The Two Heads of Smooth Muscle Myosin Are Enzymatically Independent but Mechanically Interactive*

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The interaction between the two heads of myosin II during motion and force production is poorly understood. To examine this issue, we developed an expression and purification strategy to isolate homogeneous populations of heterodimeric smooth muscle heavy meromyosins containing heads with different properties. As an extreme example, we characterized a heterodimer containing one native head and one head locked in a “weak binding” state by a point mutation in switch 2 (E470A). The in vitro actin filament motility of this heterodimer was the same as the homodimERIC control with two cycling heads, suggesting that only one head of a pair actively interacts with actin to generate maximal velocity. A second naturally occurring heterodimer contained two cycling heads with 2-fold different activity, due to the presence or absence of a 7-amino acid insert near the active site. Enzymatically this (+/−) insert heterodimer was indistinguishable from a (50:50) mixture of the two homodimers, but its motility averaged 17% less than that of the mixture. These data suggest that one head of a heterodimer can disproportionately affect the mechanics of double-headed myosin, a finding relevant to our understanding of heterogeneous mutant myosins found in disease states like familial hypertrophic cardiomyopathy.

There has been speculation for many years about whether myosin derives any advantage from having two cross-bridge heads. The double-headed structure is clearly not necessary for actin binding or enzymatic activity, because it has long been known that proteolytically derived, single-headed subfragment 1 (S1)† retains both of these abilities (1). Moreover, a single-headed molecule can move actin filaments in the in vitro motility assay (2, 3). However, the velocities sustained by one-headed fragments in this assay are generally less than that of double-headed species from the same myosin, and recent measurements in the laser trap have indicated that the unitary step size of a single-headed myosin is only half as great as that of a double-headed molecule (4). These observations indicate not only that two heads are more effective than one in moving actin but also that they may work together in some sort of coordinated fashion.

One way to gain insight into the nature of potential head-head interactions is to study myosins containing two heads that differ functionally. If each head works independently, then the activity of such a heterodimer should be halfway between that of the faster homodimer and the slower homodimer. On the other hand, if there is an interaction between the heads, the properties of the heterodimer might be closer to that of one of the homodimeric species. To study heavy meromyosin (HMM) molecules with different heads, we developed an expression strategy that involves differential labeling of the constituent heavy chains with FLAG and His tags, co-infection in the Sf9 cell system, and sequential affinity chromatography columns to isolate homogeneous preparations.

We assessed two types of smooth muscle HMM heterodimer using enzymatic and mechanical assays. The first was composed of two naturally occurring smooth muscle heavy chain isoforms that differ in vitro by a factor of two in their actin-activated ATPase activity and actin filament motility (5, 6). This difference is due to the presence or absence of a 7-amino acid insert in the surface loop near the nucleotide-binding pocket (7, 8), which has been called loop 1 (9). Such heterodimers almost certainly exist in nature, because the mRNAs for the +insert and −insert heavy chains are co-expressed in single smooth muscle cells (10). The second heterodimer contained one wild type (WT) heavy chain and a second head with a mutation in switch 2 (E470A) that prevents the formation of a salt bridge between this residue and R247 in switch 1 of the active site. This mutation slows the ATPase activity of smooth myosin by two orders of magnitude, essentially “locking” it in a weak binding configuration (11, 12). Thus, the heterodimeric E470A/WT molecule has a much greater disparity in function between the two heads.

Enzymatic assays indicated that the two heads of both heterodimers function independently. However, the in vitro motility assay showed that the heterodimeric (+insert/−insert) HMM moved actin filaments 17% more slowly than a 50:50 mixture of the corresponding homodimeric HMMs. In contrast, the heterodimeric E470A/WT HMM showed motility that was not significantly different from WT HMM. These results are consistent with the notion that myosin employs only one head at a time to perform work on the actin filament and, furthermore, suggest that one head of a heterodimer can exert a

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The abbreviations used are: S1, subfragment 1; HMM, heavy meromyosin; FHC, familial hypertrophic cardiomyopathy; MOPS, 3-[N-morpholino]propanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin, FLAG, epitope tag with amino acid sequence DYKDDDDK; His, epitope tag with amino acid sequence HHHHHH; +insert, smooth muscle myosin heavy chain containing the 7-amino acid insert in loop 1; −insert, smooth muscle myosin heavy chain lacking the 7-amino acid insert in loop 1; WT HMM, wild type homodimeric heavy meromyosin; E470A HMM, E470A homodimeric heavy meromyosin; +/−insert, heterodimeric heavy myosin with one head containing the 7-amino acid insert and the other head lacking the insert; E470A/WT HMM, heterodimeric heavy meromyosin with one wild type head and one carrying the point mutation E470A; AMP-PNP, adenosine 5′-[(β,γ-imino)triphosphate.

