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Comparison of the Binding of Heavy Meromyosin and Myosin Subfragment 1 to F-Actin†

Lois E. Greene

ABSTRACT: The binding of heavy meromyosin (HMM) to F-actin was examined at varying ionic strengths and temperatures and in the presence of ADP and AMPPNP and then compared to the binding of subfragment 1 (S-1) under identical conditions. In the absence of nucleotide (μ = 0.12–0.43 M, 22 °C), HMM binds 100–1000-fold more strongly to F-actin than does S-1. This indicates that, in the absence of nucleotide, both heads of HMM bind to F-actin, with the second head making a significant contribution to the free energy of binding. On the other hand, in the presence of ADP (μ = 0.43 M, 22 °C) or AMPPNP (μ = 0.12 M, 22 °C), the binding of HMM to F-actin is quite similar to the binding of S-1, indicating that here the second head of HMM does not make a strong contribution to the free energy of binding. In fact, in the presence of AMPPNP, HMM appears to bind to F-actin primarily with one head, while the detached head may be interfering with the binding of another HMM molecule at an adjacent actin site. With all of the different agents tested (ionic strength, temperature, and nucleotide), the effect of the agent on the binding of HMM to F-actin is approximately the square of its effect on the binding of S-1 to F-actin, results consistent with these various agents affecting the binding of *each* of the two HMM heads to the same extent as they affect the binding of an S-1 head.

The myosin molecule has a structure consisting of a long rodlike portion with two globular heads at one end (Lowey et al., 1969). The rodlike portion causes the myosin molecule to aggregate at low ionic strength and is thus integral to the formation of the thick filament. Each globular head has the ability to bind actin and hydrolyze ATP (Lowey & Luck, 1969; Nauss et al., 1969; Eisenberg & Moos, 1970; Eisenberg et al., 1972; Margossian & Lowey, 1973). The myosin molecule can be degraded by proteolysis to produce two soluble fragments, heavy meromyosin (HMM),[1] a double-headed fragment, and subfragment 1 (S-1), a single-headed fragment. Since HMM is two headed and is therefore usually considered a model for myosin, it is important to examine the binding of HMM to F-actin under different conditions.

In the absence of nucleotide, HMM binds to F-actin significantly stronger than S-1, although there is controversy over the magnitude of the actin–HMM association constant. Greene & Eisenberg (1980a) previously found that the association constant for the binding of HMM to F-actin is 3×10^9 M^{-1} at μ = 0.22 M, 22 °C, while under the same conditions, the actin–S-1 association constant is 5×10^6 M^{-1}.

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[1] Abbreviations used: S-1, subfragment 1; HMM, heavy meromyosin; acto-S-1, a complex of actin with S-1; acto-HMM, a complex of actin with HMM; DTT, dithiothreitol.

Therefore, their study indicates that HMM binds 600-fold stronger than S-1 to F-actin. In contrast, under similar conditions Margossian & Lowey (1978) and Highsmith (1978) found that HMM binds only 10-fold stronger than S-1 to F-actin. Interestingly enough, in the presence of AMPPNP (μ = 0.22 M, 22 °C), Greene & Eisenberg (1978) also found that the actin–HMM and actin–S-1 association constants were quite similar, i.e., the binding constant of HMM to F-actin was only twice the actin–S-1 association constant ($K_{S\text{-}1} \simeq 10^4$ M^{-1}). They interpreted these results as suggesting that the difference between the actin–HMM and actin–S-1 association constants depends on whether or not AMPPNP is present. On the other hand, the results of Margossian & Lowey and Highsmith would suggest that the actin–HMM and actin–S-1 association constants are similar in the presence and absence of AMPPNP.

Differences in the binding of HMM and S-1 to F-actin depend on the contribution each of the HMM heads makes to the free energy of binding. These contributions can be determined by analyzing the binding of HMM to adjacent F-actin monomers as a two-step process (Highsmith, 1978; Greene & Eisenberg, 1980a; Hill & Eisenberg, 1980). When HMM binds to F-actin, the first head binds in a second-order reaction with a binding constant which appears to be the same as the binding constant of S-1 to F-actin. This is followed by the binding of the second head at an adjacent site on F-actin in a first-order reaction. This difference in binding of the two heads of HMM to F-actin does not arise from an intrinsic difference between the heads (Inoue & Tonomura, 1976) but because the two heads bind to adjacent F-actin monomers (Craig et al., 1980). By use of this analysis, the results of Greene & Eisenberg (1978, 1980a) suggest that in the absence of nucleotide, the second head of HMM contributes significantly to the free energy of binding, whereas in the presence of AMPPNP, the second head does not contribute significantly to the free energy of binding. In fact, under these conditions, the second head may be binding weakly or not at all.

In this paper, the binding of S-1 and HMM to F-actin was studied at varying ionic strengths and temperatures and in the presence of ADP and AMPPNP to determine the contribution of the second head of HMM to the free energy of HMM binding to F-actin. This comparative study shows that in the absence of nucleotide, the second head of HMM contributes significantly to the free energy of binding, but not in the presence of ADP (μ = 0.43 M, 22 °C) or AMPPNP (μ = 0.12 M, 22 °C). The results also show that the effect of a particular agent (ionic strength, temperature, or the presence of nucleotide) on the binding of HMM to F-actin is approximately the square of its effect on the binding of S-1, suggesting that the binding of each of the two HMM heads to F-actin is affected to the same extent as the binding of S-1 to F-actin by these various agents.

Materials and Methods

Proteins. Myosin was purified from rabbit back and leg muscles according to the method of Kielley & Harrington (1960). F-Actin was prepared by using a modified method of Spudich & Watt (1971) (Eisenberg & Kielley, 1974). Chymotryptic S-1 was prepared by the method of Weeds & Taylor (1975), and tryptic HMM was made according to Eisenberg & Moos (1968). Column chromatography of the HMM gave only one peak, indicating that the amount of S-1 in the HMM is insignificant. Protein concentrations were determined from the absorbance at 280 nm with $E_{280}^{1\%}$ equal to 5.6, 6.47, 7.5, and 11.5 for myosin, HMM, S-1, and F-actin, respectively. The molecular weights used for myosin, HMM, S-1, and F-actin were 470 000, 350 000, 120 000, and 42 000, respectively.

