P *in vitro* to dissociate the actin·S-1 (acto·S-1) complex. Using the analytical ultracentrifuge, we find that, in contrast to ATP, the acto·S-1 complex only partially dissociates as the AMP-P(NH)P concentration is increased, with more than 80% of the acto·S-1 associating with AMP-P(NH)P to form an acto·S-1·AMP-P(NH)P ternary complex at 0.04 M ionic strength. Furthermore, preliminary experiments with HMM indicate that, at least *in vitro*, the second head contributes very little to the free energy of binding.

MATERIALS AND METHODS

Rabbit skeletal myosin, HMM, S-1, and actin were prepared as described previously (2, 11). The molecular weights for HMM, S-1, and actin were taken as 350,000, 120,000, and 42,000, respectively, with protein concentrations being determined by UV absorption at 280 nm (12). Sodium AMP-P(NH)P was purchased from P-L Biochemicals and ICN. Unless the AMP-P(NH)P showed greater than 90% purity, it was chromatographed on a DEAE-Sephadex column (13).

Ultracentrifuge experiments were performed in a Beckman model E analytical ultracentrifuge equipped with a photoelectric scanner as previously described (14). The reference side of the cell always contained solvent, including nucleotide, while the sample side contained actin alone, S-1 or HMM alone, or actin with either S-1 or HMM. The solutions (1 ml total volume) were stirred for 30 min at 22° and then centrifuged for 40 min at 30,000 rpm at 22°. The cells were scanned at 282, 288, or 292 nm (see figure legends) to determine the free protein concentration. The concentration of free S-1 was corrected for unpolymerized actin (<3% of the total added).

RESULTS

The binding studies measuring the dissociation of acto·S-1 by AMP-P(NH)P were initially done at 22°, 0.04 M ionic strength, at an actin concentration of 40 μM. Under these conditions ATP causes essentially complete acto·S-1 dissociation (11), but as

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Abbreviations: HMM, heavy meromysin; S-1, subfragment 1 of myosin; AMP-P(NH)P, 5′-adenylyl imidodiphosphate; acto·S-1, complex of actin with S-1; acto·HMM, complex of actin with HMM.

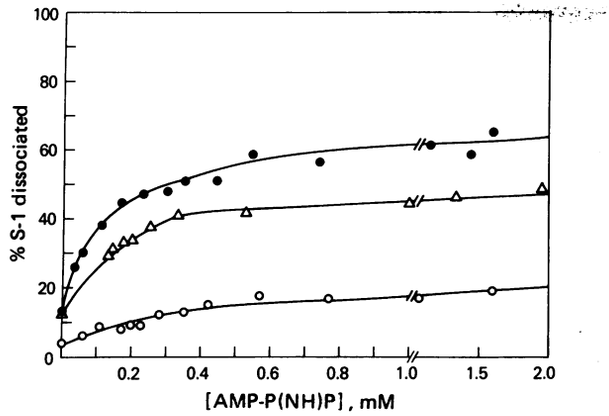
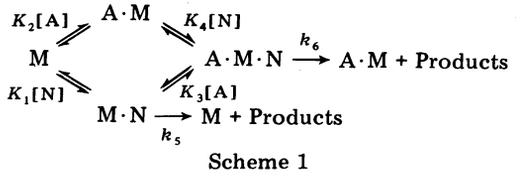


FIG. 1. Dissociation of acto·S-1 by AMP-P(NH)P. A mixture of actin, S-1, and AMP-P(NH)P was centrifuged in a Beckman model E analytical ultracentrifuge at 30,000 rpm, 22°. The concentration of dissociated S-1 was determined from the absorbance of the supernatant at 282 nm. The experimental conditions were 10 μM S-1, 40 μM actin, 5 mM $MgCl_2$, 10 mM imidazole (pH 7.0), 0.05–2.0 mM AMP-P(NH)P, and (○) 0.02 M, (△) 0.12 M, or (●) 0.2 M KCl at 22°. In the absence of AMP-P(NH)P, 5% of the S-1 did not bind to actin at low ionic strength, while at higher salt this fraction increased to 14%. The 5% inactive fraction is probably denatured S-1 and proteolytic fragments. The additional 9% unbound S-1 that appears only at higher salt may be due to a small population of weaker binding S-1 molecules, because no further dissociation was obtained when both the actin and S-1 concentrations were reduced to 5 μM or 1 μM, i.e., the 86% intact S-1 remained tightly bound to actin.

