Heavy Meromyosin Binds Actin with Negative Cooperativity†

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ABSTRACT: The association of fluorescently labeled heavy meromyosin (HMM) and F-actin was measured by time-resolved fluorescence depolarization. The effects of varying the protein concentrations, temperature, KCl concentration, and pH were determined. Measurements of HMM mobility supported a model of no interaction between the two heads in the absence of actin. Measurements of actin binding, when compared with results for myosin subfragment 1, indicated that the two heads of HMM do not bind independently in the rigor complex. This could result from actin-transmitted negative cooperativity or from steric inhibition due to the structure of HMM. For HMM and actin in 0.15 M KCl at 25 °C: $K_a = 3.9 \times 10^7$ M^{-1}, $\Delta H_c^{\circ\prime} = 36 \pm 2$ kJ M^{-1}, $\Delta S_c^{\circ\prime} = 0.26 \pm 0.02$ kJ M^{-1} K^{-1}; the slope of ln K_a vs. [KCl]$^{1/2} = -3.88$ and the pH of maximum association was 6.9.

One of the interesting questions about the structure of muscle is why does the myosin molecule have two heads? When the thick filament is solubilized in high ionic strength media, the resulting myosin has a duplex nature. Its structure is a long rod of two coiled-coil α-helices with two globular heads at one end. In nature, myosin does not occur alone but is aggregated, due to the low solubility of the rod portion, into the thick filament. Nonetheless, the proximity of the two heads has invited speculation about their possible interactions and has stimulated many experiments.

The results of these experiments have not determined unambiguously what, if any, interactions occur between the heads. With regard to myosin ATPase, the two heads have been reported by some to act equally and independently (Murphy and Morales, 1970; Taylor, 1977) and by others to be different in their ATPase mechanisms (Tonomura and Inoue, 1977). In the case of results from experiments on actomyosin ATPase, superprecipitation and contractile threads, the conclusions vary from assertion that the heads are structurally and functionally different (Inoue and Tonomura, 1976) to assertion that they are an interacting pair (Tokiwa and Morales, 1971), to assertion that they are equal and independent (Margossian and Lowey, 1973a; Gadasi et al., 1974; Cooke and Franks, 1977). Unfortunately in x-ray diffraction and electron microscopy studies of muscle fibers, the interpretation of the results is hampered because one cannot be sure if the "crossbridge" is composed of one or two heads.

In the present work, the simplest interaction of myosin with actin, the formation of the rigor bond in solution, has been investigated. The rigor bond corresponds to the energetic minimum of the contractile cycle (Morales, 1975). The approach was to measure the effects of protein concentration, temperature, KCl concentration, divalent ions, and pH on the association constant K_a for HMM[1] (the duplex fragment of myosin which is soluble under physiological conditions) and actin. These results were interpreted and compared with earlier results for S-1 (the single-headed fragment) and actin under equivalent conditions (Highsmith, 1977). By using time-resolved fluorescence depolarization to measure the binding (Highsmith et al., 1976), additional information about the mobility of the two S-1 moieties of HMM was obtained. In the case of free HMM, i.e., with no actin present, this information was interpreted in terms of a possible intramolecular equilibrium between self-associated heads and dissociated heads.

