Both Heads of Tissue-derived Smooth Muscle Heavy Meromyosin Bind to Actin in the Presence of ADP*

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The effect of ADP and phosphorylation upon the actin binding properties of heavy meromyosin was investigated using three fluorescence methods that monitor the number of heavy meromyosin heads that bind to pyrene-actin: (i) amplitudes of ATP-induced dissociation, (ii) amplitudes of ADP-induced dissociation of the pyrene-actin-heavy meromyosin complex, and (iii) amplitudes of the association of heavy meromyosin with pyrene-actin. Both heads bound to pyrene-actin, irrespective of regulatory light chain phosphorylation or the presence of ADP. This behavior was found for native regulated heavy meromyosin prepared by proteolytic digestion of chicken gizzard myosin with between 5 and 95% heavy chain cleavage at the actin-binding loop, showing that two-head binding is a property of heavy meromyosin with uncleaved heavy chains. These data are in contrast to a previous study using an uncleaved expressed preparation (Berger, C. E., Fagnant, P. M., Heizmann, S., Trybus, K. M., and Geeves, M. A. (2001) J. Biol. Chem. 276, 23240–23245), which showed that one head of the unphosphorylated heavy meromyosin-ADP complex bound to actin and that the partner head either did not bind or bound weakly. Possible explanations for the differences between the two studies are discussed. We have shown that unphosphorylated heavy meromyosin appears to adopt a special state in the presence of ADP based upon analysis of actin-heavy meromyosin association rate constants. Data were consistent with one head binding rapidly and the second head binding more slowly in the presence of ADP. Both heads bound to actin at the same rate for all other states.

Smooth muscle myosin (SMM), like other members of the myosin II family, has two heads connected by a coiled-coil tail. SMM and the double-headed subfragment HMM are regulated by phosphorylation of the two regulatory light chains, one on each head (1–3). In contrast, single-headed SMM (4, 5) and the single-headed S1 (6–8) are not regulated by phosphorylation.

Non-muscle HMM IIB is also regulated by phosphorylation, and constructs lacking one motor domain have been shown to be unregulated (9). Therefore, two motor domains are required for regulation.

Structural differences between unphosphorylated and phosphorylated HMM have been demonstrated by a number of studies. Reconstruction of images of expressed unphosphorylated HMM in the presence of ATP in two-dimensional crystalline arrays (10, 11) shows an asymmetrical structure with the converter domain of one head bound to the actin-binding site of the other head. No interaction was seen between the motor domains of phosphorylated HMM. This model is supported by data from Berger et al. (12), who demonstrated that only one of the heads of an unphosphorylated expressed smooth muscle HMM-ADP complex binds to actin. Either the binding of the first head prevented the binding of the second head or the second head bound weakly such that no signal was observed for its binding. Both heads bound to actin in the phosphorylated state. These data are inconsistent with two studies of the effect of ADP and phosphorylation in intact gizzard muscle, which are consistent with binding of both heads of myosin to actin irrespective of ADP or phosphorylation (13, 14).

We investigated the actin binding properties of HMM derived from chicken gizzards. Digestion of SMM by Staphylococcus aureus V8 protease or chymotrypsin generates HMM with varying degrees of internal cleavage at loop 2 (the actin-binding loop), but the cleavage products remain associated under non-denaturing conditions (6, 15–19). Based upon the model discussed previously, it was possible that the extent of internal heavy chain cleavage at the actin-binding loop could alter the actin binding behavior. Therefore, we produced HMM with between 5 and 95% heavy chain cleavage for this study. Measurements of the fluorescence changes upon binding of HMM to pyrene-actin and upon ATP-induced dissociation from pyrene-actin were used to determine the stoichiometry of HMM binding to actin in the unphosphorylated and thiophosphorylated states. We show that both heads of tissue-derived HMM bind to actin. This two-headed binding was observed irrespective of the extent of internal heavy chain cleavage, the presence or absence of ADP, or the phosphorylation state of the RLC. These experiments were consistent with the fact that ADP did not induce dissociation of the pyrene-actin HMM complex irrespective of the phosphorylation state. As these data contrast those for an expressed HMM construct (12), explanations for the differences between the two protein preparations are presented.

We also measured the actin-activated ATPase activity of unphosphorylated and thiophosphorylated HMM by single turnover assays with both ATP and FTP. All HMM preparations used in this study were found to be fully regulated as defined by a slow turnover rate in the presence of actin for the unphosphorylated protein. Therefore, we have shown that the...
native tissue-derived unphosphorylated HMM-ADP complex binds to actin with two heads. The one-headed actin binding mode of an unphosphorylated HMM-ADP complex predicted by the model of Wendt et al. (10, 11) is not a property required for down-regulation.