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disproportionate effect on mechanical properties in molecules where both heads are actively cycling.

EXPERIMENTAL PROCEDURES

DNA Engineering and Production of Recombinant Baculoviruses—

The baculovirus expression vector pAcGS2 (Pharmingen) was modified for the expression of HMM heavy chains with two different epitope tags. For FLAG-tagged species, both N- and C-terminal variants were created. For C-terminal tagging, a BamHI restriction site was added 3' to an NcoI site in the polyclinker, followed by in-frame sequence coding for FLAG (DYKDDDK) and a stop codon. For an N-terminal tag, nucleotide sequence encoding the initiating ATG and the FLAG tag was added 5' to the NcoI site. To produce species fused to an N-terminal hexa-histidine (His) tag, an initiating ATG codon followed by six histidine diones and an NcoI site were added 5' to a BglII site in the pAcGS2 polyclinker. cDNAs encoding the HMM portion of the chicken gizzard myosin heavy chain clone (13) were ligated into these three vectors to express four different HMM heavy chains. First, a C-terminal FLAG tag was attached to a clone encoding the first 1175 amino acids of the insert heavy chain, whereas a mutant 1186-residue HMM from which the loop 1 insert had been removed (insert 6) was ligated into the N-terminally His-tagged vector, using the NcoI and BglII sites. Glutamic acid 470 of the 1175-residue cDNA was converted to alanine by site-directed mutagenesis, and this was then cloned into the vector that adds the C-terminal FLAG tag. Finally a wild-type (WT) + insert N-terminally His-tagged construct was also synthesized.

Each construct in pAcGS2 was transfected and amplified in SF9 cells by established methods (14). To produce protein, myosin heavy chain viruses were co-infected with a recombinant virus encoding both the smooth muscle myosin essential and regulatory light chains (15). In the case of heterodimers, an N-terminally His-tagged heavy chain virus was co-infected with the appropriate C-terminally FLAG-tagged heavy chain, along with the dual light-chain virus. Preliminary trials were conducted to optimize relative viral ratios so that the expression levels of the FLAG- and His-tagged heavy chains were nearly equal.

Purification of HMM Proteins—After 3 days of infection, SF9 cells were harvested and lysed, and proteins were fractionated by two successive ammonium sulfate precipitations, 0–40% and 40–70%. For the homodimers of C-terminally FLAG-tagged + insert and E470A HMM, as well as for the two types of heterodimer, the 40–70% pellet was dialyzed overnight versus a buffer containing 90 mM NaCl, 10 mM imidazole-HCl (pH 7.4 at 4°C), 1 mM EDTA, 1 mM NaN3, 1 mg/ml BSA (15), and 1 μM/ml leupeptin. MgATP was added to a final concentration of 4 mM, the material was clarified by centrifugation, and the supernatant was applied to an anti-FLAG affinity column (M2 antibody, Sigma-Aldrich Chemical). After washing, HMM was eluted using a large molar excess of FLAG peptide (0.1 mg/ml), and peak fractions were pooled. This was the end point for the preparation of FLAG-labeled homodimers. In the case of the two heterodimer preparations, NaCl was added to the FLAG eluate to give a final concentration of 300 mM, and the pH was increased to 8.0 by adding 35 mM MOPS, pH 8.55. This was the end point for the preparation of FLAG-labeled heterodimers. In the case of heterodimers, an N-terminally His-tagged heavy chain virus was co-infected with the appropriate C-terminally FLAG-tagged heavy chain, along with the dual light-chain virus. Preliminary trials were conducted to optimize relative viral ratios so that the expression levels of the FLAG- and His-tagged heavy chains were nearly equal.