Experimental Section

HMM was prepared from myosin by cleavage with trypsin which had been treated with tosylphenylalanine chloromethyl ketone (trypsin-Tos-PheCH$_2$Cl, Worthington Biochemicals). The method was a modification of that of Margossian and Lowey (1973b). The digestion consisted of 120 s at 25 °C in 0.50 M KCl, 0.050 M K$_2$HPO$_4$, pH 6.2. The molar ratio of trypsin-Tos-PheCH$_2$Cl to myosin was 1:350. The reaction was stopped with a 3:1 molar excess of soybean trypsin inhibitor. Myosin and LMM were removed by dialyzing to 0.02 M KCl, 0.002 M K$_2$HPO$_4$, pH 6.2, and sedimenting at 150 000*g* for 60 min. HMM was isolated from the supernate by (NH$_4$)$_2$SO$_4$ precipitation (70% saturated at 4 °C). HMM prepared this way moved as a single peak on Sephadex G-200 equilibrated and eluted with 0.15 M KCl, 0.010 M Tes, pH 7.0, and showed a major (>80%) heavy chain band at about 110 000 amu and three light chains on sodium dodecyl sulfate–polyacrylamide gels. Its Ca^{2+}-ATPase activity at 25 °C in 0.6 M KCl was 72 mol of PO$_4^{2-}$ mol^{-1} s^{-1} and its Ca^{2+}-ITPase activity was 96 mol of PO$_4^{2-}$ mol^{-1} s^{-1} when determined by the method of Fiske and SubbaRow (1925). Actin activated ATPase in 30 mM KCl, 5 mM Mg-ATP, pH 7.0, at 22 °C was 3.3 μmol of PO$_4^{2-}$ g^{-1} s^{-1}. HMM was labeled with (*N*-iodoacetyl-*N*′-1-sulfo-5-naphthyl)ethylenediamine (1,5-IAEDANS) by incubating the HMM at 4 °C in the dark for 24 h with a 1.5 molar excess of dye.

S-1 was prepared by the method of Cooke (1972); its properties were described by Highsmith et al. (1976).

Actin was prepared by the method of Spudich and Watt (1971) and generously supplied by Dr. Roger Cooke of the University of California at San Francisco. Residual ATP was removed as described earlier (Highsmith, 1977).

All protein stock solutions were stored at 20 μM concentrations at 4 °C and were diluted immediately before making a measurement. Repeated measurements on solutions of

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[1] Abbreviations used: HMM, heavy meromysin; S-1, myosin subfragment 1; EDTA, ethylenediaminetetraacetic acid; EGTA, 2,2′-ethylenedioxybis(ethyliminodiacetic acid); TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tos-PheCH$_2$Cl, tosylphenylalanine chloromethyl ketone; EPR, electron paramagnetic resonance; 1,5-IAEDANS, (*N*-iodoacetyl-*N*′-1-sulfo-5-naphthyl)ethylenediamine.

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HMM and actin gave the same results within experimental error; so actin does not appear to depolymerize significantly under these conditions. All measurements were made on solutions of proteins in 0.15 M KCl, 3 mM $MgCl_2$, 3 mM EGTA, 10 mM Tes, pH 7.0, at 4 °C unless stated otherwise.

The time-resolved fluorimeter (Mendelson et al., 1975) and the precision and limitations of this method of measuring association constants have been detailed in earlier publications (Highsmith et al., 1976).

Data Analysis

The expression for the anisotropy of a solution of fluorophores with equal intensities which are in i different states was given by Wahl (1969) as:

$$r(t) = \sum_i x_i r_i(t) \tag{1}$$

where $r_i(t)$ is the anisotropy and x_i is the mole fraction of fluorophores in state i. The determination of association constants using this expression was described for actin and S-1 (Highsmith et al., 1976). In order to obtain the mole fractions of free and bound HMM, a model is required for the acto-HMM interaction. A priori, one must consider HMM in three states: one with both heads free of actin, one with one head free and one head immobilized, and one with both heads immobilized. Fortunately, there is evidence that, when one head binds, the second one has a high probability of binding. Mendelson et al. (1973) have shown by time-resolved fluorescence depolarization that both heads of HMM and myosin are immobilized by actin. Thomas et al. (1975) have confirmed this result by the method of saturation transfer EPR. Recently Lin (1977) has published preliminary results for HMM binding to labeled actin indicating that the second head binds more strongly than the first. The results reported here confirm these earlier ones and the acto-HMM interaction has been analyzed as a two state system—either unbound with both heads free to rotate or bound with both heads immobilized. Thus, eq 1 becomes:

$$r(t) = x_F r_F(0) e^{-t/\Phi_F} + (1 - x_F) r_B(0) e^{-t/\Phi_B} \tag{2}$$

where $r(0)$ is the anisotropy before any rotation has occurred, Φ is the rotational correlation time (the time required for $r(t)$ to reach $(1/e)r(0)$), and the subscripts F and B refer to free and bound states. Values for $r_F(0)$ and Φ_F were obtained from measurements on solutions of HMM with no actin present. Φ_B was large enough for e^{-t/Φ_B} to be approximated as 1.0 for the time scale of a measurement. The parameters x_F and $r_B(0)$ were obtained by least-squares curve fitting of the fluorescence polarization decay data.