Sulfhydryl Modification. The SH_1 groups of myosin were blocked with [^{14}C]iodoacetamide (sp act. $\approx 2 \times 10^{12}$ cpm/mol) by using the labeling procedure described previously (Greene & Eisenberg, 1980a). After the myosin was labeled (1 ± 0.1 mol of iodoacetamide/mol of heads), either tryptic HMM or chymotryptic S-1 was made.

Competition and Binding Experiments. In the binding studies in the presence of nucleotide, S-1, or HMM, F-actin and nucleotide (ADP or AMPPNP) in a 4-mL volume were mixed for several minutes at 22 °C. Three-milliliter aliquots were incubated for 30 min at 22 °C and then centrifuged for 1 h at 22 °C in a Beckman L2-65B ultracentrifuge at 80000*g*. The supernatant was carefully removed. The concentrations of the total and unbound SH_1-blocked fragment were determined from radioactive counts by using 0.5 mL of the solution prior to centrifugation and 0.5 mL of the supernatant after centrifugation, respectively.

The competition experiments were conducted as described previously (Greene & Eisenberg, 1980a). S-1, SH_1-blocked HMM, and F-actin in a 4-mL volume were mixed for 5 min at 22 °C. The solution was then incubated for 75 min at 22 °C to ensure equilibrium in the system at time of centrifugation. Three milliliters of solution was then centrifuged (1 h; 80000*g* at 22 °C) and the supernatant was carefully removed. The solutions prior to centrifugation and after centrifugation (0.5 mL of each) were counted in a Beckman LS250 liquid scintillation counter to determine protein concentrations of the total and unbound SH_1-blocked HMM. The concentration of native S-1 was calculated from the absorbance at 280 nm, after subtracting the absorbance contribution of the SH_1-blocked fragment and depolymerized actin. The latter correction was quite small (1–5%), determined from the absorbance remaining in the supernatant after centrifugation of F-actin alone.

In both the binding studies and the competition studies, there was a correction made for HMM and S-1 that sedimented in the absence of actin, as well as for enzyme which did not bind to actin under optimal conditions. These controls were performed under the identical conditions as described above for the binding and competition experiments. At least 95% of the HMM and 97% of the S-1 remained in the supernatant after centrifugation. There was also a small correction made in the concentrations of unbound S-1 and unbound HMM (~5% of the total) for denatured enzyme which did not bind to actin under optimal conditions (low ionic strength and a large free actin concentration). Competition experiments (at μ = 0.43 M) were also conducted where the proteins were incubated only for 30 min prior to centrifugation. These experiments gave the same results as those with the 75-min incubation time.

All binding experiments in the presence of ADP contained ~2 μM diadenosine pentaphosphate to inhibit myokinase activity (Lienhard & Secemski, 1973).

Data Analysis. Linear regression was used to determine the slopes of the Scatchard plot of S-1 binding to F-actin in the presence of nucleotides. In the competition experiments the line drawn through the data was the best line intersecting the origin. This was determined by computing for each data point a line intersecting the origin, and the mean of all these lines was then determined. All competition studies were repeated at least 3 times with different preparations and the results agreed within 10%.

Chemicals. ADP and diadenosine pentaphosphate were from P-L Biochemicals and AMPPNP was from ICN. The

purity of the ADP and AMPPNP was analyzed by both PEI–cellulose chromatography in 0.75 M KH_2PO_4 (pH 3.4) and phosphate analysis (Yount et al., 1971). The ADP was >95% pure and the AMPPNP was >90% pure. The [^{14}C]-iodoacetamide was from Amersham/Searle.

Results

In this study, the binding constant of the second head of HMM to F-actin was determined under a variety of conditions: varying ionic strengths and temperatures and in the presence of ADP and AMPPNP. This was done by analyzing the binding of the two heads of HMM to adjacent F-actin monomers as a two-step process as shown by

$$K_{2\text{-HMM}} = K^{\alpha}_{\text{HMM}}\, K^{\vartheta}_{\text{HMM}} \quad (1)$$

where

$$K^{\alpha}_{\text{HMM}} = 2K_{\text{S-1}} \quad (2)$$

$K_{2\text{-HMM}}$ is the binding constant of HMM (or HMM·nucleotide) to F-actin where two actin sites are occupied; K^{α}_{HMM} is the binding constant of the first head of HMM to F-actin; $K^{\vartheta}_{\text{HMM}}$ is the binding constant of the second head of HMM to F-actin; $K_{\text{S-1}}$ is the binding constant of S-1 (or S-1·nucleotide) to F-actin. When HMM binds to F-actin, the first head binds in a second-order reaction, followed by the binding of the second head to an adjacent F-actin monomer in a first-order reaction. This difference in the manner in which the two HMM heads bind to F-actin originates from the heads binding to adjacent F-actin monomers; the heads themselves are assumed to be equivalent. As shown by eq 2, it is assumed that the binding constant of the first head of HMM to F-actin is twice the binding constant of S-1 to F-actin. This is based on the results of White & Taylor (1976), who found the same rate of attachment of S-1 and HMM (on a per head basis) to F-actin; most likely, the rates of detachment are also similar (Hill & Eisenberg, 1980). The theoretical analysis of Hill & Eisenberg (1980) on the binding of the myosin fragments to F-actin is also consistent with the suggestion that $K^{\alpha}_{\text{HMM}} \simeq K_{\text{S-1}}$. It is important to recognize that, based on eq 1 and 2, HMM will bind to F-actin with two heads only if $K^{\vartheta}_{\text{HMM}} \gg 1$, i.e., $K_{2\text{-HMM}} \gg K^{\alpha}_{\text{HMM}}$ (or $2K_{\text{S-1}}$). In this study, the actin–S-1 and actin–HMM association constants ($K_{\text{S-1}}$ and $K_{2\text{-HMM}}$) were first measured, and then the binding constant of the second head of HMM to F-actin ($K^{\vartheta}_{\text{HMM}}$) was calculated according to eq 1 and 2.