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For all motility experiments, 1.2–3.5 μM HMM was mixed with a 2.5-fold molar excess of F-actin in the presence of 1 mM MgATP, and centrifuged for 25 min at 350,000 × g to remove ATP-insensitive cross-bridge heads. The protein concentration of the supernatant was determined by Bradford (17), and dilutions were made to the appropriate concentrations for either measurement of unloaded maximum velocity, or average force (see below). Polyacrylamide SDS gels run on these supernatants confirmed the accuracy of the concentrations and indicated that the amount of contaminating actin was less than 5%. HMMs were anchored to the coverslip using antibody S2.2 (18), and actin filament movement was measured as described previously (16, 19). The basic buffer typically used for actin treatment and washing in the flow cell (see below) contained 90 mM KCl, 25 mM imidazole-HCl (pH 7.4 at 30°C), 4 mM MgCl₂, 1 mM EGTA, and 10 mM DTT (buffer A). The final assay buffer additionally contained 0.7% methylene cellulose, an oxygen scavenger mixture (containing 0.1 mg/ml glucose oxidase, 0.018 mg/ml catalase, and 2.3 mg/ml glucose), and 1 mM MgATP. In some experiments, the concentration of KCl in the buffer was changed to 25 or 90 mM. The concentrations of average filamentary structure, using α-actinin as a load, which impeded the movement of actin (20). α-Actinin (Sigma) was dialedyzed into buffer A with 1 rather than 10 mM DTT and clarified for 25 min at 350,000 × g. Its concentration was determined using BSA as a standard (17). The following components were added sequentially to the flow cell: 1) 25 μg/ml S2.2 antibody for 1 min, wash with buffer A; 2) α-actinin for 1 min, wash with buffer A, including 0.5 mg/ml BSA; 3) HMM for 1 min, wash with buffer A; and finally 4) actin for 2 × 30 s. Finally, buffer A containing methylene cellulose, oxygen scavengers, and ATP was added, and the filaments were observed under fluorescent illumination.

The concentration of α-actinin at which just stopped filament movement on the motility surface was determined for different HMM concentrations. The “stopping concentration” of α-actinin was defined by the long, straight, “taut” appearance of the filaments, as well as their failure to move. The precision of these measurements was defined as one-half the concentration difference between the stopping point as defined above and the closest lesser α-actinin concentration at which long, taut filaments were not observed.

ADP Release from Acto-HMM—The rate of ADP release was determined by mixing acto-HMM(100 μM ADP) with 2 mM MgATP, and measuring acto-HMM dissociation either by the decrease in light scattering or the increase in pyrene actin fluorescence. Actin was labeled with pyrene-iodoacetamide as described previously (19). Experiments were done in 10 mM HEPES (pH 7.0), 0.1 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM NaN₃, 1 mM DTT at 20°C using a Kin-Tek stopped-flow spectrophotometer and a 100-watt mercury lamp. For 90° light scattering, the exciting beam was passed through a 294-nm interference filter, and the emission was detected with a 294-nm interference filter. Pyrene fluorescence was measured using an interference filter (370–430 nm) placed at 90° to the excitation beam, and detected with a 400-nm cutoff filter. Transients are the average of at least three independent mixings. The signal averaging and fitting was done using Kin-Tek software.

RESULTS

Isolation of Homogeneous Heterodimer Preparations—By placing unique epitope tags on the two species of heavy chain included in a given heterodimer, we were able to isolate homogeneous preparations of HMM with two different heads. We prepared and characterized two heterodimers. One was com-
posed of two naturally occurring smooth muscle heavy chain isoforms that differ in their cycling rates by a factor of two due to the presence or absence of a seven amino acid insert in loop 1. The second heterodimer contained the WT inserted heavy chain along with a non-cycling head created by a point mutation in switch 2 (E470A/WT HMM) (11, 12).

The flowchart in Fig. 1 illustrates the stages of this purification for the (+insert/−insert) heterodimer, along with Western blots depicting the amounts of FLAG- and His-reactive material in each step. The sensitivities of our anti-FLAG and anti-His probes were adjusted to give approximately the same amount of staining when reacted with equal amounts of the respective control proteins (Fig. 1B, lane S). The protein that was loaded onto the FLAG column (Fig. 1B, lane 1) contained nearly equal amounts of FLAG-labeled (+insert) heavy chain and His-labeled (−insert) heavy chain. As expected, the flowthrough from the anti-FLAG column contained predominantly His-tagged molecules (Fig. 1B, lane 2). The small amount of FLAG immunoreactivity in this fraction indicated that the column was loaded with more FLAG-labeled material than it had the capacity to bind. Protein eluted from this column contained both FLAG-labeled homodimers of the +insert heavy chain and heterodimers (Fig. 1A, step 3 and Fig. 1B, lane 3). After loading this mixture onto the poly-histidine binding column, a small amount of the His-reactive material was seen in the flowthrough due to supersaturation of the column, along with a large excess of FLAG-tagged protein (Fig. 1B, lane 4). After washing with loading buffer, a more stringent wash with buffer containing 10 mM imidazole-HCl was employed to release any nonspecifically bound FLAG-FLAG homodimer (data not shown). Elution with 300 mM imidazole buffer liberated the final, homogeneous preparation of heterodimeric HMM (Fig. 1B, lane 5), which had equal amounts of FLAG- and His-reactive material in it. Western blots conducted on preparations of the heterodimeric E470A/WT HMM gave the same results (21).