The reaction of HMM and actin in a two state equilibrium is:

$$\text{HMM} + \text{AA} \rightleftharpoons \text{HMM}{=}\text{AA} \tag{3}$$

where AA is the concentration of HMM binding sites on actin and the double line in HMM=AA indicates that HMM has both heads immobilized. Peller (1975) has developed a model assuming that AA is composed of two neighboring HMM binding sites on the F-actin. In the present case where there is low occupancy of the sites on actin, AA equals the concentration of actin monomer, assuming the binding sites on actin to be equivalent. The association constant is then calculated as:

$$K_a = \frac{1 - x_F}{x_F([A]_0 - (1 - x_F)[HMM]_0)} \tag{4}$$

where $[A]_0$ and $[HMM]_0$ are the total concentrations of actin monomers and HMM in moles/liter.

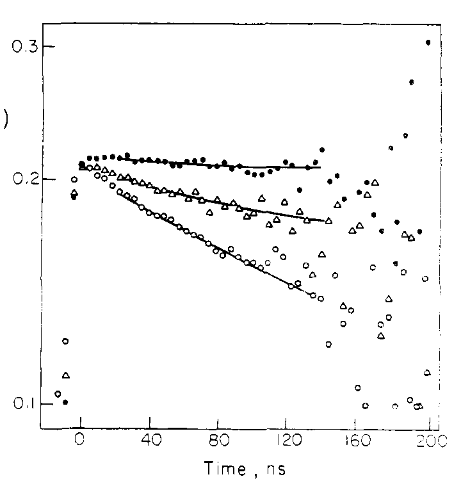

FIGURE 1: Anisotropy decay. The symbols are experimental data for the fluorescence depolarization of 1,5-IAEDANS-labeled HMM. The lowest curve is for 1.96 × 10^{-7} M HMM and was analyzed as a single exponential to obtain $r_F(0)$ and Φ_F. The upper data are for HMM with 2.99 × 10^{-7} M and 8.78 × 10^{-7} M F-actin in ascending order and were fitted by least squares to eq 2 to obtain X_F and $r_B(0)$. Time equal zero was taken as the peak of the lamp intensity (not shown). The solid lines indicate the region which was fitted to eq 2.

One of the parameters used to probe for possible interactions between the heads of HMM in the absence of actin was the hydrodynamic volume, V_h. It would be expected to be different for HMM with both heads free or with them self-associated. The hydrodynamic volume was obtained using the equation (Tanford, 1961)

$$\Phi_F = \frac{3\eta V_h}{kT} \tag{5}$$

where η is the viscosity of water at temperature T and k is the Boltzmann constant. Applying this equation implies that HMM is a sphere, which is a poor approximation. However, since only *changes* in V_h were being sought, eq 5 was used, to get qualitative results: Φ_F was plotted against η/T. A straight line for such a plot shows that V_h is constant and that no global conformational change is occurring in that temperature range.

Results

HMM Mobility

Labeling with 1,5-IAEDANS. The rotational mobility of HMM at 4 °C in 0.15 M KCl, 0.003 M $MgCl_2$, 0.003 M EGTA, 0.010 M Tes, pH 7.0, was the same within experimental error whether it was labeled on one head (0.3:1 dye to HMM) or both heads (1.5:1 dye to HMM).

Divalent Cations and Chelators. When neither cations nor chelators were added to HMM in 0.15 M KCl, 0.010 M Tes, pH 7.0, at 4 °C, Φ_F was 386 ± 20 ns and $r_F(0)$ was 0.21 ± 0.01. The addition of Mg^{2+}, Ca^{2+}, or Mn^{2+} in the millimolar range did not affect Φ_F or $r_F(0)$ measurably. When up to 3 mM EDTA or EGTA was added, there was no effect.