shown by the open circles in Fig. 1, AMP-P(NH)P had no such effect. As the AMP-P(NH)P concentration was increased to 0.5 mM, 20% of the acto·S-1 initially dissociated, but above 0.5 mM very little further dissociation occurred. Presumably then, at 1.5 mM AMP-P(NH)P, 80% of the S-1 was complexed with both actin and AMP-P(NH)P in a ternary complex. At higher ionic strength, a similar phenomenon occurred: maxima of 48% and 62% of the S-1 were dissociated by AMP-P(NH)P at 0.14 M and 0.22 M ionic strength, respectively. Therefore, our data suggest that even at 0.22 M ionic strength with 40 μM actin and 1.5 mM AMP-P(NH)P present, one-third of the S-1 is associated with actin and AMP-P(NH)P in a ternary complex. Of course under similar conditions, ATP would completely dissociate acto·S-1, not only physically as at lower ionic strength, but enzymatically as well, because almost no actin-activated ATPase would occur. Clearly, AMP-P(NH)P and ATP differ markedly in their ability to dissociate acto·S-1 *in vitro*.

If a ternary acto·S-1·AMP-P(NH)P complex is indeed formed as our data suggest, then it should be possible to analyze the interaction of S-1 with actin and AMP-P(NH)P in terms of the general scheme for the interaction of enzyme, substrate, and modifier first described by Botts and Morales (15). This was applied to the actin·myosin·ATP interaction by Eisenberg and Moos (16) as shown in Scheme 1:



Scheme 1

K_1, K_2, K_3, and K_4 are association constants, k_5 and k_6 are rate constants, M is S-1, A is actin, and N is ATP. As suggested by Eisenberg and Moos, this model is based on separate binding sites for actin and ATP on S-1. The actin-activated ATPase cycle was later shown to be much more complex than indicated by

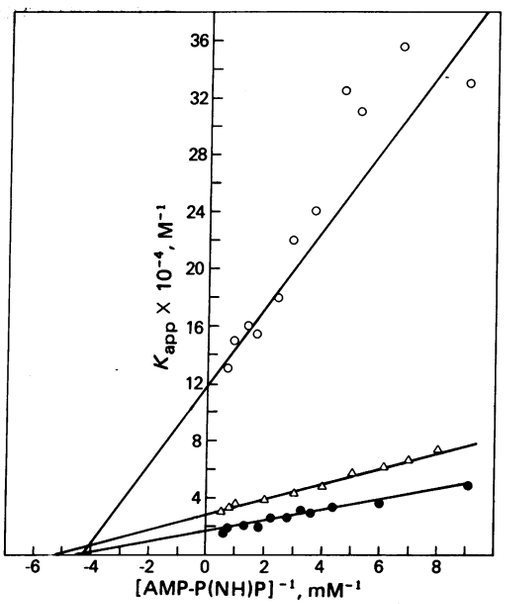


FIG. 2. Reciprocal plot measuring the dissociation of acto·S-1 as a function of 1/[AMP-P(NH)P]. The data shown in Fig. 1 for (○) 0.02 M, (△) 0.12 M, and (●) 0.2 M KCl were replotted. K_{app} at each given AMP-P(NH)P concentration was calculated after the $[M]_{bound}$ and $[M]_{free}$ were measured in the analytical centrifuge and after the total actin concentration was corrected for actin bound to S-1. This correction was made by assuming a 1:1 binding of actin to S-1, both in the absence and presence of AMP-P(NH)P. (The latter was verified in Fig. 3.) This altered K_{app} at most by 25%, and linear plots were obtained with or without the correction. The calculated values for K_{app} were obtained without correcting the data in Fig. 1 for S-1 that did not bind actin in the absence of AMP-P(NH)P. Such a correction would increase the values of K_{app} by about 14%.

