Heavy Meromyosin: Evidence for a Refractory State Unable to Bind to Actin in the Presence of ATP

(muscle/myosin/enzyme kinetics)

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ABSTRACT The binding of actin to heavy meromyosin (HMM) in the presence of ATP was studied by analytical ultracentrifugation and ATPase studies. At 0°C, at very low ionic strength, the double-reciprocal plot of HMM ATPase against actin concentration is linear. If one assumes that all of the HMM is bound to actin when the ATPase activity equals V_max, then, at an actin concentration where the actin-HMM ATPase is 85% of V_max all but 15% of the HMM should be complexed with actin. However, when the binding of HMM to actin in the presence of ATP was measured with the analytical ultracentrifuge, more than 60% of the HMM was not bound to actin. From experiments with EDTA- and Ca-ATPases it seemed unlikely that the unbound HMM was denatured. It is thus possible that during the steady-state hydrolysis of ATP, HMM spends more than 50% of its cycle of interaction with actin and ATP in a "refractory state," unable to bind to actin, i.e., while an HMM molecule goes through one cycle of interaction with actin and ATP, an actin monomer could bind and release several HMM molecules so that the turnover rate per mole of added actin would be considerably greater than that per mole of added HMM. Comparison of the rate of ATPase activity at very high actin concentration with that at very high HMM concentration shows that this is indeed so. Therefore, both kinetic and ultracentrifugation studies suggest that the HMM exists in a refractory state during a large part of its cycle of interaction with actin and ATP.

The sliding filament theory of muscle contraction proposes that contraction is caused by the cyclic formation and breakage of links between actin and myosin filaments, accompanied by the hydrolysis of ATP (1). One of the key questions in the biochemistry of muscle is, therefore, the nature of the interaction of actin, myosin, and ATP in vitro. Under in vitro conditions, at low ionic strength, both F-actin and myosin are filaments and, therefore, the interaction between the two proteins is very difficult to study quantitatively. However, the enzymatically active portion of the myosin molecule known as heavy meromyosin (HMM), obtained by proteolytic digestion, does not form filaments at low ionic strength but, nevertheless, retains the basic structure of the myosin molecule, having two identical enzymatic sites or "heads," each of which is capable of hydrolyzing ATP and binding to actin (2, 3).

Recent studies with the analytical ultracentrifuge on the binding of actin to HMM in the absence of ATP have shown that the binding constant is greater than $2 \times 10^8$ M and the molar binding ratio is 2 mol of actin monomer per mol of HMM (4, 5). These studies are consistent with the situation in vivo where in muscle in rigor, i.e., in the absence of ATP, it is thought that most of the myosin bridges bind to actin (1, 6).

On the other hand, in the presence of ATP a completely different situation obtains. First, x-ray diffraction studies of contracting muscle suggest that less than half of the myosin bridges bind to actin at any one time (7, 8). Second, viscosity and turbidity, which show marked increases when actin and HMM interact in the absence of ATP, show no increase in its presence under conditions where enzymatic studies give clear evidence of interaction, with the actin markedly increasing the ATPase activity of HMM (HMM ATPase) (9-11). Third, in a kinetic study at 6°C, the data suggested that one HMM molecule binds to one actin monomer in contrast to the one-to-two ratio observed in the absence of ATP (12).

In the light of these differences in the interaction of actin with HMM in the presence and absence of ATP, we have investigated this interaction in the presence of ATP using both ATPase (ATP phosphohydrolase EC 3.6.1.3) studies and analytical ultracentrifugation. Unexpectedly both of these methods indicate that under conditions where the actin has maximally activated the HMM ATPase, more than 50% of the HMM is in fact dissociated from the actin. It therefore appears that during the cycle of actin--HMM interaction in the presence of ATP, much of the HMM exists in a "refractory" state in which, even at very high actin concentration, it cannot bind to actin.