Effect of Ionic Strength. The effect of ionic strength on the binding of the second head of HMM to F-actin was calculated after first measuring the ionic strength sensitivity of the binding of S-1 and HMM to F-actin. The actin–S-1 association constant was determined at ionic strengths of 0.12 and 0.43 M. Since the binding of S-1 to actin at low ionic strength is very strong, large errors are encountered in directly measuring very low concentrations of unbound S-1. The binding of S-1 to F-actin was measured in the presence of ADP or AMPPNP to avoid this problem. The change in the association constant of the S-1·nucleotide complex to actin provides a measure of the effect of ionic strength on the actin–S-1 association constant since the binding of nucleotide to S-1 and acto·S-1 appears rather insensitive to changes in ionic strength (Lowey & Luck, 1969; Kodama et al., 1977; Bagshaw et al., 1972; Schliselfeld, 1974; Wagner & Yount, 1975; Greene & Eisenberg, 1978).

The binding of S-1·ADP or S-1·AMPPNP to F-actin was plotted as K_{app} vs. [nucleotide]$^{-1}$ in Figure 1. The abscissa intercept, a measure of the association constant of nucleotide to acto·S-1, does not change significantly with a change in ionic

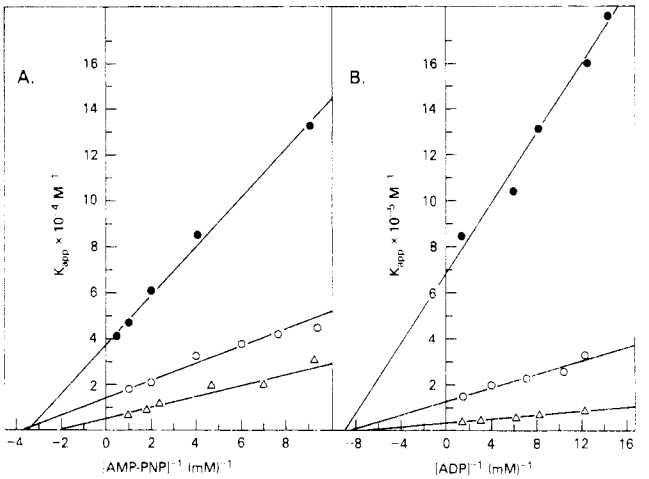


FIGURE 1: Binding of S-1 to F-actin in the presence of AMPPNP or ADP at varying ionic strengths, 22 °C. Varying concentrations of AMPPNP (100 μM–2 mM) or ADP (60 μM–1 mM) were added to a fixed concentration of SH_1-blocked S-1 and actin in parts A and B, respectively. In part A, 20 μM actin and 6 μM SH_1-blocked S-1 were used at μ = 0.12 M (●), 40 μM actin and 15 μM SH_1-blocked S-1 were used at μ = 0.22 M (○), and 60 μM actin and 11 μM SH_1-blocked S-1 were used at μ = 0.43 M (△). In part B, 3 μM actin and 3 μM SH_1-blocked S-1 were used at μ = 0.12 M (●), 15 μM actin and 10 μM SH_1-blocked S-1 were used at μ = 0.22 M (○), and 13 μM actin and 10 μM SH_1-blocked S-1 were used at μ = 0.43 M (△). Conditions were 12 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 0.5 mM DTT, and either 0.1 M KCl (●), 0.2 M KCl (○), or 0.4 M KCl and 5 mM KP_i (△).

strength with either AMPPNP (Figure 1A) or ADP (Figure 1B). The association constant of the S-1·nucleotide complex to actin is obtained from the ordinate intercepts in parts A and B of Figures 1 for AMPPNP and ADP, respectively. With both AMPPNP and ADP, the association constant of the nucleotide complex to actin changes 10–15-fold with the change in ionic strength: from μ = to 0.12 to 0.22 M, the association constant decreases ~3-fold and from μ = 0.22 to 0.43 M, there is ~4-fold decrease. Since Greene & Eisenberg (1980b) determined the actin–S-1 association constant ($K_{\text{S-1}}$) to be 5×10^6 M^{-1} at μ = 0.22 M, 22 °C, it follows that $K_{\text{S-1}} \simeq 2 \times 10^7$ M^{-1} at μ = 0.12 M and $K_{\text{S-1}} \simeq 2 \times 10^6$ M^{-1} at μ = 0.43 M.

The effect of ionic strength on the actin–HMM association constant ($K_{2\text{-HMM}}$) was determined from the competition of HMM and S-1 for sites on F-actin both at μ = 0.12 and 0.43 M, 22 °C. This method was used because it is not necessary to measure very low concentrations of unbound HMM (<10^{-6} M) when the actin–HMM association constant is quite strong (>10^6 M^{-1}). A detailed study of the competition method and analysis of the equations has been presented previously (Greene & Eisenberg, 1980a). The equations, derived by Hill (1978), describing the binding of HMM and S-1 to F-actin in a competition experiment are

$$\theta_{\text{HMM}} = [(1 + K_{1\text{-HMM}}[\text{HMM}] + K_{\text{S-1}}[\text{S-1}] + \vartheta)K_{1\text{-HMM}}[\text{HMM}] + 2K_{2\text{-HMM}}[\text{HMM}]]/[(1 + K_{1\text{-HMM}}[\text{HMM}] + K_{\text{S-1}}[\text{S-1}] + \vartheta)\vartheta] \quad (3)$$

$$\theta_{\text{S-1}} = K_{\text{S-1}}[\text{S-1}]/\vartheta \quad (4)$$

where

$$\vartheta = [(1 + K_{1\text{-HMM}}[\text{HMM}] + K_{\text{S-1}}[\text{S-1}])^2 + 4K_{2\text{-HMM}}[\text{HMM}]]^{1/2}$$