EXPERIMENTAL PROCEDURES

**Protein Preparations**—SMM was prepared from frozen chicken gizzards (20) obtained from Pel-Freez Biologicals (Rogers, AR). HMM and S1 with between 50 and 60% cleaved heavy chain was obtained by digestion in the absence of ATP. HMM with <25% or less than 5% heavy chain cleavage was prepared by limited digestion with chymotrypsin (Sigma) as described (18) except that digestion was performed with 0.05 mg/ml chymotrypsin at 25 °C for 3 min. Chymotryptic digestion also results in cleavage of the RLC. Therefore, cleaved RLC was exchanged for intact, wild-type RLC using a standard exchange procedure followed by purification of fully exchanged HMM (with two intact RLC) as described previously (21). Myosin, HMM, and S1 concentrations were determined using the following 0.1% extinction coefficients: 0.56, 0.65, and 0.75, respectively. The extent of heavy chain cleavage of HMM and S1 preparations was estimated by densitometric scanning and analysis of SDS gels (Novex precast 4–20% acrylamide gradient Tris-glycine (Invitrogen)) using an EPI Chem II darkroom and Labworks image analysis software (UVP Laboratory Products, San Gabriel, CA). All HMM preparations were stored on ice and used within 2 weeks of purification. F-actin was prepared from rabbit muscle according to the method of Spudich and Watt (22) and was labeled with pyrene iodacetamide (Molecular Probes) (23). MLCK was prepared from frozen chicken gizzards by the method of Adelstein and Klee (24) except that Superdex 200 (Amersham Biosciences) was used for gel filtration, and Super Q (Toso-Hase) was used for anion exchange.

**N-terminal Sequencing**—Protein bands were transferred from an SDS gel to a PVDF membrane, and the sequences of the largest HMM fragment (see Fig. 1, heavy chain) and the N-terminal fragment (see Fig. 1, N) were determined using an Applied Biosystems Procise-HT 492. The transfer efficiency was >80%.

**Thiophosphorylation of Proteins**—HMM was thiophosphorylated by incubation in 50 mM NaCl, 2.5 mM MgCl₂, 2.5 mM CaCl₂, 4 μg/ml calmodulin (Sigma), 1 mM ATP-γS (Roche Molecular Biochemicals), and 20–40 μM MLCK. Excess ATP-γS was removed by Sephadex G-50 (Sigma) centrifugal gel filtration (25). The extent of thiophosphorylation was >95% as verified by gel electrophoresis as described previously (26) except that protein samples were applied to Novex precast 10% acrylamide Tris-glycine gels (Invitrogen). Thiophosphorylation also results in cleavage of the RLC. Therefore, cleaved RLC was exchanged for intact, wild-type RLC using a standard exchange procedure followed by purification of fully exchanged HMM (with two intact RLC) as described previously (21).

**Gel Filtration**—SMM was prepared from frozen chicken gizzards by the method of Adelstein and Klee (24) except that Superdex 200 (Amersham Biosciences) was used for gel filtration, and Super Q (Toso-Hase) was used for anion exchange.

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**Transient Kinetics**—Experiments were carried out using a Hi-Tech SF-61 BX2 stopped-flow spectrophotometer equipped with a 150-watt xenon arc lamp and an electronic shutter to avoid photolysis over prolonged time courses. All HMM and S1 concentrations refer to the concentration of heads. All pyrene-actin solutions contained equimolar phallolidin (Sigma) to stabilize the F-actin form. Pyrene-actin fluorescence was excited at 365 nm, and emission was detected after passing through a KV-399 cut-off filter (Schott).

ATP-induced dissociation experiments were performed using the method of Kurzawa and Geeves (27). All concentrations quoted refer to the concentration of heads. Mixtures of pyrene-actin and HMM for dissociation experiments were preincubated for 30 min at 20 °C to ensure that the mixture had reached equilibrium. For association experiments, all concentrations quoted refer to the concentration of heads in the cuvette after mixing. Data were analyzed by fitting the average from three to four shots (with a variance of between ±0.3% and ±5%) to a single or double exponential model using the KinetAsyst 2 curve fitting software (Hitech Scientific, Salisbury, UK). To allow comparison between different data sets, fluorescence changes for dissociation experiments were expressed as the ratio of the fluorescence amplitude change to the final pyrene-actin fluorescence (ΔFΔ/Δt). Fluorescence changes for association experiments were expressed as the ratio of the fluorescence amplitude change to the initial pyrene-actin fluorescence (ΔFΔ/Δt). Since all plots of ΔFΔ/Δt versus time showed a clear plateau, the data were analyzed by fitting the average from three to four shots (with a variance of between ±0.3% and ±5%) to a single or double exponential model using the KinetAsyst 2 curve fitting software (Hitech Scientific, Salisbury, UK). To allow comparison between different data sets, fluorescence changes for dissociation experiments were expressed as the ratio of the fluorescence amplitude change to the final pyrene-actin fluorescence (ΔFΔ/Δt). Since all plots of ΔFΔ/Δt versus time showed a clear plateau, the data were analyzed by fitting the average from three to four shots (with a variance of between ±0.3% and ±5%) to a single or double exponential model using the KinetAsyst 2 curve fitting software (Hitech Scientific, Salisbury, UK).