MATERIALS AND METHODS

Myosin was prepared by the method of Kielley and Harrington (13) and HMM was prepared from the myosin as described (11). Actin was prepared with a Sephadex G-200 column as described by Adelstein, et al. (14). All protein concentrations were determined (11) by UV absorption at 280 nm. ATPase was measured at pH 7.0 and 0.5°C with a Radiometer pH-stat (11). Temperature was monitored during the reaction by a Yellowstone thermistor and no. 427 Teflon-coated probe. The centrifuge experiments were performed in a model E Analytical Ultracentrifuge equipped with a photodensit electric scanner. A two-hole aluminum rotor and 12-mm double sector cells were used, all precooled to 0°C. The reference side of the cell always contained solvent, including
nucleotide, while the sample side contained either actin, HMM, or both. In samples containing both actin and HMM, the ATP was rapidly hydrolyzed so it was crucial that the centrifuge reach our running speed of 30,000 rpm as quickly as possible. In general it took 6.5 min from the time the reaction was started, by the addition of HMM to a sample stirring in an ice bath, for the rotor to reach 30,000 rpm. All samples were centrifuged at 0–1°C, and scans were taken at 290 nm to minimize absorption by ATP. For purposes of calculation the molecular weights of myosin, HMM, and actin were taken to be 500,000 (3), 350,000 (15), and 45,000 (16), respectively.

RESULTS

It has been shown that under almost all conditions studied, double-reciprocal plots of HMM ATPase against actin concentration are linear and that the intercepts of the ordinate and abscissa yield, respectively, the turnover rate of the actin–HMM–ATP complex and the apparent binding constant of the HMM–ATP complex to actin (12, 17, 18). The simplest interpretation of such plots is that the binding of the HMM–ATP complex to actin follows a normal hyperbolic curve, with essentially all of the HMM–ATP complex binding to actin at high actin concentrations. To test whether this is indeed the case, one can use an analytical ultracentrifuge equipped with a photoelectric scanner to directly measure the binding of actin to HMM in the presence of ATP, making use of the fact that F-actin rapidly sediments in an ultracentrifuge under conditions where HMM does not.

The optimal conditions for such an experiment are: (a) a high actin concentration and a low ionic strength so that as much actin as possible is bound (12, 17); (b) as high an HMM concentration as possible so that any free HMM present can be accurately measured; and (c) sufficient ATP concentration so that the actin sediments to a significant degree before all of the ATP is hydrolyzed. To maximize all of these conditions we performed our experiment at 0°C with 3 mM Mg–ATP, 5.7 μM HMM, and 45 μM actin. In addition 2 mM Pi was added to inhibit actin depolymerization. Under these conditions it took 20 min for all of the ATP to be hydrolyzed. Fig. 1 shows a double-reciprocal plot of ATPase against actin concentration under these conditions. As can be seen at 45 μM actin, the actin–HMM ATPase activity was 88% of $V_{\text{max}}$, i.e., if we assume that all of the HMM is bound to actin when the ATPase activity equals $V_{\text{max}}$, all but 15% of the HMM should be complexed with actin.

Fig. 2 shows the ultracentrifuge scanner patterns for HMM alone, actin alone, and HMM and actin together under these conditions. As can be seen, the HMM alone (top) essentially did not sediment at all, whereas the actin (bottom) clearly pulled away from the meniscus leaving no protein behind. Surprisingly, however, in the sample with a mixture of actin and HMM (middle), 60% of the HMM remained behind as the actin and actin–HMM complex sedimented. This experiment was repeated numerous times and always gave the result that 55–65% of the HMM remained free. Therefore, under conditions where the reciprocal plot of ATPase against actin concentration suggests that almost all of the HMM

![Figure 1](image1.png)

**Fig. 1.** Double-reciprocal plot of the activity of actin–HMM ATPase against actin concentration. All samples contained 3 mM MgCl₂, 3 mM ATP, 3 mM imidazole (pH 7.0), 2 mM Pi, and 5.8 μM HMM. Temperature was 0.5°C. As described for this type of plot (26), ATPase activity of the HMM in the absence of actin was subtracted from the measured ATPase rate to give the activity of actin–HMM ATPase, the reciprocal of which is plotted on the ordinate. (HMM ATPase = 0.017 sec⁻¹.)