$\theta_{\text{S-1}}$ and θ_{HMM} are the number of moles of S-1 or HMM bound per mole of F-actin monomer, respectively, [HMM] and [S-1] are the concentrations of unbound HMM and unbound S-1, respectively, and $K_{1\text{-HMM}}$ is the binding constant of HMM (or

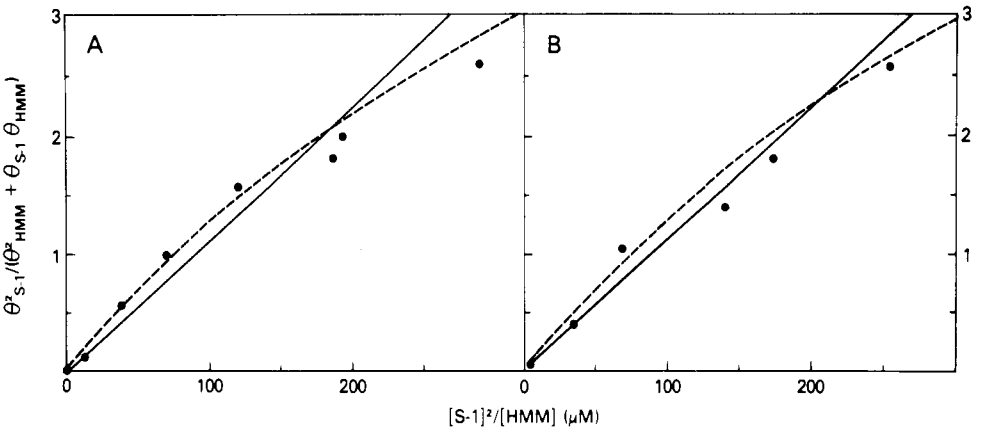


FIGURE 2: Competition of SH_1-blocked HMM and S-1 for sites on F-actin. SH_1-blocked HMM (~7 μM) and varying concentrations of S-1 (5–50 μM) were added to F-actin (11 μM). In part A, the conditions were 0.1 KCl, 12 mM imidazole (pH 7.0), 5 mM $MgCl_2$, and 0.5 mM DTT at 22 °C. The curved line (dashed line) is obtained by using eq 3 and 4 with $K_{S\text{-}1} = 2 \times 10^7$ M^{-1}, $K^*_{1\text{-}HMM} = 2 \times 10^7$ M^{-1}, and $K^*_{2\text{-}HMM} = 2 \times 10^{10}$ M^{-1}. The straight line (solid line) is obtained with $K_{S\text{-}1} = 2 \times 10^7$ M^{-1}, $K^*_{1\text{-}HMM} = 0$, and $K^*_{2\text{-}HMM} = 4 \times 10^{10}$ M^{-1}. In part B, the conditions were 0.4 M KCl, 12 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 5 mM KP_i, and 0.5 mM DTT at 22 °C. The curved line (dashed line) is obtained by using eq 3 and 4 with $K_{S\text{-}1} = 2 \times 10^6$ M^{-1}, $K^*_{1\text{-}HMM} = 2 \times 10^6$ M^{-1}, and $K^*_{2\text{-}HMM} = 2 \times 10^8$ M^{-1}. The straight line (solid line) is obtained with $K_{S\text{-}1} = 2 \times 10^6$ M^{-1}, $K^*_{1\text{-}HMM} = 0$, and $K^*_{2\text{-}HMM} = 4 \times 10^8$ M^{-1}. $K^*_{1\text{-}HMM}$ was calculated by using eq 2 where the value used for $K^*_{S\text{-}1}$ was obtained by reducing the value of $K_{S\text{-}1}$ by 45%. This is to account for the effect of SH_1 modification on the ability of S-1 to bind to actin (Greene & Eisenberg, 1980a). $\theta_{S\text{-}1}$ and θ_{HMM} are the number of moles of S-1 and HMM bound per mole of F-actin monomer, respectively.

HMM·nucleotide) to F-actin where only one actin site is occupied (see eq 1 for the definition of the other binding constants). As discussed previously, the complexity of these equations arises because of the "parking problem" which occurs because HMM binds to two actin sites rather than one.

Equations 3 and 4, used in analyzing the results from the competition experiment, assume independent binding of HMM and S-1 to sites on F-actin with no interaction between the heads of HMM, i.e., when HMM binds with only one head, the unbound head has no effect on the binding of another HMM molecule to the actin site adjacent to the site where the first HMM is bound. If this is indeed the case, then the binding constant for HMM occupying only one actin site would be equivalent to the binding constant of the first HMM head to an F-actin monomer ($K_{1\text{-}HMM} = K^{\alpha}_{HMM} = 2K_{s\text{-}1}$). On the other hand, it is possible that the unbound HMM head totally inhibits the binding of another HMM molecule to the site adjacent to the one where the first HMM head is bound. In this case, all bound HMM molecules would occupy two actin sites and $K_{1\text{-}HMM}$ would be zero. Since the degree of interference between the heads is not known, the data from the competition experiments were analyzed in two different ways to calculate the minimum and maximum values of $K_{2\text{-}HMM}$: $K_{1\text{-}HMM}$ was either equated to K^{α}_{HMM}, assuming no interference between the heads, or $K_{1\text{-}HMM}$ was equated to zero, assuming maximum interference. Of course, $K_{1\text{-}HMM}$ may well have a value between these two extremes. If this is the case, it seems very likely that $K_{2\text{-}HMM}$ would also have a value between the two calculated values, even though eq 3 and 4 would no longer be applicable in analyzing the results of the competition experiment.