Scheme 1 (1, 2). However, if N is AMP-P(NH)P and k_5 and k_6 are 0, this model can be applied to the binding of AMP-P(NH)P to acto·S-1, as was done by Highsmith (10).

With the actin and AMP-P(NH)P concentrations used in these experiments, there will be essentially no free S-1 present because it will be complexed with actin, AMP-P(NH)P, or both. Therefore, Scheme 1 can be simplified to:

$$A\cdot M \overset{K_4[N]}{\rightleftharpoons} A\cdot M\cdot N \overset{K_3[A]}{\rightleftharpoons} M\cdot N$$

Scheme 2

from which Eq. 1 can be derived:

$$K_{app} = \frac{[M]_{bound}}{[M]_{free}[A]_{free}} = \frac{[A\cdot M\cdot N + A\cdot M]}{[M\cdot N][A]} = \frac{K_3}{K_4 N} + K_3 \quad [1]$$

in which $[M]_{bound}$ and $[M]_{free}$ refer to S-1 complexed with and free of actin, respectively. On the basis of this equation, myosin complexed with actin can occur either as A·M or A·M·N. As the AMP-P(NH)P concentration is increased, A·M·N will increase relative to A·M until at infinite nucleotide concentration no A·M will occur and $K_{app} = K_3$. K_4 determines the AMP-P(NH)P concentration needed to saturate the acto·S-1 complex with nucleotide. Both K_3 and K_4 can be obtained from Eq. 1 by plotting K_{app} as a function of 1/N. This gives a linear plot with an ordinate intercept K_3 and abscissa intercept $-K_4$.

The data in Fig. 1 were replotted on this basis to determine whether Scheme 1 for the formation of a ternary complex is applicable to the actin·S-1·AMP-P(NH)P system. In these experiments, a 4-fold molar excess of actin over S-1 was used so that the free actin concentration was essentially equal to the total actin. Thus, only a small correction for bound actin was necessary (see caption to Fig. 2). On this basis, K_{app} at each

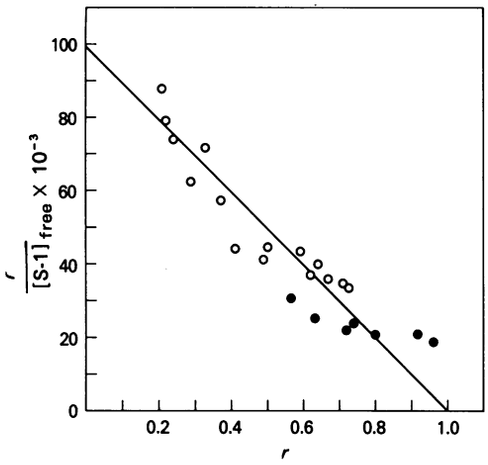


FIG. 3. Scatchard plot of S-1 binding to actin in the presence of AMP-P(NH)P. The conditions were 22°, 30 μM actin, 1.5 mM AMP-P(NH)P, 20 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7.0) and from 10 to 80 μM S-1. The concentration of dissociated S-1 was determined from the absorbance at 288 nm (○) and at 292 nm (●). r is the number of moles of S-1 bound per mole of F-actin monomer.

given AMP-P(NH)P concentration was calculated after the $[M]_{bound}$ and $[M]_{free}$ were measured in the analytical ultracentrifuge. Using this method, we find that, as shown in Fig. 2, our results fit a straight line at each ionic strength used, suggesting that Scheme 1 is applicable to this system. The abscissa intercepts of these plots give K_4, the binding constant of AMP-P(NH)P to the acto·S-1 complex. As can be seen, within experimental error, K_4 is unaffected by ionic strength and is about 4.5×10^3 M^{-1}. Therefore, at 220 μM AMP-P(NH)P, half of the acto·S-1 will be complexed with AMP-P(NH)P, while at 1.5 mM AMP-P(NH)P more than 85% of the acto·S-1 will be complexed with nucleotide. The relatively strong binding constant of AMP-P(NH)P for the acto·S-1 complex explains why, as shown in Fig. 1, the maximum dissociation caused by AMP-P(NH)P occurs at a relatively low AMP-P(NH)P concentration with very little further dissociation occurring as the AMP-P(NH)P concentration is increased above 1 mM.