**RESULTS**

Preparation of HMM with Various Degrees of Heavy Chain Cleavage—HMM prepared proteolytically from SMM contains heavy chains cleaved internally at the actin-binding loop. However, cloned, expressed HMM contains only intact, full-length heavy chains. To examine what effect, if any, heavy chain cleavage at the actin-binding loop had upon the mode of actin binding, we prepared tissue-derived HMM with between 95 and 5% heavy chain cleavage (Fig. 1). Lanes 1–3 show three preparations of HMM with 95, 60, or 50% of the heavy chain cleaved by V8 protease. Chymotryptic digestion was required to achieve preparations with 25 and 5% heavy chain cleavage (Fig. 1, lanes 4 and 5).

The largest fragment (Fig. 1, HC) and the N-terminal fragment (Fig. 1, N) were transferred to a membrane and sequenced. These data showed that residues 1–9 and 1–27 for V8 and chymotryptic HMM, respectively, were cleaved from the N terminus. It was difficult to determine the extent of this cleavage since a gel shift was not observed (Fig. 1). For 5% cleaved chymotryptic HMM (lane 5, HC), we measured 2 pmol of the sequence beginning at residue 28. If all of the heavy chain were N-terminally cleaved, we would have measured about 50 pmol. Therefore, we estimate that only 5–10% of the N terminus is cleaved in this preparation.

**ATP-induced Dissociation of the acto-HMM Complex**—Binding of smooth muscle S1 to pyrene-labeled actin quenches the pyrene fluorescence by ~75% (28), as has been found for skeletal muscle S1 (23, 27). Addition of ATP to the acto-HMM complex dissociates the heads from pyrene-actin, resulting in an increase in pyrene fluorescence; the amplitude of the fluorescence change is dependent upon the amount of pyrene-actin-HMM complex present before the addition of ATP. Fig. 2 shows representative traces of ATP-induced dissociation of pyrene-actin complexed with u-HMM or u-HMM-ΔDP showing an

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**Fig. 1. SDS-PAGE of HMM preparations with various extents of heavy chain cleavage.** Gel 1 (lanes 1–3): lane 1, HMM (95% cleaved) prepared with V8 protease in the absence of ATP; lanes 2 and 3, HMM (60 and 50% cleaved, respectively) prepared with V8 protease in the presence of ATP. Gel 2 (lanes 4 and 5): lanes 4 and 5, HMM (25 and 5% cleaved, respectively) prepared with chymotrypsin followed by RLC exchange. Uncleaved heavy chain (HC), N-terminal (N) and C-terminal (C) fragments generated by cleavage, RLC, and essential light chain (ELC) are indicated.
were independent of the HMM concentration. The proteins studied, a plateau in the not shown), and these data are tabulated in Table I. For all were also measured, in the presence and absence of ADP (plots were shown). The u-HMM-ADP complex behaves in a similar manner. The HMM concentration dependence of fluorescence changes that the u-HMM-ADP complex showed biphasic actin association with actin and that all of the actin-binding sites eventually become filled with heads. Fig. 3 shows the HMM concentrations less than 1.5 \text{M} actin. The maximal fluorescence changes (\Delta F/F_{\text{final}}) were plotted against the concentration of HMM. The intersecting lines were drawn by eye through the data for HMM (squares). The buffer conditions are 100 \text{mM} KCl, 20 \text{mM} MOPS (pH 7.0), 5 \text{mM} MgCl$_2$, 0.1 mM EGTA, 1 mM DTT.