The results of the competition experiments at ionic strengths of 0.12 and 0.43 M are shown in parts A and B of Figure 2, respectively. In these experiments, varying concentrations of S-1 were added to a fixed concentration of SH_1-blocked HMM and F-actin, and the amount of S-1 and HMM bound to F-actin was determined after centrifugation. At both ionic strengths, the data were first fitted to eq 3 and 4 by setting $K^*_{1\text{-}HMM} = K^{\alpha*}_{HMM} = 2K^*_{S\text{-}1}$ where K^* denotes a binding constant of an SH_1-blocked fragment to F-actin. This was done by calculating the values of $K^*_{1\text{-}HMM}$ from the values of $K_{S\text{-}1}$ obtained above, after correcting for the effect of SH_1 modification (see the legend for Figure 2). The best fit is given by the dashed curves in Figure 2. At $\mu = 0.12$ M, the value of $K^*_{2\text{-}HMM}$ was determined to be 2×10^{10} M^{-1} ($K^*_{1\text{-}HMM} = 2 \times 10^7$ M^{-1} and $K_{S\text{-}1} = 2 \times 10^7$ M^{-1}), while at $\mu = 0.43$ M, the value of $K^*_{2\text{-}HMM}$ was determined to be 2×10^8 M^{-1} ($K^*_{1\text{-}HMM} = 2 \times 10^6$ M^{-1} and $K_{S\text{-}1} = 2 \times 10^6$ M^{-1}). On the other hand, when $K^*_{1\text{-}HMM}$ was assumed to be zero, the results were analyzed by using the following equation, obtained by simplifying eq 3 and 4:

$$\frac{\theta_{S\text{-}1}^2}{\theta_{HMM}^2 + \theta_{S\text{-}1}\theta_{HMM}} = \frac{K_{S\text{-}1}^2[S\text{-}1]^2}{K_{2\text{-}HMM}[HMM]} \qquad (5)$$

This equation is applicable in analyzing the results since the competition experiments were conducted at an S-1 concentration where $K_{S\text{-}1}[S\text{-}1] > 1$ [see Greene & Eisenberg (1980a)]. The data in Figure 2 were fitted to a straight line according to eq 5. At both ionic strengths, these lines had almost identical slopes of 1×10^4 M^{-1}. Since the slope is equivalent to $K_{S\text{-}1}^2/K^*_{2\text{-}HMM}$ and the value of $K_{S\text{-}1}$ was determined above, the binding constant of SH_1-blocked HMM to F-actin can then be calculated. At $\mu = 0.12$ M, 22 °C, $K^*_{2\text{-}HMM} = 4 \times 10^{10}$ M^{-1}, and at $\mu = 0.43$M, $K^*_{2\text{-}HMM} = 4 \times 10^8$ M^{-1}.

This analysis shows that the binding constant of SH_1-blocked HMM to F-actin ranges between 2×10^{10} and 4×10^{10} M^{-1} at $\mu = 0.12$ M and between 2×10^8 and 4×10^8 M^{-1} at $\mu = 0.43$ M, depending on whether or not there is interference between HMM molecules when they bind to F-actin. In contrast, S-1 binds to F-actin with a binding constant of 2×10^7 M^{-1} at $\mu = 0.12$ and 2×10^6 M^{-1} at $\mu = 0.43$ M. Therefore, at both ionic strengths, HMM binds much more strongly to F-actin than does S-1. By use of eq 1 and 2, the binding constant of the second head of HMM to F-actin (K^{θ}_{HMM}) can be calculated to be ~10^3 at $\mu = 0.12$ M and ~10^2 at $\mu = 0.43$ M. Therefore, at both $\mu = 0.12$ M and $\mu = 0.43$ M, HMM is primarily binding with two heads to F-actin with the second head contributing significantly to the free energy of binding. Furthermore, between $\mu = 0.12$ M and $\mu = 0.43$ M, where there is a 10-fold decrease in the value of $K_{S\text{-}1}$, there is a 100-fold decrease in the value of $K^*_{2\text{-}HMM}$. Therefore, the change in ionic strength affects the binding of HMM to F-actin much more than the binding of S-1.

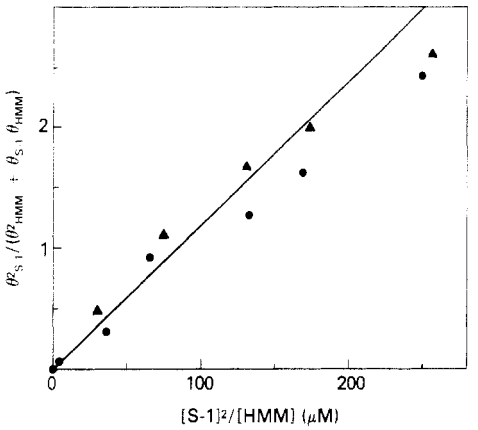


FIGURE 3: Competition of SH_1-blocked HMM and S-1 for sites on F-actin at 4 and 22 °C (μ = 0.12 M). The same enzyme concentrations were used as in Figure 2. Conditions were 0.1 M KCl, 12 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 4 mM KP_i, and 0.5 mM DTT at 4 °C (▲) or at 22 °C (●).