The ordinate intercepts of these plots give K_3, the binding constant of actin to the S-1·AMP-P(NH)P complex. K_3 equals 12×10^4 M^{-1}, 3×10^4 M^{-1}, and 2×10^4 M^{-1} at 0.04 M, 0.14 M, and 0.22 M ionic strength, respectively. Therefore, unlike the binding of AMP-P(NH)P to acto·S-1, the binding of actin to S-1·AMP-P(NH)P depends on ionic strength, becoming about 6-fold weaker as the ionic strength is increased from 0.04 to 0.22 M.

The values of K_{app}, and by extrapolation K_3, presented in Fig. 2 are determined at a single S-1 and actin concentration, with a 4-fold molar excess of actin over S-1. In using these plots, we are assuming that the S-1 molecules bind independently along the actin filament, which may not necessarily be the case (17). Therefore, to determine if S-1 was binding independently to actin, we measured the binding of S-1·AMP-P(NH)P to actin over a wide range of S-1 concentration (10–80 μM) and then analyzed the data using the Scatchard equation (18). These experiments were performed at nearly saturating AMP-P(NH)P concentration (1.5 mM) and at 0.04 M ionic strength, where extensive ternary complex formation occurs. As can be seen in Fig. 3, within experimental error the Scatchard plot appears nearly linear. Some nonlinearity may occur at high r values, but points obtained at larger r values tend to be relatively inaccurate (19). Therefore, the S-1·AMP-P(NH)P molecules appear to bind

Table 1. The turbidity of the ternary complex

Solution	OD_{350}
Acto·S-1	0.22
Acto·S-1 + 1.5 mM AMP-P(NH)P	0.21
Acto·S-1 + 4.3 mM ATP	
Initial	0.11
After ATP hydrolysis	0.21
Sum of actin and S-1 measured individually	0.09

The measurements of turbidity were done at 350 nm, using a Cary 14 spectrophotometer. The reaction conditions were 10 μM actin, 100 μM S-1, 20 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole·HCl (pH 7.0), 22°. The solutions (1.25 ml total volume) were added directly into a cuvette with the S-1 last, mixed by inverting the cuvette, and then degassed under vacuum. The aliquots of nucleotide were from concentrated stock solutions, 20 mM AMP-P(NH)P and 38 mM ATP, so there were only minimal changes in the volume after their addition. The addition of AMP-P(NH)P followed the same mixing and degassing procedure as with acto·S-1, but ATP was inserted into the spectrophotometer cuvette without degassing. The same acto·S-1 solution was used with AMP-P(NH)P added first and then ATP.

essentially independently to the actin filament, except for possible slight positive cooperativity when most of the sites on the actin filament are saturated. The abscissa intercept of 1 shows that at saturation the ternary complex is composed of 1 mol of S-1·AMP-P(NH)P binding per mol of actin monomer in the F-actin filament. The slope of the Scatchard plot gives a value for K_3 of 1.0×10^5 M^{-1}, which is identical within experimental error to the value of K_3 obtained from the plot in Fig. 2. Thus, over a wide range of S-1 concentration, the S-1, actin, and AMP-P(NH)P appear to interact in accordance with Scheme 1.

The ultracentrifuge experiments thus far presented in this paper have demonstrated that below 40 mM ionic strength it is quite easy to work at actin, S-1, and AMP-P(NH)P concentrations at which almost all of the S-1 is complexed with actin and AMP-P(NH)P in a ternary complex. This provides a useful opportunity to determine if the turbidity of the ternary acto·S-1·AMP-P(NH)P complex is identical to the turbidity of acto·S-1 itself. This is a question of considerable interest because, in interpreting pre-steady-state kinetic studies employing ATP, the critical assumption is generally made that the turbidity of the acto·HMM·ATP ternary complex is identical to the turbidity of acto·HMM alone (1, 2).