\begin{figure}
\centering
\includegraphics[width=0.45\textwidth]{Fig3.png}
\caption{Fluorescence amplitudes for ATP-induced dissociation of u-HMM from pyrene-actin versus u-HMM concentration. Pyrene-actin (80 \text{nm}) and the indicated concentrations of 5% cleaved chymotryptic u-HMM (squares) or the u-HMM-ADP complex (circles; formed by addition of 200 \mu\text{M} ADP) were mixed with 80 \mu\text{M} ATP. All concentrations are before mixing. The amplitudes of the pyrene-actin fluorescence changes (\Delta F/F_{\text{final}}) are consistent with stoichiometric binding of both heads to pyrene-actin, in the presence or absence of ADP. Therefore, we observed the same stoichiometry that was observed for the dissociation experiment (Fig. 3) as expected. We did the same experiment for u-HMM preparations with 50\% heavy chain cleavage at three different KCl concentrations, for u-HMM with between 25 and 95\% heavy chain cleavage, and for tp-HMM and S1 with 60\% heavy chain cleavage (plots not shown). For all proteins and conditions studied, a plateau in the \Delta F/F_{\text{final}} was reached at a \Delta F/F_{\text{initial}} of 0.62–0.90 as tabulated in Table II, at HMM concentrations less than 1.5 \times \text{actin concentration}. These data are consistent with stoichiometric binding of both heads to pyrene-actin (Table I). The $k_{obs}$ values of u-HMM and tp-HMM dissociation from pyrene-actin were monophasic and were independent of the HMM concentration. The $k_{obs} = 29 \text{s}^{-1}$ and 28 \text{s}^{-1} in the absence of ADP, and the $k_{obs} = 2.2 \text{s}^{-1}$ and 1.6 \text{s}^{-1} in the presence of ADP, for u-HMM and tp-HMM, respectively.

\textbf{Effect of ADP upon the acto-HMM Complex—} There was no change in fluorescence upon mixing the pyrene-actin-HMM complex (either tp-HMM or u-HMM) with saturating concentrations of ADP (Table I). This result means that the extent of actin binding was not significantly affected by ADP under these conditions. These data are consistent with the ATP-induced dissociation results (Fig. 3) and suggest that both heads of HMM bind to actin in the presence of ADP.

\textbf{Association of HMM with Pyrene-Actin—} Fig. 4 shows representative traces of u-HMM or u-HMM-ADP (60\% cleaved heavy chains) association with pyrene-actin. Fig. 5 shows the fluorescence decreases versus HMM concentration for u-HMM or u-HMM-ADP (with 5\% heavy chains cleaved). The amplitudes of the fluorescence changes were linearly dependent upon the HMM concentration until a plateau was reached at $-0.5 \mu\text{M}$ heads in the presence of 0.5 \mu\text{M} actin. The maximal fluorescence changes (Table II, $\Delta F/F_{\text{final}}$) are consistent with stoichiometric binding of both heads to pyrene-actin, in the presence or absence of ADP. Therefore, we observed the same stoichiometry that was observed for the dissociation experiment (Fig. 3) as expected. We did the same experiment for u-HMM preparations with 50\% heavy chain cleavage at three different KCl concentrations, for u-HMM with between 25 and 95\% heavy chain cleavage, and for tp-HMM and S1 with 60\% heavy chain cleavage (plots not shown). For all proteins and conditions studied, a plateau in the $\Delta F/F_{\text{final}}$ was reached at a $\Delta F/F_{\text{initial}}$ of 0.62–0.90 as tabulated in Table II, at HMM concentrations less than 1.5 \times the actin concentration, consistent with binding of both heads of HMM to pyrene-actin. As the extent of heavy chain cleavage increased, the HMM concentration at which a plateau was reached also increased (plots not shown), consistent with weakening of the actin binding affinity as shown previously for proteolytic smooth muscle S1 by Ikebe et al. (19).

In the absence of ADP, association of u-HMM and tp-HMM was monophasic at the lowest HMM concentrations tested in Fig. 5 (data not shown). This was expected if the association process for each head was equivalent under conditions in which actin is not limiting. A biphasic process was observed at the higher u-HMM or tp-HMM concentrations as expected under conditions of limiting actin availability. The tp-HMM-ADP complex behaved as described above for tp-HMM. In contrast, the u-HMM-ADP complex showed biphasic actin association kinetics with similar amplitudes for each rate, even at the lowest HMM concentrations in which actin was not limiting.

\textbf{Single Turnover of MgF\textsuperscript{3}P in the Presence of Actin—} A number of recent studies have demonstrated that steady-state ATPase assays are an unsatisfactory method for determining the true degree of regulation of smooth and nonmuscle myosins (21, 29, 30). We therefore assessed regulation by using a single turnover stopped-flow assay with the fluorescent ATP analog, FTP, for all HMM preparations used in this study (data not shown). The rate of FTP single turnover for all preparations was monophasic at $-0.001 \text{s}^{-1}$ at 5 \mu\text{M} actin, consistent with
Interaction of Tissue-derived HMM with Actin