![Figure 2](image2.png)

**Fig. 2.** Ultracentrifuge scanner traces showing binding of actin to HMM in the presence of ATP. The solution meniscus in the three traces have been aligned, and the negative absorbance just above the solution meniscus in each trace is due to ATP absorption on the solvent side of the cell. For all three runs the conditions were identical to those of Fig. 1, and all scans were taken about 8 min after reaching 30,000 rpm. (See Methods for further details.) Upper and middle trace, HMM concentration = 5.8 μM; middle and lower trace, actin concentration = 45 μM.
should be complexed with actin, the analytical ultracentrifuge shows that 60% of it is free.

To further investigate this phenomenon, we performed the experiments in Table 1. If all of the residual absorbance in the supernatant is indeed HMM, then when the HMM concentration is halved, with the actin concentration held constant, the residual absorbance should also be halved; as seen in samples 3 and 4 of Table 1, this is indeed the case. This experiment also shows that the amount of residual HMM is not dependent on the specific amount of ATP remaining in the sample, since with half the HMM added, the ATPase rate will be half as fast and, therefore, about twice as much ATP will be present when the scan is taken.

Table 1 also shows that no residual HMM is observed in the supernatant unless ATP is present. In samples 6 and 7, if the sample was centrifuged after all of the ATP was hydrolyzed to ADP and P_i, then essentially all of the protein sedimented, i.e., all of the HMM was bound to actin. As can be seen by comparing samples 5, 6, and 7, the small amount of protein remaining was apparently almost all depolymerized actin, since the absorbance was essentially independent of the concentration of the added HMM. In the absence of ATP, at this low salt and temperature, it is not surprising that a small amount of actin depolymerized. Therefore, it is clear that ATP is required for the dissociation of the actin and HMM we observe in the ultracentrifuge—ADP and P_i in the millimolar range will be dependent on the concentration of the actin, and, therefore, about twice as much ATP will be present when the scan is taken.

Finally sample 8 of Table 1 shows that when the actin concentration is halved, significantly less HMM is complexed with actin. Of course the ATPase also decreases when the actin concentration is halved and, therefore, as might be expected, the ATPase correlates with the amount of bound HMM. However, there must be some reason why even at high actin concentration, where the ATPase activity is very close to V_max, a significant fraction of the HMM remains dissociated from the actin.

One possibility is that a large fraction of the HMM is de-natured. We tested this possibility by comparing the EDTA- and Ca-ATPase of the HMM and the parent myosin from which it was made. Table 2 shows that the ATPase rates per mol of myosin or HMM were almost identical, and these rates are very similar to those obtained by Seidel for myosin under similar conditions (19). Furthermore, as noted above, this HMM shows essentially complete binding to actin in the absence of ATP and the same level of actin activation of its ATPase as obtained by Barouch and Moos at this temperature (18). Therefore, it seems very unlikely that the explanation for our centrifuge results is that the HMM is half denatured.

Another possible reason why a large fraction of the HMM is dissociated from actin is the effect of pressure in the ultracentrifuge. However, since we took our reading very close to the meniscus and worked at a centrifuge speed of only 30,000 rpm, the pressure head at the point where the actin and HMM separated was only about 6 kg/cm^2, only 1% of the pressure necessary for complete inhibition of the actin–HMM ATPase (20). Therefore, it seems only remotely possible that the actin–HMM is being dissociated by pressure. We are thus led to the possibility that in the steady state of ATPase activity, the HMM spends part of its cycle of interaction with actin and ATP in a “refractory” state in which it is unable to bind to the actin. In an effort to test this possibility with a completely different approach from the ultracentrifuge, we turned to ATPase studies.