We therefore compared the turbidity of acto·S-1 with and without 1.5 mM AMP-P(NH)P present under conditions where more than 90% of the actin monomers will be complexed with S-1 and AMP-P(NH)P in a ternary complex. Lines 1 and 2 of Table 1 show that the ternary acto·S-1·AMP-P(NH)P complex has almost the same turbidity as the acto·S-1 complex itself. In contrast, as a control, lines 3 and 4 show that when ATP is added under the same conditions the turbidity drops almost to the sum of the turbidity of the S-1 and actin measured individually (line 5), rising to its original value only after all of the ATP is hydrolyzed. Therefore, our data support the postulate that the binding of nucleotide to the acto·S-1 complex does not itself cause a change in turbidity. W. Hofmann and R. S. Goody (personal communication) have obtained data similar to that presented in this paper by using turbidity as a measure of ternary complex formation.

Having demonstrated that the interaction of S-1 with actin and AMP-P(NH)P is consistent with Scheme 1, we turned to an investigation of the interaction of the two-headed myosin fragment, HMM, with actin and AMP-P(NH)P. We were particularly interested in whether HMM·$[AMP\text{-}P(NH)P]_2$,

which has two actin-binding sites, shows stronger affinity for actin than does S-1·AMP-P(NH)P. Of course, studying the interaction of HMM with actin and AMP-P(NH)P is more complex than studying this same interaction using S-1. First, more species occur than with S-1, i.e., both HMM and acto·HMM can bind either one or two AMP-P(NH)P molecules. Second, as the two-headed HMM molecules bind along the actin filament, either positive (17) or negative cooperativity may occur. Indeed, we found that if saturating AMP-P(NH)P and excess actin were *not* present, the experimental results were quite complex and will require further work to interpret. Therefore, plots similar to those we did with S-1 where either actin or AMP-P(NH)P were varied (Figs. 2 and 3) are not presented. However, by working at saturating AMP-P(NH)P with the actin present in excess over HMM, it is possible to measure the binding of HMM·[AMP-P(NH)P]$_2$ to actin under conditions in which the HMM molecules bind independently to the actin filament.

This experiment was performed at varied actin concentrations to make certain that in the range where we worked the molar ratio of HMM to actin has no effect on the results. Because the actin was always present in excess, the free actin concentration was essentially equal to the total actin concentration. Therefore, K'_3, the binding constant of HMM·[AMP-P(NH)P]$_2$ to actin, could be calculated directly after measuring the free and bound HMM with only a small correction for the bound actin. As shown in Table 2, we find only a 2-fold difference between K'_3 obtained with HMM and K_3 obtained with S-1. Although we have not proven that acto·HMM is saturated with AMP-P(NH)P at 1.5 mM AMP-P(NH)P, if the AMP-P(NH)P concentration was increased, the value of K'_3 obtained with HMM would presumably only decrease, bringing it still closer to the value of K_3 obtained with S-1. Therefore, these data strongly suggest that, if binding of the second HMM head to actin occurs at all at saturating AMP-P(NH)P concentration, it must contribute very little to the free energy of binding of the HMM·[AMP-P(NH)P]$_2$ complex to actin.

Table 2. Association constants of S-1·AMP-P(NH)P and HMM·[AMP-P(NH)P]$_2$ to actin

[Actin], μM	$K_3 \times 10^{-4}$, M^{-1} *	$K'_3 \times 10^{-4}$, M^{-1} †	
		Assumption 1‡	Assumption 2§
25	1.5	2.5	3.1
40	1.3	2.3	2.7
55	1.5	2.2	2.5
70	1.1	2.3	2.5
85	1.0	2.0	2.1
Mean	1.3	2.3	2.6

Experimental conditions were 1.5 mM AMP-P(NH)P, 0.2 M KCl, 5 mM $MgCl_2$, 10 mM imidazole (pH 7.0), 22°, and either 14.5 μM S-1 or 6 μM HMM.

* Association constant of S-1·AMP-P(NH)P to actin. These values for K_3 were calculated after correcting the total actin concentration for actin bound to S-1 as was done in Fig. 2.