**Table I**

Maximal fluorescence changes upon mixing nucleotide (ATP or ADP) with the complex of pyrene-actin and S1, u-HMM, or tp-HMM

| Protein     | Nucleotide | Protease   | Heavy chain cleavage | \(\Delta F_{\text{max}}/F_{\text{final}}\) + ADP | \(\Delta F_{\text{max}}/F_{\text{final}}\) - ADP |
|-------------|------------|------------|----------------------|-----------------------------------------------|-----------------------------------------------|
| u-HMM      | ATP        | chymotrypsin| 5                    | 0.78                                          | 0.60                                          |
| S1         | ATP        | V8         | 50                   | 0.68                                          | 0.54                                          |
| u-HMM      | ATP        | V8         | 50                   | 0.70                                          | 0.50                                          |
| tp-HMM     | ATP        | V8         | 50                   | 0.65                                          | 0.75                                          |
| u-HMM      | ADP        | V8         | 50                   | NA                                            | \(\Delta F = 0\)                               |
| tp-HMM     | ADP        | V8         | 50                   | NA                                            | \(\Delta F = 0\)                               |

\(^{a}\) Data were obtained from an experiment similar to that shown in Fig. 3. \(\Delta F_{\text{max}}/F_{\text{final}}\) was determined from the average fluorescence change (\(\Delta F/F_{\text{final}}\)) at 200 nM HMM heads.

\(^{b}\) Pyrene-actin (200 nM) was mixed with 100 nM HMM and equilibrated for 30 min. This mixture was then mixed in the stopped-flow with 300 \(\mu M\) ADP that had been treated with hexokinase (20 units/ml), glucose (1 mg/ml), and AP \(5\)A (0.5 \(\mu M\)), respectively, for 30 min to remove trace amounts of ATP. All concentrations are before mixing. The observed change in fluorescence (\(\Delta F\)) was zero. NA means not applicable. V8 is \(S. aureus\) protease.

![Fig. 4. Association of u-HMM with pyrene-actin in the presence and absence of ADP. Representative traces are shown for the fluorescence changes upon mixing 0.5 \(\mu M\) u-HMM (with 60% cleaved heavy chain), in the presence and absence of 100 \(\mu M\) ADP, with 0.5 \(\mu M\) pyrene-actin. All concentrations are after mixing.](image)

![Fig. 5. Fluorescence amplitudes for association of u-HMM with pyrene-actin versus HMM concentration. Pyrene-actin (0.5 \(\mu M\)) and the indicated concentrations of 5% cleaved chymotryptic u-HMM (squares) or the u-HMM-ADP complex (circles; formed by addition of 100 \(\mu M\) ADP) were mixed. All concentrations are after mixing. The amplitudes of the pyrene-actin fluorescence changes (\(\Delta F_{\text{max}}/F_{\text{initial}}\)) are plotted against the concentration of HMM. The intersecting lines were drawn by eye through the data for u-HMM (squares). The buffer conditions were 100 mM KCl, 20 mM MOPS (pH 7.0), 5 mM MgCl\(_2\), 0.1 mM EGTA, 1 mM DTT.](image)

Our previous measurements (21). In addition, we measured the rate of FTP single turnover versus actin concentration for u-HMM and tp-HMM with 40% heavy chain cleavage (Fig. 6). Data for tp-HMM fit best to a double exponential model, whereas data for u-HMM fit best to a single exponential model. The fast rate of tp-HMM was fit to the Michaelis-Menten equation, giving a \(V_{\text{max}}\) of 2.9 \(\pm 0.2\) \(s^{-1}\) and a \(K_{\text{atpase}}\) of 40 \(\pm 8\) \(\mu M\). The rates for u-HMM were low over a wide range of actin concentrations and could not be fit to the Michaelis-Menten equation. At 90 \(\mu M\) actin, the rate for u-HMM was 0.005 \(s^{-1}\), whereas the rate for tp-HMM was 2 \(s^{-1}\), representing a 400-fold increase in activity upon thiophosphorylation. In a previous study, we measured the steady-state MgATPase activities of tp-HMM (21). The \(V_{\text{max}}\) and \(K_{\text{atpase}}\) of tp-HMM were 2.3 \(\pm 1\) and 31 \(\mu M\), respectively, consistent with the single turnover values presented here. Our results (Fig. 6) are similar to single turnover data obtained for an expressed thiophosphorylated non-muscle HMM IIB construct that has no heavy chain cleavage (9).

As an alternative method to assess regulation, the turnover rate of the physiological nucleotide, ATP, was measured for u-HMM with 60% cleaved heavy chain (Fig. 7). Data were fit to a single exponential plus a line and gave a rate of 0.0027 \(s^{-1}\) at 5 \(\mu M\) actin. The linear process had a slope of 0.0008. A faster phase was not detected. With FTP as the nucleotide substrate (Fig. 6), the equivalent rate at 5 \(\mu M\) actin for u-HMM was 0.001 \(s^{-1}\). Therefore, the FTP and ATP turnover rates were within a factor of 3 and were consistent with full regulation of turnover with both ATP and FTP.