Temperature, KCl, and pH. The mobility of HMM changed in a regular fashion between 0 and 25 °C. When Φ_F was plotted against η/T, the points were well fitted by a straight line (not shown) indicating that HMM does not undergo any global conformational change in this temperature range.

KCl in the range of 0.05 to 0.60 M and pH in the range of 6.3 to 8.6 had no effect on the mobility of HMM at 4 °C.

Actin. Incremental additions of F-actin increasingly reduced the mobility of HMM. Both heads were completely immobilized when there was an 8× molar excess of total actin over 0.20

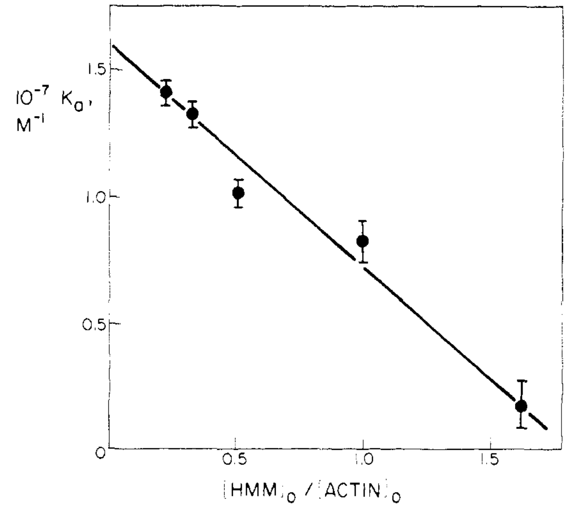

FIGURE 2: The association constant K_a, for HMM and actin as a function of the ratio of total HMM to total actin in the presence of 3 mM $MgCl_2$, 3 mM EGTA. The solutions also contained 0.15 M KCl, 0.010 M Tes, pH 7.0, at 4 °C.

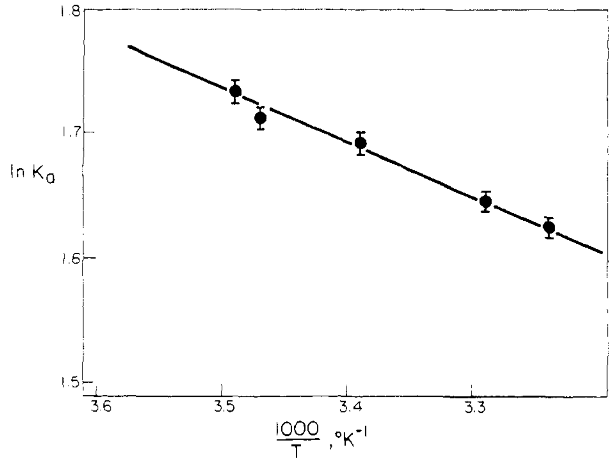

FIGURE 3: The temperature dependence of ln K_a for HMM and actin in the presence of 3 mM $MgCl_2$, 3 mM EGTA. The solutions were 0.184 μM HMM, 0.823 μM actin, 0.15 M KCl, 0.010 M Tes, pH 7.0.

μM HMM. Typical results for additions of actin are shown in Figure 1.

HMM-Actin Binding

Measurements were made on solutions of HMM and actin in the presence of 3 mM $MgCl_2$ and 3 mM EGTA. Solution also contained 10 mM Tes, pH 7.0, and 0.15 M KCl except when the pH or KCl concentration was being varied. The effect of labeling the HMM was tested by measuring the mole fraction free for label to HMM ratios between 0.25 and 0.75. No measurable difference could be detected.

Concentration Dependence. The association constant varied with the ratio of total HMM to total actin. At 4 °C, K_a increased as the ratio was increased by adding actin, as shown in Figure 2. The value of K_a for $[\text{HMM}]_0/[\text{actin}]_0$ extrapolated to zero was 1.6×10^7 M^{-1}; this value will be compared with the K_a for S-1 and actin.

Temperature Effects. All measurements were made on solutions of 0.18×10^{-6} M HMM and 0.84×10^{-6} M actin. This ratio of protein concentrations gave K_a close enough to the extrapolated value for differences between the measured and extrapolated values to be ignored.