† Association constant of HMM·[AMP-P(NH)P]$_2$ to actin.

‡ These values for K'_3 were calculated by assuming that the free actin concentration approximately equals the total actin concentration.

§ These values for K'_3 were calculated by assuming that the free actin concentration equals the total actin concentration minus the actin bound to HMM in the ternary complex (2 F-actin monomers/HMM).

DISCUSSION

In the present study, we carried out a detailed investigation of the dissociation of acto·S-1 by AMP-P(NH)P. Our results establish that, at saturating levels of AMP-P(NH)P, extensive formation of the ternary acto·S-1·AMP-P(NH)P complex occurs at low ionic strength, 22°. Our results also indicate that the mechanism of binding of AMP-P(NH)P and actin to S-1 is compatible with the simple model shown in Scheme 1. In this model, the bindings of AMP-P(NH)P and actin occur on separate sites, but the binding of AMP-P(NH)P weakens the binding of actin and vice versa.

Because AMP-P(NH)P clearly did not completely dissociate acto·S-1, why has the ternary acto·S-1·AMP-P(NH)P complex not been more commonly observed *in vitro*? Yount *et al.* (3) found that AMP-P(NH)P dissociated actomyosin at 0.6 M KCl, pH 7.4, 20°, probably because of the high ionic strength. Highsmith (10) examined the binding of acto·S-1 in the presence of AMP-P(NH)P at 0.15 M KCl, pH 7.0, 4°, using fluorescence depolarization. Although his data were analyzed according to Scheme 1 for the formation of a ternary complex, he found that $K_3 < 100\ M^{-1}$ and $K_4 < 50\ M^{-1}$, i.e., ternary complex formation could be observed only if the actin concentration approached 10 mM. Highsmith's failure to detect a ternary complex may be due to the low temperature and the very low concentration of actin (1 μM) used in this study. One other study of the binding of S-1·AMP-P(NH)P to actin *in vitro* was briefly reported by Marston *et al.* (7), who stated that at 0.1 M ionic strength, 25°, K_3 was about $3 \times 10^5\ M^{-1}$. This is about 5-fold higher than our value for K_3 under similar conditions.

Turning to the *in vivo* data, Dos Remedios *et al.* (4) found that 5 mM AMP-P(NH)P reduces the tension and stiffness of rabbit psoas fibers in rigor (at 40 mM KCl, 5 mM $MgCl_2$, pH 7.9, 22°), indicating dissociation of the actomyosin cross-bridges. In addition, Lymn (5) found that rabbit psoas muscle in the presence of AMP-P(NH)P could be slowly stretched at 0°, pH 8.0. However, the existence of a ternary complex in muscle fibers was suggested by x-ray diffraction studies in which the patterns produced by addition of AMP-P(NH)P to rabbit psoas (5, 8) and *Lethocerus* (6, 9) muscle fibers did not resemble either the relaxed or rigor state. More conclusive evidence for the existence of a ternary complex in these muscle fibers was later reported by Marston *et al.* (7), who found that, even after addition of saturating levels of AMP-P(NH)P to muscle fibers in rigor (at 20 mM potassium phosphate, 5 mM $MgCl_2$, pH 7.1, 18°), fiber stiffness and tension were restored to their initial value, providing the muscle fibers were subjected to a slight re-extension. This suggests that almost all of the cross-bridges in the fiber are attached to actin filaments at saturating AMP-P(NH)P concentration.

Our value for the binding constant of S-1·AMP-P(NH)P to actin *in vitro* (K_3) cannot be directly compared with the binding of the cross-bridge to actin *in vivo*, because *in vivo* the myosin cross-bridge is held in a fixed position relative to the actin filament so that the actin "concentration" is unrelated to the fraction of attached cross-bridges (20). Nevertheless, the first-order rate of attachment of the cross-bridge to actin *in vivo* is probably at least as fast as the pseudo first-order rate of attachment of S-1 to actin at the relatively low actin concentration that was used *in vitro*. Therefore, the observation of Marston *et al.* (7) that all of the cross-bridges are attached *in vivo* at saturating AMP-P(NH)P concentrations is certainly consistent with our *in vitro* results.