**DISCUSSION**

We have demonstrated that both heads of tissue-derived HMM and HMM-ADP bind to actin, irrespective of the phosphorylation state of the RLC. These findings are consistent with the effects of ADP and phosphorylation upon measurements of RLC mobility (13) and tension (14) in intact smooth muscle. In contrast, Berger et al. (12) found that only one head of expressed smooth muscle u-HMM-ADP bound to actin, whereas tp-HMM, tp-HMM-ADP, and u-HMM bound with two heads. The buffer conditions and protein concentrations used in this study and the Berger et al. (12) study were similar. Berger et al. (12) used the same ATP-induced dissociation method that we used here. Therefore, it appears that the HMM preparations used in the two studies are different.

One obvious difference between the HMM preparations is the presence of internal heavy chain cleavage at the actin-binding loop in tissue-derived HMM. In our HMM preparation with 5% heavy chain cleavage, a maximum of 10% of all molecules would contain at least one cleaved head with at least 90% of all molecules containing two uncleaved heads. If these uncleaved u-HMM-ADP molecules behaved in a manner similar to the expressed u-HMM-ADP, the \(\Delta F_{\text{max}}/F_{\text{final}}\) (Table I) or \(\Delta F_{\text{max}}/F_{\text{initial}}\) (Table II) for u-HMM-ADP would be 45% lower than that for u-HMM. Our data for u-HMM with 5% heavy
Interaction of Tissue-derived HMM with Actin

Table II
Maximal fluorescence changes upon association of S1, u-HMM, and tp-HMM with pyrene-actin

| Protein   | Protease | Heavy chain cleavage | [KCl] | $\Delta F_{\text{max}}/F_{\text{initial}}$ + ADP | $\Delta F_{\text{max}}/F_{\text{initial}}$ – ADP |
|-----------|----------|----------------------|-------|-----------------------------------------------|-----------------------------------------------|
| S1        | V8       | 60                   | 100   | 0.90                                          | 0.90                                          |
| u-HMM     | V8       | 95                   | 100   | 0.87                                          | 0.87                                          |
| u-HMM     | V8       | 60                   | 100   | 0.85                                          | 0.85                                          |
| u-HMM     | V8       | 50                   | 50    | 0.83                                          | 0.83                                          |
| u-HMM     | V8       | 50                   | 200   | 0.86                                          | 0.81                                          |
| u-HMM     | chymotrypsin | 25               | 100   | 0.62                                          | 0.70                                          |
| u-HMM     | chymotrypsin | 5               | 100   | 0.74                                          | 0.73                                          |
| tp-HMM    | V8       | 60                   | 100   | 0.70                                          | 0.72                                          |

Fig. 6. FTP single turnover rates for u-HMM and tp-HMM versus actin concentration. Rates of phosphate release from the acto-HMM-FDP-P complex were measured for an HMM preparation with 40% cleaved heavy chain. Final conditions for the turnovers were: 0.4 $\mu$M HMM heads, 0.4 $\mu$M FTP, 100 $\mu$M ATP, 0.4 mM MgCl$_2$, and various actin concentrations in 10 mM MOPS (pH 7.0), 50 mM NaCl, 0.1 mM EGTA, 1 mM DTT, with NaCl concentrations as indicated. V8 is S. aureus protease.

Chain cleavage show no significant differences between maximal fluorescence amplitude changes obtained in the presence or absence of ADP using two different methods (Fig. 3 and Table I, Fig. 5 and Table II). We conclude that the extent of heavy chain cleavage at the actin-binding loop is not the reason for the differences between expressed and tissue-derived HMM.

In addition to the actin-binding loop cleavage, chymotrypsin and V8 protease cleave a small number of residues from the N terminus of the heavy chain. The V8 protease preparation was missing only 9 residues and is unlikely to explain the different actin binding behavior. For 5% cleaved chymotryptic HMM, 27 residues are cleaved, but we estimated that only 5–10% of the N terminus is cleaved. This suggests that N-terminal cleavage is not likely to explain the differences in actin binding behavior by the same reasoning described above for the actin-binding loop.

Our tissue-derived HMM was not frozen at any stage of the preparation, whereas the expressed HMM was frozen in liquid nitrogen in the presence of sucrose and stored at $-80^\circ$C. We have found that freezing tissue-derived HMM in this manner causes loss of regulation. However, both Berger et al. (12) and this study (Figs. 6 and 7) showed that HMM preparations were regulated using single turnover approaches.