Increasing the temperature increased the binding. A plot of ln K_a against T^{-1} was linear between 0 and 25 °C as shown in Figure 3. The standard enthalpy change was calculated to be 36 ± 2 kJ M^{-1}. At 25 °C, the standard free energy change was $\Delta G_c^{\circ\prime} = -43 \pm 2$ kJ M^{-1}. The standard entropy change was calculated from $\Delta G_c^{\circ\prime}$ and $\Delta H_c^{\circ\prime}$ to be 0.26 ± 0.02 kJ M^{-1} K^{-1}.[2]

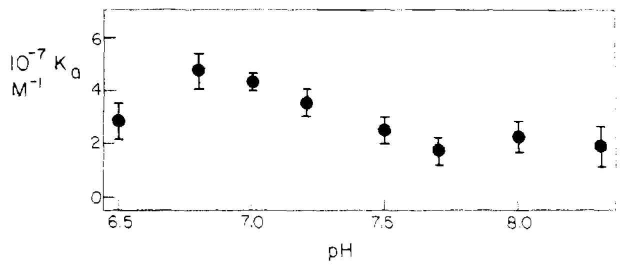

FIGURE 4: The association constant, K_a, for HMM and actin as a function of pH. The solutions were 0.183 μM HMM, 0.544 μM actin, 0.15 M KCl, 4 °C (see text for buffers).

KCl Concentration Dependence. All measurements were made on solutions of 0.18×10^{-6} M HMM, 0.84×10^{-6} M actin, 3 mM $MgCl_2$, 3 mM EGTA, 0.010 M Tes, pH 7.0, at 4 °C. KCl was varied between 0.05 and 0.60 M. Increasing the KCl concentration decreased K_a. When K_a was plotted against [KCl], a straight line was obtained (not shown). This gave a somewhat better fit than ln K_a vs. [KCl] or $[\text{KCl}]^{1/2}$. No rigorous theoretical treatment of the KCl dependence was attempted. The slope for a plot of ln K_a vs. $[\text{KCl}]^{1/2}$ was −3.88 and is reported for comparison to the acto-(S-1) value.

pH Dependence. All measurements were made on solutions of 0.20×10^{-6} M HMM, 0.47×10^{-6} M actin at 4 °C with 3.0×10^{-5} M buffer: histidine for pH 6.5; Tes for pH 6.8, 7.0, 7.2, and 7.5; and Tris for pH 7.7, 8.0, and 8.3. The pH was checked in the cuvette before and after each experiment. Measurements at lower and higher pH values were attempted but HMM had spurious Φ_F values which were not reversed when the pH was returned to 7.0. This was probably due to protein denaturation at the pH extremes. The binding was maximum near pH 7.0 as shown in Figure 4.

Discussion and Conclusions

HMM Mobility. If the two heads of actin-free HMM were in equilibrium between a self-associated state and a dissociated state, one would expect the mobility of the heads in the associated state to be different from the dissociated state. In such a case, the perturbations of temperature, KCl concentration, and pH would very likely affect the equilibrium and likewise the mobility. The absence of any measurable changes in Φ_F for variations of KCl and pH or any change in V_h between 0 and 25 °C argues against there being such an equilibrium. The heads appear to remain unassociated.

HMM-Actin Binding. The association constant for HMM and actin at 4 °C obtained by extrapolating to conditions of excess actin in 0.15 M KCl was 1.6×10^7 M^{-1}. Margossian and Lowey (1973a) found $K_a = 4 \times 10^7$ M^{-1} at 7 °C in 0.10 M KCl pH 7.0, using sedimentation methods, which is in good agreement with the results presented here. Eisenberg et al. (1972), also using the ultracentrifuge, found $K_a > 2 \times 10^6$ M^{-1} at 4 °C in 20 mM KCl, pH 7.0. Takeuchi and Tonomura

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[2] All thermodynamic parameters are given using the notation [(1976) *J. Biol. Chem. 251*, 6879]: $\Delta G_c^{\circ\prime}$ indicates the standard free energy change at pH 7.0 obtained from equilibrium constants calculated from concentrations. The concentration of water was not introduced into the calculations.