Our *in vitro* findings for the binding constant of AMP-P(NH)P to acto·S-1 (K_4) to form the ternary complex also agree qualitatively with the *in vivo* results of Marston *et al.* (7) and Goody *et al.* (21). In *Lethocerus* fibers, approximately 100 μM AMP-P(NH)P (7, 21) was needed to half saturate the cross-bridges with nucleotide, while in rabbit skeletal fibers half of

the cross-bridges were saturated at 30 μM AMP-P(NH)P. On the other hand, we found that half of the acto-S-1 was saturated at 220 μM AMP-P(NH)P. Further investigation will be required to determine if these differences are significant.

Relating the *in vivo* and *in vitro* effects of AMP-P(NH)P will also require an understanding of the way the two heads of myosin interact with actin. Several recent studies have reported that, in the absence of AMP-P(NH)P, HMM binds only about 10-fold stronger than S-1 to actin (22–24), which opens up the possibility that the binding of the second HMM head to actin contributes relatively little to the free energy of binding. Our results with HMM in the presence of AMP-P(NH)P are not only consistent with this result but, in fact, are even more pronounced. HMM·[AMP-P(NH)P]$_2$ binds only twice as strongly as S-1·AMP-P(NH)P to actin (at 0.22 M ionic strength, pH 7.0, 22°). This suggests that, in the presence of AMP-P(NH)P, HMM may bind to actin with only one head, with the other head binding weakly or not at all. It is possible a similar effect occurs *in vivo*, but much more work with the acto·HMM·AMP-P(NH)P system will be necessary before we can fully understand the interaction of the two HMM heads with actin and AMP-P(NH)P.

One of the advantages of obtaining accurate values for K_3 and K_4 is that it allows an accurate determination of the much stronger binding constant of S-1 to actin (K_2), a value of considerable importance that is difficult to measure directly. K_2 is calculated to be 2×10^7 M^{-1} at 0.14 M ionic strength, pH 7.0, 22°, determined by substituting our values of K_3 (3×10^4 M^{-1}) and K_4 (4.5×10^3 M^{-1}) as well as the value of 3×10^6 M^{-1} for K_1 reported by Bagshaw *et al.* (25) in the detailed balance equation $K_1K_3 = K_2K_4$. Under comparable conditions, both Marston and Weber (26) and White and Taylor (27) obtained a value of 1 to 2×10^7 M^{-1} for K_2, and Highsmith (28) obtained a value of 7×10^6 M^{-1}. These are similar to our value for K_2, but about an order of magnitude greater than the value of 1×10^6 M^{-1} obtained by Margossian and Lowey (22).

The major findings of this paper is that the ability of AMP-P(NH)P to dissociate acto-S-1 is very different from that of ATP. This is perhaps not surprising because, while the binding constant of AMP-P(NH)P to myosin is about 10^6 M^{-1} (29), the binding constant of ATP to myosin is about 10^{11} M^{-1} (30, 31), demonstrating a clear difference in the way ATP and AMP-P(NH)P bind to myosin. In contrast, the binding constant of ADP to myosin has been reported to range from 2×10^5 M^{-1} to 2×10^6 M^{-1} (32–34), approximately within an order of magnitude of the reported value of K_1 for AMP-P(NH)P. Furthermore, the binding constant of ADP to acto-S-1 has been reported by White (35) to be 5×10^3 M^{-1}, identical within experimental error to the value of K_4 we obtained for AMP-P(NH)P. Therefore, *in vitro* AMP-P(NH)P may be more similar to ADP than it is to ATP in its ability to dissociate acto-S-1. On the other hand, *in vivo* x-ray diffraction studies suggested that ADP caused almost no change in the rigor pattern of *Lethocerus* muscle, while AMP-P(NH)P caused a marked change in this pattern (21). To relate the *in vivo* and *in vitro* effects of these nucleotides, it is necessary to investigate the ability of ADP to dissociate acto-S-1 *in vitro*.

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