Berger et al. (12) found that dissociation of u-HMM heads from pyrene-actin by ATP resulted in a $\Delta F_{\text{max}}/F_{\text{final}}$ of $-0.4$ in the absence of ADP and $-0.2$ in the presence of ADP. We wondered why they did not observe a $\Delta F_{\text{max}}/F_{\text{final}}$ of $-0.8$ in the absence of ADP, as would be expected from previous studies with tissue-derived smooth muscle S1 (28), and consequently $-0.4$ in the presence of ADP. A lower than expected pyrene-actin quenching by expressed HMM might be due to the following. First, it could be an inherent property of the molecule, although the amino acid sequence of the expressed HMM is known post-translational modifications specific to the tissue-derived HMM. Second, it is possible that there are unknown post-translational modifications specific to the tissue-derived HMM. Third, a significant population of rigor heads (heads that bind irreversibly to actin) (21, 31) would lower the $\Delta F_{\text{max}}/F_{\text{final}}$ without altering the observed stoichiometry. Berger et al. (12) showed that ADP could dissociate $\sim40\%$ of the heads from an acto-u-HMM complex but not from an acto-tp-HMM complex. The rate of this process for the acto-u-HMM complex was much slower than the rate of ADP binding and thus was consistent with a rearrangement. This suggested that these ADP heads initially bound to actin but eventually found a thermodynamically more stable place to bind or somehow lost their normal tight actin binding properties ($K_d < 40$ nM). This result would be obtained if dead heads were abundant, as we suggested previously, and if the surface of dead heads had an extremely tight binding site for the actin-binding site of a functional partner ADP head (perhaps in...
a structure similar to that proposed by Wendt et al. (10, 11). Formation of this nonphysiological structure might be expected to be slow, as Berger et al. (12) observed, as it would involve dissociation of a functional ADP head from actin followed by binding (nearly irreversibly) to the partner dead head. The lack of such a motor-motor domain interaction in a tp-HMM-ADP preparation containing dead heads would be compatible with the structural data of Wendt et al. (10, 11) showing no interaction between motor domains in tp-HMM. Our results, under identical conditions to Berger et al. (12), showed that ADP could not dissociate heads from the acto-HMM complex regardless of the phosphorylation state (Table I). Therefore, the results from both studies are internally consistent, suggesting that the HMM preparations are different.

Berger et al. (12) reported that ~25% of u-HMM heads bound ADP with an affinity of 2 μM, ~25% bound ADP with a much weaker affinity, and the remaining ~50% did not respond to ATP at maximal ADP concentrations. These nonresponsive heads may be attributed to dead heads behaving as described above. Nevertheless, their data strongly suggest that functional HMM binds to ADP with two different affinities. We are currently characterizing the ADP binding properties of our preparations.

A test for the presence of dead heads is to compare the ΔFmax/Finitial and ΔFmax/Ffinal values for association and dissociation experiments, respectively. These values should be the same in the absence of dead heads. The ΔFmax/Finitial from an association experiment should not be affected by the presence of dead heads, whereas the ΔFmax/Ffinal from a dissociation experiment would be lowered. In our study, both dissociation (Table I) and association (Table II) of HMM heads from/to pyrene-actin resulted in maximal fluorescence changes consistent with earlier studies with tissue-derived smooth S1 (28). This agreement between dissociation and association data is strong evidence that our preparations do not contain a significant fraction of dead heads. Furthermore, the single turnover measurements in Fig. 6 are consistent with previous steady-state measurements from our laboratory (21). Dash and Hackney (31) estimated that the V8-cleaved tissue-derived preparation contains ~88% of dead heads, consistent with the study of Ellison et al. (21).

Our data do not rule out the possibility that the tissue-derived u-HMM-ADP complex can adopt a conformation like that described by Wendt et al. (10, 11). Indeed, our association rate data are consistent with the idea that the heads interact in some manner in the presence of ADP but not in its absence. Our association rate data are in agreement with a previous study by Rosenfeld et al. (32). They measured the rates of pyrene-actin binding of tissue-derived u-HMM and tp-HMM with and without ADP at high actin/HMM ratios. The binding was monophasic except for the u-HMM-ADP complex, which bound in a biphasic manner with two phases of similar amplitude. They interpreted these results to indicate that both heads of the u-HMM-ADP complex bound to actin but that an interaction between the heads slowed the binding of the second head. Under conditions similar to theirs (at the highest actin/HMM ratios of Fig. 5), we made the same observations. Therefore, our data, like those of Rosenfeld et al. (32), are consistent with an interaction between the two heads of the u-HMM-ADP complex, which must be broken to allow the second head to bind to actin. It is possible that this interaction is between the two motor domains, as described in the model of Wendt et al. (10, 11), but our data do not address this structural issue. Our data suggest that for tissue-derived HMM, if such an interaction is occurring, it is not strong enough to compete with actin to prevent binding of both heads to actin. We have also shown that one-headed actin binding behavior for u-HMM-ADP is not a requirement for down-regulation of smooth muscle myosin and that two-headed actin binding in the presence of ADP is a property of the native, undamaged, fully regulated molecule.