As we noted above, double-reciprocal plots of ATPase activity against actin concentration are linear and, when extrapolated to infinite actin concentration, give the ATP turnover rate per mol of added HMM (V_max). Likewise it is possible at a fixed concentration of actin to vary the HMM concentration and, by extrapolation to infinite concentration of HMM, to obtain the ATP turnover rate per mol of added actin. If it is assumed that all of the HMM binds to actin at infinite actin concentration, then a comparison of the ATP turnover rate per mol of HMM with that per mol of actin should yield the binding ratio of HMM to actin in the presence of ATP. On the other hand, if the HMM spends more than 50% of its time in a refractory state, it might be expected that during the time an HMM molecule goes through one cycle of interaction with actin and ATP, an actin monomer could bind and release several HMM molecules, so that the turnover rate per mol of added actin would be considerably greater than that per mol of added HMM.

One of the authors (E.E.) found that the turnover rate per mol of actin equalled that per mol of HMM, but in that experiment the HMM concentration was only varied over a 4-fold range (12). We therefore repeated the experiment with much higher HMM concentrations, and the results are shown in Fig. 3. As can be seen, at the highest protein concentration used, rather than being equal, the turnover rate per mol of added actin was twice that per mol of added HMM, and extrapolation of this data yielded an ATP turn-

### Table 1. Binding of HMM to actin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Actin (mg/ml)</th>
<th>HMM (mg/ml)</th>
<th>Nucleotide</th>
<th>A_260</th>
<th>Fraction of HMM</th>
<th>1 - (V/V_max)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>2</td>
<td>ATP</td>
<td>0.75</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0</td>
<td>ATP</td>
<td>0.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
<td>ATP</td>
<td>0.44</td>
<td>0.59</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1</td>
<td>ATP</td>
<td>0.21</td>
<td>0.56</td>
<td>0.15</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>0</td>
<td>ADP</td>
<td>0.09</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2</td>
<td>ADP</td>
<td>0.12</td>
<td>0.043†</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>1</td>
<td>ADP</td>
<td>0.10</td>
<td>0.01†</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>2</td>
<td>ATP</td>
<td>0.53</td>
<td>0.71</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Conditions as in Fig. 1, except ADP rather than ATP was added to sample 5. ATP was initially added to samples 6 and 7, but the samples were centrifuged after the ATP was completely hydrolyzed to ADP and P_i.

* From data in Fig. 1.

† For this calculation, the A of the actin alone (Sample 5) was subtracted from the measured A.

### Table 2. Comparison of myosin and HMM ATPases

<table>
<thead>
<tr>
<th>Protein</th>
<th>EDTA (sec⁻¹)</th>
<th>Ca⁺ (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>20</td>
<td>3.2</td>
</tr>
<tr>
<td>HMM</td>
<td>21</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Conditions: 0.5 M KCl, 2 mM ATP, 2 mM Tris (pH 7.5), and either 1 mM EDTA or 5 mM CaCl₂; temperature, 25°.
over rate per mol of added actin three times that per mol of added HMM. Unlike the double-reciprocal plot of ATPase against actin concentration, that of ATPase against HMM shows some curvature that might perhaps be caused by crowding of the HMM molecules on the actin at high HMM concentration. Despite this curvature, however, it is clear from Fig. 3 that in the actin–HMM–ATP system, the ATP turnover rate per mol of actin is much greater than that per mol of HMM, implying that the HMM does indeed exist in a refractory state during a large part of its cycle of interaction with actin and ATP.

DISCUSSION

In the first attempt to measure the physical interaction of actin and HMM in the presence of ATP, Perry and his associates found no increase in turbidity or viscosity under conditions where actin activated the HMM ATPase and, therefore, concluded that in some way, the actin activated the HMM ATPase without actually binding to it (9, 10). In our study, unlike Perry and his associates, we do find that binding occurs between actin and HMM in the presence of ATP, and, furthermore, we find that qualitatively at least (as shown in samples 3 and 8 of Table 1) the amount of binding correlates with the amount of ATPase activity. It is probably safe to conclude, therefore, that actin activation of the HMM ATPase occurs through the direct binding of actin to HMM.