Effect of Temperature. Competition experiments were also conducted at 4 °C (μ = 0.12 M) to determine the effect of temperature on the binding of the second head of HMM to F-actin. As shown in Figure 3, when the data were plotted according to eq 5, there was no significant difference in results at 4 and 22 °C. Assuming maximum interference between the heads ($K^*_{1\text{-HMM}} = 0$), the best line drawn through the data had a slope, $K_{S\text{-}1}{}^2/K^*_{2\text{-HMM}}$, of 1×10^4 M^{-1} at both 4 and 22 °C. Over this temperature range, $K_{S\text{-}1}$ has been reported to change at least 3-fold (Highsmith, 1977; Woledge, 1977), although other workers have reported a 10-fold change (Chantler & Gratzer, 1976; Margossian & Lowey, 1978). Assuming a 3-fold change in $K_{S\text{-}1}$ with temperature and the value of 2×10^7 M^{-1} obtained above for $K_{S\text{-}1}$ at 22 °C, $K^*_{2\text{-HMM}}$ at 4 °C is calculated from the slope to be 5×10^9 M^{-1}, while assuming a 10-fold change in $K_{S\text{-}1}$ with temperature, $K^*_{2\text{-HMM}}$ is calculated to be 4×10^8 M^{-1}. Therefore, at 4 °C (μ = 0.12 M) the binding constant of the second head of HMM to F-actin (K^β_{HMM}) is on the order of 10^2. These results would not change significantly if it was assumed that there was no interference between the heads ($K_{1\text{-HMM}} = K^\alpha_{HMM}$). Therefore, at 4 °C ($\mu$ = 0.12 M), HMM is binding to F-actin primarily with two heads with the second head contributing significantly to the free energy of binding. In addition, since $K^*_{2\text{-HMM}}$ was determined to be 4×10^{10} M^{-1} at 22 °C (μ = 0.12 M), the effect of temperature on the binding of HMM to F-actin is the square of its effect on the binding of S-1 to F-actin, indicating that the change in enthalpy is the same for the binding of each HMM head and S-1 to F-actin.

Effect of Nucleotide. Although increasing the ionic strength and decreasing the temperature both decreased the binding of S-1 to actin, adding ADP or AMPPNP has an even greater effect on S-1 binding. It was therefore of interest to determine the effect of saturating concentrations of ADP and AMPPNP on the binding of the second head to HMM to F-actin. This binding constant (K^β_{HMM}) was again calculated by using eq 1 and 2 after measuring the binding of S-1 and HMM to F-actin under identical conditions in the presence of saturating concentrations of ADP or AMPPNP. Saturation was established by showing that doubling the nucleotide concentration produced no significant difference in the measured actin–HMM and actin–S-1 association constants. However, the general conclusions obtained from this study would apply even if a small population of the heads did not have bound nucleotide.

The binding of SH_1-blocked S-1 to F-actin was first examined in the presence of saturating concentrations of ADP (2 and 4 mM) at μ = 0.43 M, 22 °C. As shown in Figure 4A, a linear Scatchard (1949) plot is obtained with a slope, $K^*_{S\text{-}1}$, of 4×10^4 M^{-1} and an abscissa intercept, n, of 1.0 (correlation coefficient = 0.93). Similar results were obtained in the binding studies conducted in the presence of saturating concentrations of AMPPNP (1 and 2 mM) at μ = 0.12 M (Figure 4B). (These experiments were conducted at lower ionic strength so as to reduce the dissociation of the acto-S-1 complex by AMPPNP.) Here $K^*_{S\text{-}1} = 3 \times 10^4$ M^{-1} and n = 0.9 (correlation coefficient = 0.92).

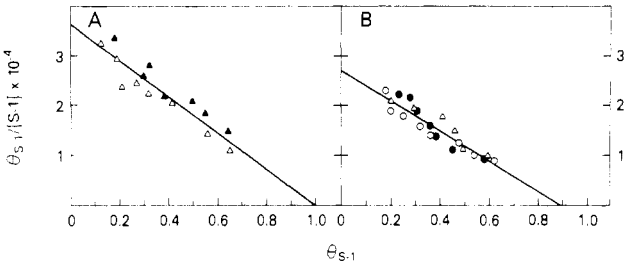


FIGURE 4: Binding of SH_1-blocked S-1 to F-actin in the presence of saturating concentrations of ADP or AMPPNP. Varying concentrations of SH_1-blocked S-1 (5–80 μM) were added to F-actin (10 μM). In part A, the binding experiments in the presence of ADP, either 2 mM (▲) or 4 mM (△), were conducted under the same conditions as in Figure 2B. In part B, the binding experiments in the presence of AMPPNP, 1.1 mM (○), 1.8 mM (△), or 2.0 mM (●), were conducted under the same conditions as in Figure 2A. With each different concentration of nucleotide, a different preparation of SH_1-blocked S-1 was used.

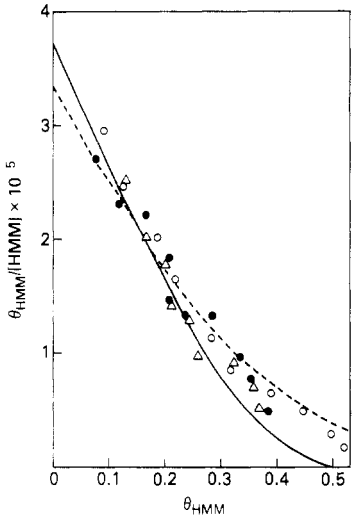


FIGURE 5: Binding of SH_1-blocked HMM to F-actin in the presence of saturating concentrations of ADP. Varying concentrations of SH_1-blocked HMM (1–30 μM) were added to 10 μM F-actin in the presence of ADP: 2 mM (●), 3 mM (○), or 4 mM (△). The conditions were the same as in Figure 2B plus 2 μM diadenosine pentaphosphate was present. The solid line is the theoretical plot obtained from eq 3 and 4 when $K^*_{1\text{-HMM}} = 0$ and $K^*_{2\text{-HMM}} = 3.7 \times 10^5$ M^{-1}, and the dashed line is obtained when $K^*_{1\text{-HMM}} = 8 \times 10^4$ M^{-1} and $K^*_{2\text{-HMM}} = 2.5 \times 10^5$ M^{-1}. A different preparation of SH_1-blocked HMM was used with each different concentration of ADP.