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REFERENCES

1. Sellers, J. R. (1991) Curr. Opin. Cell Biol. 3, 98–104
2. Sellers, J. R., and Goodson, H. (1995) in Motor Protein 2: Myosin (Sheterline, P., ed) Vol. 2, Protein Profile, Academic Press Limited, London
3. Hartshorne, D. J. (1987) in Biochemistry of the Contractile Process in Smooth Muscle (Johnson, L. R., ed) pp. 423–481, Second Ed., Physiology of the Gastrointestinal Tract, Raven Press, New York
4. Cremo, C. R., Sellers, J. R., and Facemeyer, K. C. (1995) J. Biol. Chem. 270, 2171–2175
5. Konishi, K., Katoh, T., Morita, F., and Yazawa, M. (1996) J. Biochem. (Tokyo) 124, 165–170
6. Ikeda, M., and Hartshorne, D. J. (1985) Biochemistry 24, 2380–2387
7. Sellers, J. R., Eisenberg, E., and Adelstein, R. S. (1982) J. Biol. Chem. 257, 12880–12883
8. Konishi, K., Kujima, S., Katoh, T., Yazawa, M., Kato, K., Fuijiwara, K., and Onishi, H. (2001) J. Biol. Chem. (Tokyo) 129, 365–372
9. Cremo, C. R., Wang, F., Facemeyer, K., and Sellers, J. R. (2001) J. Biol. Chem. 276, 41465–41472
10. Wendt, T., Taylor, D., Messier, T., Trybus, K. M., and Taylor, K. A. (1999) J. Cell Biol. 147, 1385–1399
11. Wendt, T., Taylor, D., Trybus, K. M., and Taylor, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4361–4366
12. Berger, C. E., Fagnant, P. M., Heimann, S., Trybus, K. M., and Greeves, M. A. (2001) J. Biol. Chem. 276, 23240–23245
13. Gollub, J., Cremo, C. R., and Cooke, R. (1999) Biochemistry 38, 10107–10118
14. Dantzig, J. A., Barsotti, R. J., Manz, S., Sweeney, H. L., and Goldman, Y. E. (1999) Biophys. J. 77, 386–397
15. Ikeda, M., and Hartshorne, D. J. (1986) Biochemistry 25, 6177–6185
16. Bonet, A., Momet, D., Audemard, E., Derancourt, J., Bertrand, R., and Kassab, R. (1997) J. Biol. Chem. 272, 15624–15630
17. Sellers, J. R. (1999) Myosins (Sheterline, P., ed) 2nd Ed., Protein Profiles, Oxford University Press, Oxford
18. Seidel, J. C. (1980) J. Biol. Chem. 255, 4355–4361
19. Ikeda, M., Mitra, S., and Hartshorne, D. J. (1993) J. Biol. Chem. 268, 25948–25951
20. Ikeda, M., and Hartshorne, D. J. (1985) J. Biol. Chem. 260, 13146–13151
21. Ellison, P. A., Sellers, J. R., and Cremo, C. R. (2000) J. Biol. Chem. 275, 15142–15151
22. Spudich, J. A., and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871
23. Cridle, A. H., Greeves, M. A., and Jeffries, T. (1985) Biochem. J. 232, 343–349
24. Adelstein, R. S., and Klee, C. B. (1982) Methods Enzymol. 85, 298–308
25. Penevsky, H. S. (1977) J. Biol. Chem. 252, 2891–2899
26. Facemeyer, K. C., and Cremo, C. R. (1992) Bioconjugate Chem. 3, 408–413
27. Kurzawa, S. E., and Greeves, M. A. (1996) J. Muscle Res. Cell Motil. 17, 669–676
28. Cremo, C. R., and Greeves, M. A. (1989) Biochemistry 28, 4871–4877
29. Jackson, A. P., and Bagshaw, C. R. (1988) Biochem. J. 251, 519–526
30. Sellers, J. R. (1985) J. Biol. Chem. 260, 15815–15819
31. Dash, P. K., and Hackney, D. D. (1991) Biochem. Int. 25, 1013–1022
32. Rosenfeld, S. S., Xing, J., Cheung, H. C., Brown, P., Kur, S., and Sweeney, H. L. (1998) J. Biol. Chem. 273, 28682–28690