(1971) using light scattering methods found $K_a = 4 \times 10^6\ \mathrm{M}^{-1}$ at 4 °C and 0.1 M KCl, pH 8.8.

The value of K_a for HMM, when compared with that for S-1, justifies having analyzed the binding as a simple two-step reaction. The details of the binding probably involve one head binding followed by the second head binding. This reaction is shown in eq 6

$$\mathrm{HMM} + \mathrm{AA} \overset{K_1}{\rightleftharpoons} \mathrm{HMM{-}AA} \overset{K_2}{\rightleftharpoons} \mathrm{HMM{=}AA} \qquad (6)$$

where HMM—AA and HMM=AA are HMM bound by one and two heads, respectively. The K_a measured in these experiments equals K_1K_2. If one assumes that K_1 is the same as the association constant for S-1 (Highsmith et al., 1976), then K_2 equals 10 and 90% of the HMM has both heads bound. Actually, K_a for S-1 is probably an upper limit for K_1 and 90% is a lower limit for the percentage of HMM bound by two heads.

The slope of the plot of K_a against $[\mathrm{HMM}]_0/[\mathrm{actin}]_0$ is negative (Figure 2). This could result from one HMM, when bound, inhibiting the binding of a second. Another explanation, predicted by Peller's treatment of the binding as linear Ising system (1975), is that, as the actin sites are filled, single sites which cannot accommodate duplex HMM appear and reduce the effective concentration of actin. Given the low occupancy of actin in these experiments for all but the highest ratios of HMM to actin, this seems unlikely if the HMM is distributed randomly over the actin. Finally, the possibility that HMM binds to two actin filaments at high actin to HMM ratios cannot be excluded as an explanation of the negative slope.

Next consider what interactions may be occurring between the heads when HMM is bound. The potential complication of myosin having heads with different properties (Inoue and Tonomura, 1976) is not a problem in this analysis. Differences between the heads do not affect the following discussion because S-1 data represent an average which may be compared with the HMM data. Furthermore, the two S-1 species isolated by Yagi and Otani (1974) and Weeds and Taylor (1975) appear to have the same rigor association constant (Wadzinski et al., 1977). A priori, several modes of binding are possible for HMM. Both heads could bind in a manner identical with that for S-1. One head could bind like S-1 and the other bind differently or not at all. Or, both heads could bind but differently from S-1. The last two catagories include many variations. In order to reduce the number of possible modes of binding for HMM, the data at hand were compared with data for S-1 and actin collected earlier in this laboratory using the same instrument and identical conditions where the only difference was that S-1 was replaced by HMM. (Highsmith, 1977) These results are compiled in Table I to facilitate the following discussion.

The association constant for HMM and actin is only tenfold greater than for S-1 and actin. The value for HMM is several orders of magnitude smaller than one would predict if both heads behaved energetically as independent S-1's (Chantler and Gratzer, 1976). In that case the free energy change upon binding would be additive for the two heads and be near 80 kJ M^{-1}; instead, it is 43 kJ M^{-1}. A more rigorous test for independent binding is to see if $\Delta H_c^{\circ\prime}$ for HMM is double that for S-1. As seen in Table I, clearly it is not double. The fact that $\Delta H_c^{\circ\prime}$ for HMM is not double that for S-1 is also inconsistent with the model developed by Peller (1975) where the difference in binding is rationalized purely by the entropic contribution of having the second head close to actin when the first is bound. Woledge (1977) measured ΔH° calorimetrically and obtained a value of 38 kJ M^{-1} for HMM in excellent agreement with the results presented here. However his value for S-1 was 22 kJ M^{-1}, much smaller than the value in Table I and close to half the value for HMM. However, for reasons detailed in his report. Woledge also concludes that independent and identical binding of the two heads is not occurring.