Interestingly enough, however, in partial confirmation of the findings of Perry and his associates, we find, by two independent techniques—the analytical ultracentrifuge and ATPase studies—that during the steady-state hydrolysis of ATP, 55–65% of the HMM is unable to bind to actin no matter how high the actin concentration is. Recognizing that either the ultracentrifuge or ATPase studies might be in error, we are encouraged that both techniques yield the same result. Denaturation of more than half of the HMM might account for this result, but it seems to be very unlikely (see Results). Therefore, apparently during the steady-state hydrolysis of ATP, the HMM spends 55–65% of its time in a refractory state unable to bind to actin.

There are many kinetic models that might explain these results, and we certainly do not have enough information to write a complete steady-state model for the actin–HMM–ATP system that explains not only the results in this paper but also the previous data on the dependence of the HMM ATPase on both actin and ATP concentrations (17). We, therefore, present the following cyclic pathway simply as one possible mechanism that might explain our results:

where $M = HMM$, $S = ATP$, $A = actin$, $M(S)$ = one head of the HMM in the refractory state, and $MS^*$ = one head of the HMM in the nonrefractory state.

In this model we assume that a saturating ATP concentration is present, and that both heads of the HMM are acting independently. The key postulate in the model is that both $k_1$ and $k_2$ are relatively slow compared to the other steps in the cycle, so that at saturating actin concentration two species are present in significant concentration, the refractory state of the HMM that cannot bind to actin, and the complex of the nonrefractory state with actin. In a first-order process, an HMM head in the refractory state (I) transforms to the nonrefractory state (II). Presumably in most cases, simply on a random basis, as one head of the HMM goes through the cycle the other head will remain refractory, so that in the model we have shown only one head of the HMM binding to actin at a time. After transformation to the nonrefractory state, the HMM head forms a complex with actin (III), and the actin then causes hydrolysis and release of the products. Although relatively slow, this step ($k_3$) is much faster than the corresponding step in the absence of actin (not shown in the model), thus accounting for the actin activation of the HMM ATPase. After release of the products, as proposed by Finlayson et al. (21), ATP again binds to the actin–HMM complex (IV) and rapidly and irreversibly causes dissociation of the complex leading to reformation of the refractory state. Since this dissociation ($k_4$) is postulated to be essentially irreversible, HMM in the refractory state is unable to bind to actin.

In this model we have not attempted to identify the true nature of the refractory and nonrefractory states. However, it is of interest to consider the steady-state kinetic studies on myosin and HMM (22–25). Taylor et al. (25) have proposed that the ATP on myosin is very rapidly hydrolyzed to products in an “initial burst” of ATPase activity and then, in
discussions during state is thus far state contraction (7, 8), might only that identify not as amount rate at 00 should be be ever, the and i.e., in our model of to identify the refractory Actin then activates the myosin ATPase by increasing the rate of release of products. From this analysis it is tempting to identify the refractory state as the myosin–ATP complex and the nonrefractory state as the myosin–product complex, i.e., in our model $S^*$ is the bound product. In that case, however, the rate of the initial burst of ATP hydrolysis by myosin at 0° should be identical to $k_1$ in our model; and since $k_1$ must be a slow step in our cycle in order that there be a significant amount of HMM in the refractory state, it follows that the rate of the initial burst of ATP hydrolysis should be of the same order of magnitude as the rate of our overall cycle at 0°. However, the initial burst of ATP hydrolysis at 0°, as measured by Lyman and Taylor (24), is at least 30-times faster than the ATP turnover rate at infinite actin concentration as determined in our experiments. Therefore, we cannot identify the refractory and nonrefractory states of HMM.

Despite our ignorance, it is interesting to speculate that the x-ray diffraction studies on living muscle, which suggest that only a fraction of the myosin bridges bind to actin during contraction (7, 8), might be explained by the existence of a refractory state in vivo. However, it must be emphasized that thus far we have obtained evidence for the existence of a refractory state only with actin–HMM at 0°, and more work will be required to determine if the existence of the refractory state is indeed a generalized phenomenon, involved in the mechanism of muscle contraction in vivo.

We would like to thank Dr. Carl Moos for many helpful discussions during the course of this work.