The binding of SH_1-blocked HMM to F-actin in the presence of nucleotide was then measured under the same conditions. In contrast to the determination of $K_{2\text{-HMM}}$ in the absence of nucleotide, here $K_{2\text{-HMM}}$ could be determined directly since the binding of HMM to actin is relatively weak in the presence of nucleotide. The results are shown in Figures 5 and 6 for experiments conducted in the presence of saturating concentrations of ADP (μ = 0.43 M, 22 °C) and AMPPNP (μ = 0.12 M, 22 °C), respectively. The data in these figures are plotted by using the same axes as in the Scatchard plots (Figure 4). However, the Scatchard equation cannot be used

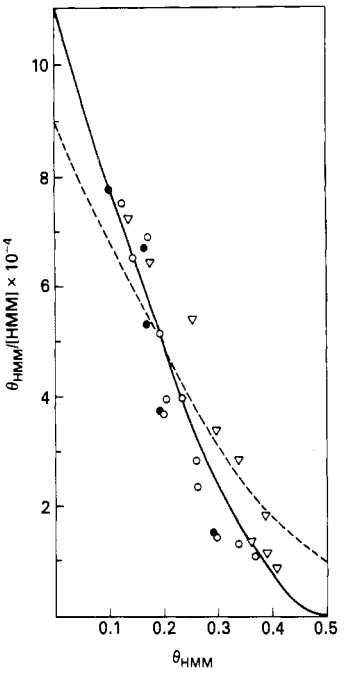


FIGURE 6: Binding of SH_1-blocked HMM to F-actin in the presence of saturating concentrations of AMPPNP. Varying concentrations of SH_1-blocked HMM (2–50 μM) were added to F-actin (11 μM) in the presence of AMPPNP: 2 mM (▽), 3 mM (○), and 4 mM (●). The conditions were the same as in Figure 2A. The solid line is the theoretical plot obtained from eq 3 and 4 when $K^*_{1\text{-HMM}} = 0$ and $K^*_{2\text{-HMM}} = 1.1 \times 10^5$ M^{-1}, and the dashed line is obtained when $K^*_{1\text{-HMM}} = 2 \times 10^4$ M^{-1} and $K^*_{2\text{-HMM}} = 7 \times 10^4$ M^{-1}. A different preparation of SH_1-blocked HMM was used with each different concentration of AMPPNP.

to analyze these results because as the sites on the F-actin filament become occupied with HMM, the number of points where two adjacent sites are available for binding decreases more rapidly than the total number of free sites (parking problem). The binding of HMM to F-actin must be analyzed by using eq 3 with [S-1] = 0. As in the analysis of the competition experiments, $K_{1\text{-HMM}}$ was either equated to K^{α}_{HMM} (no interference between the heads) or equated to zero (maximum interference between the heads).

The results obtained for the binding of SH_1-blocked HMM to F-actin in the presence of ADP were first analyzed. Assuming maximum interference between the heads ($K^*_{1\text{-HMM}} = 0$), the data were fitted with a value for $K^*_{2\text{-HMM}}$ of 3.7×10^5 M^{-1} (solid curve in Figure 5). Assuming no interference between the heads, ($K^*_{1\text{-HMM}} = 2K^*_{S\text{-}1} = 8 \times 10^4$ M^{-1}), the data were fitted with a value for $K^*_{2\text{-HMM}}$ of 2.5×10^5 M^{-1} (dashed curve in Figure 5). Since $K^*_{S\text{-}1} = 4 \times 10^4$ M^{-1} under these conditions (Figure 4A), the binding constant of the second head of HMM to F-actin (K^{β}_{HMM}) is calculated from eq 2 to have a value between 3 and 5, depending on whether or not there is interference between the heads. Therefore, the second head of HMM does not contribute significantly to the free energy of HMM binding to F-actin in the presence of ADP at $\mu = 0.43$ M, although HMM is binding primarily with two heads.

The binding of HMM to F-actin in the presence of AMPPNP was similarly analyzed. Assuming $K^*_{1\text{-HMM}} = 0$ (maximum interference between the heads), the data fit the solid curve in Figure 6 with a value for $K^*_{2\text{-HMM}}$ of 1.1×10^5 M^{-1}. Under the same conditions, $K^*_{S\text{-}1} = 3 \times 10^4$ M^{-1} so that the second head of HMM is binding very weakly to F-actin ($K^{\beta}_{HMM} = 2$). On the other hand, if it is assumed that there is no interference between the heads and $K^*_{1\text{-HMM}} = 2K^*_{S\text{-}1}$, the data in Figure 6 cannot be reasonably fitted with any value for $K^*_{2\text{-HMM}}$; i.e., there is experimentally much less one-headed binding of HMM to F-actin observed than is predicted by eq 3 in the absence of interference. These results suggest that there may be some interference between the heads in the binding of HMM to F-actin. However, if it is assumed that $K_{1\text{-HMM}} \simeq K_{S\text{-}1}$ and not $2K_{S\text{-}1}$, which is certainly possible, a reasonable fit to the data can be obtained with $K^*_{1\text{-HMM}} = 2 \times 10^4$ M^{-1} and $K^*_{2\text{-HMM}} = 7 \times 10^4$ M^{-1} (dashed curve in Figure 6). Therefore, it is not certain that in the presence of AMPPNP there is significant interference between the HMM heads when they bind to F-actin. Nevertheless, the results definitely show that the second head is binding very weakly or not at all to F-actin in the presence of AMPPNP.

Table I: Effect of Nucleotide on the Binding of HMM and S-1 to F-Actin at 22 °C

added nucleotide	μ (M)	$K^*_{S\text{-}1}$ (M^{-1})	$K_{S\text{-}1}$ (M^{-1})	$K^*_{2\text{-HMM}}$ [b] (M^{-1})	$K_{2\text{-HMM}}$ [a] (M^{-1})
0	0.12		2×10^7	4×10^{10}	5×10^{10}
AMPPNP	0.12	3×10^4 [c]	5×10^4 [a]	1.1×10^5	1.5×10^5
0	0.43		2×10^6	4×10^8	5×10^8
ADP	0.43	4×10^4 [c]	7×10^4 [a]	3.7×10^5	5×10^5

[a] Values of $K_{S\text{-}1}$ and $K_{2\text{-HMM}}$ were calculated by increasing the values of $K^*_{S\text{-}1}$ and $K^*_{2\text{-HMM}}$ by 45% and 25%, respectively (Greene & Eisenberg, 1980a). [b] Values of $K^*_{2\text{-HMM}}$ were obtained from the competition experiments (Figure 2) and binding experiments (Figures 5 and 6) when $K^*_{1\text{-HMM}} = 0$. [c] Values of $K^*_{S\text{-}1}$ were obtained from the data in Figure 4.