TABLE I: Data from Measurements of the Association of F-Actin and HMM or S-1.[a]

	HMM	S-1
K_a (at 4 °C)	$1.6 \times 10^7\ \mathrm{M}^{-1}$	$1.7 \times 10^6\ \mathrm{M}^{-1}$[b]
$\Delta G_c^{\circ\prime}$	-43 ± 2 kJ M^{-1}	-39 ± 1 kJ M^{-1}[b]
$\Delta H_c^{\circ\prime}$	36 ± 2 kJ M^{-1}	44 ± 2 kJ M^{-1}[c]
$\Delta S_c^{\circ\prime}$	0.26 ± 0.02 kJ $\mathrm{M}^{-1}\ \mathrm{K}^{-1}$	0.28 ± 0.02 kJ $\mathrm{M}^{-1}\ \mathrm{K}^{-1}$
ln K_a vs. $[\mathrm{KCl}]^{1/2}$ slope	−3.88	−8.36
K_a vs. $[\mathrm{HMM}]_0/[\mathrm{actin}]_0$ slope	Negative	Positive
Φ_B	∞	∞

[a] See text for conditions. [b] Highsmith et al. (1976). [c] Highsmith (1977).

The highly specific array of actin and myosin in muscle allows the possibility that the two S-1 moieties of a myosin may interact with two different thin filaments. This possibility must also be considered for the case at hand of HMM and F-actin in solution. The increase in K_a as $[\mathrm{HMM}]_0/[\mathrm{actin}]_0$ decreases suggests possible binding to two filaments, but the extrapolated value for K_a with actin in great excess would be much larger (see above) if the two heads bound independently. The absence of any apparent binding of HMM to two actin filaments or to two thin filaments in electron micrographs (Huxley, 1967) also argues against HMM binding two filaments in solution. Given the large number of binding sites on an F-actin molecule, if HMM bound different F-actins, gels would form at very low concentrations of proteins. Solutions of HMM and actin remained quite liquid during these experiments in the micromolar concentration range.

The K_as for S-1 and HMM are similar enough to consider the possibilities of one head of HMM binding and the other not interacting with actin at all or only very weakly. The first possibility was eliminated by the fact that Φ_B is virtually infinite for HMM and the second made unlikely by $\Delta H_c^{\circ\prime}$ and the slope of the ln K_a vs. $[\mathrm{KCl}]^{1/2}$ plot both being very different for S-1 and HMM. If HMM binding was predominantly due to one head, the change in enthalpy and the semiquantitative effect of KCl reflected by the slopes of ln K_a vs; $[\mathrm{KCl}]^{1/2}$ in Table I would be similar for S-1 and HMM. Thus, none of the modes of binding where HMM is bound like S-1 (with the first head), but with an appendage (the second head) which is free or interacts weakly with actin or with the first head, is consistent with these data.

There are still several modes of binding which are consistent with the results in Table I. These have both heads involved in binding but not energetically identical with S-1. Given the lack of any evidence for an interaction between the heads in the absence of actin and nucleotide, it seems reasonable to assume both heads interact directly with actin rather than with one another. The difference between the binding of HMM and what one would predict for two S-1's could result from a cooperative interaction of the actin filament when one head binds or from a passive mechanism where the heads of HMM are sterically unable to simultaneously bind. Steric inhibition to achieving two S-1 bonds with actin could have energetic as well as attitudinal manifestations. Energetically, the change in standard free energy of binding could be distributed equally or unequally between the heads. Electron micrographs of actin decorated with myosin, HMM and S-1 have nearly identical appearance (Moore et al., 1970) implying that both heads of HMM are attitudinally very similar to S-1 when bound to actin. Likewise, the results of polarization measurements on thin filaments decorated with fluorescently labeled HMM (Borejdo and Putnam, 1977) and S-1 (Tregear and Mendelson, 1975) show [illegible] within experimental error HMM and S-1 have equal parallel and perpendicular polarizations when bound to actin. If this is interpreted to mean that both heads of HMM are attitudinally equal to S-1, then energy from the binding must have been utilized to overcome the geometric inhibition. In other words, to get both heads of HMM bound in positions identical with that of S-1, strain must be introduced into the complex. The two models involving steric inhibition, with or without strain, and the one involving actin cooperatively are consistent with the data presented here.

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