Discussion

In this paper the effects of ionic strength, temperature, ADP, and AMPPNP on the binding of S-1 and HMM to F-actin were studied. The results show that the contribution of the second head of HMM to the free energy of binding depends upon the experimental conditions. In the absence of nucleotide ($\mu = 0.12$ M, 22 °C, $\mu = 0.43$ M, 22 °C, and $\mu = 0.12$ M, 4 °C), the second head of HMM contributes significantly to the free energy of binding. However, in the presence of ADP ($\mu = 0.43$ M, 22 °C) or AMPPNP ($\mu = 0.12$ M, 22 °C), the second head of HMM does not contribute significantly to the free energy of binding. The results also show that a change in conditions (ionic strength, temperature, or the presence of nucleotide) affects the binding of HMM to F-actin much more than the binding of S-1. In fact, as shown in Table I, the effect of a particular agent on the binding of HMM to F-actin is approximately the square of its effect on the binding of S-1 to F-actin. Increasing the ionic strength from 0.12 to 0.43 M in the absence of nucleotide weakens the binding of S-1 to F-actin ~10-fold, but it weakens the binding of HMM to F-actin ~100-fold. Even more striking, at $\mu = 0.43$ M ADP weakens the binding of S-1 to F-actin ~30-fold, but it weakens the binding of HMM ~1000-fold. Likewise, at $\mu = 0.12$ M, AMPPNP weakens the binding of S-1 to F-actin ~400-fold, but it weakens the binding of HMM almost 300 000-fold.

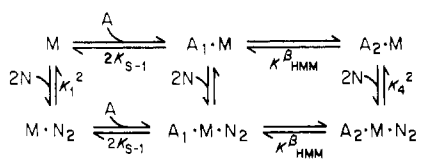
As noted above (eq 2) there is evidence that $K^{\alpha}_{HMM} = 2K_{S\text{-}1}$; i.e., the binding constant of the first head of HMM to F-actin is affected to the same extent as the binding constant of S-1 to F-actin by a change in conditions. On this basis, the results in Table I suggest that the various agents which affect the binding of S-1 to F-actin affect the binding of the second head of HMM to the same extent. This would explain why the effect of these agents on HMM binding is always the square of the effect on S-1 binding.

Based on the analysis of Hill & Eisenberg (1980) on the equilibrium binding of myosin fragments to F-actin, the best explanation for the finding that the agents employed in this paper appear to affect the binding of each of the HMM heads

and S-1 in an identical manner is that these agents are affecting only the binding energy of the heads. The binding energy very likely has the same value for the binding of each of the HMM heads, as well as for the binding of S-1. On the other hand, these agents do not appear to affect the various motions of the HMM molecule or the distortion which probably occurs in the HMM molecule when both heads are bound. If these agents affected the motion of the heads or the distortion in the HMM molecule, they would not be expected to have an identical effect on the binding of S-1 and each of the HMM heads since the motion and the distortion are different for S-1 and the HMM heads.

It is not surprising that ionic strength and the binding of ADP and AMPPNP have their major effect on the binding energy of the heads. However, changes in temperature might be expected to affect the motion of the heads as well. Therefore, it may be fortuitous that changes in temperature affect the binding of each of the HMM heads and S-1 in an identical fashion (Hill & Eisenberg, 1980).

Greene & Eisenberg (1980a) previously presented a scheme for the binding of the HMM to F-actin both in the presence and in the absence of nucleotide. This scheme has been expanded to include the two-step binding of HMM to F-actin described in eq 1 and 2:



M is HMM, A is actin, and N is nucleotide. A_x and N_x indicates that x molecules of F-actin monomer and nucleotide, respectively, are bound to HMM. K_1 is the association constant of nucleotide to S-1 and K_4 is the association constant of nucleotide to acto-S-1 (see eq 1 for the definition of other association constants). This model assumes independent binding of the myosin fragments to F-actin and no interaction between the heads of HMM. The above scheme explains the similarity between the actin–HMM and actin–S-1 association constants in the presence of nucleotide and the difference between these association constants in the absence of nucleotide. However, this scheme is unable to account for the results obtained in the presence of AMPPNP. As discussed above, the data for the binding of HMM to F-actin in the presence of AMPPNP (Figure 6) could not be fitted by using eq 3 with a value of $K_{2\text{-HMM}}$ when $K_{1\text{-HMM}} = 2K_{S\text{-}1}$. Therefore, the above scheme must be modified. A change as simple as making $K_{1\text{-HMM}} \simeq K_{S\text{-}1}$ instead of $2K_{S\text{-}1}$ would enable the data obtained in the presence of AMPPNP to fit the scheme. On the other hand, it is quite possible that HMM is binding to F-actin in the presence of AMPPNP with only one head, while the detached head is either partially or completely interfering with the binding of another HMM molecule at the available adjacent actin site. To determine whether this is the case, it will be necessary to test whether both actin sites "occupied" by HMM actually have HMM heads bound to them in the presence of AMPPNP. This might be possible through the use of fluorescent probes attached to the HMM heads.

In conclusion, this study shows that the contribution each of the HMM heads makes to the free energy of binding depends upon the experimental conditions. In the absence of nucleotide, both heads of HMM contribute significantly to the free energy of binding, but not in the presence of ADP (μ = 0.43 M, 22 °C) or AMPPNP (μ = 0.12 M, 22 °C). Furthermore, the results suggest that the binding of each of the two HMM heads is affected to the same extent as the binding of S-1 to F-actin by a change in conditions, e.g., ionic strength, temperature, or the presence of nucleotide.

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