TABLE I

| HMM                  | Rate (s⁻¹) |
|----------------------|------------|
| E470A HMM            | 0.01       |
| E470A/WT HMM         | 12.0 ± 0.8 |
| WT HMM               | 24.2 ± 2.8 |

NH₄⁺-ATPase activity of HMMs with WT and E470A heads

Expressed as moles of P_i hydrolyzed per head per second. The assay was conducted at 37 °C. Values for E470A/WT HMM and WT HMM are average ± S.D. of three samples.

FIG. 2. Actin-activated ATPase activity of different HMM species at 40 μM actin concentration. The homodimeric +insert and −insert HMMs (black and white bars, respectively) were compared with an equal mixture of these species (gray bar), as well as the heterodimeric HMM (cross-hatched). Actin-activated activity was calculated as the total activity in the presence of actin minus the basal activity in the absence of actin.
ADP was used to measure the rate of ADP release by a given type of myosin (22). The release of ADP from the nucleotide-binding pocket is affected by the other head, both in the presence or absence of actin.

Rate of ADP Release from Acto-HMM by Two Techniques—The release of ADP from the nucleotide-binding pocket is thought to be the step in the cross-bridge cycle that limits the velocity of actin movement by a given type of myosin (22). Pyrene-labeled actin was used to measure the rate of ADP release from acto-HMM. When pyrene actin-HMM-ADP is mixed with excess ATP, pyrene fluorescence increases at the rate at which ADP leaves the active site and is replaced with ATP (23). The time course of this fluorescence increase was best fit by a single exponential that was 4-fold faster for the +insert HMM than the −insert HMM (Table II). Phosphorylation had little effect on this rate. When measured over a range of temperatures, the Q10 value for this kinetic step was 3. Although our ADP release measurements showed a 4-fold difference between the +insert and −insert species, our current and previous (6) comparisons of velocities by the motility assay indicate a 2-fold difference between these isoforms. These results suggest that other steps in the cross-bridge cycle in addition to ADP release may limit the velocity of actin filament movement.

The difference in ADP release rates of the homodimers allowed us to probe the properties of heterodimeric (+insert−insert) HMM (Table II). As expected, the fluorescence time course of the 50:50 mixture of dephosphorylated homodimers was better fit by an equation with two exponential terms than when only the single fit is listed, its $\chi^2$ value was significantly lower than that of a single exponential. Values for (+/−) heterodimer are averages ± S.D. of four preparations for DeP and three preparations for Phos. Phos refers to phosphorylated HMM; DeP, dephosphorylated. The value for Phos (+/−) heterodimer is the average of two preparations.

| Protein | Phos state | Fit | Rate (s$^{-1}$) | $\chi^2$ |
|---------|------------|-----|----------------|---------|
| (−/−) Homodimer | DeP | S | 9.5 ± 0.5 | 0.09 |
| (+/−) Homodimer | DeP | S | 52.3 ± 0.3 | 0.02 |
| 50:50 Homodimer mixture | DeP | D | 10.5 ± 0.06 | 0.15 |
| (+/−) Heterodimer | DeP | D | 9.4 ± 0.16 | 1.10 |
| 50:50 Homodimer mixture | DeP | D | 33.6 ± 0.67 | 0.52 |

All measurements conducted at 25°C. Raw data were fit with either a single (S) or double exponential (D) equation. Bold values are rate constant(s) (±S.D.) from the better fit, based upon $\chi^2$ values. When only the single fit is listed, its $\chi^2$ value was significantly lower than that of a double exponential. Phos refers to phosphorylated HMM; DeP, dephosphorylated. The value for Phos (+/−) heterodimer is the average of two preparations.

Comparison of the homodimers with the heterodimer (Fig. 2) shows that the rate of ADP release is approximately half that of homodimeric (+insert) HMM, as we have shown previously (6). The rate of the heterodimer was the same as an equal mixture of the two homodimers, at a value intermediate between the rates shown by the homodimers. Taken together with the NH$_2$-ATPase results of Table I, these data indicate that each of the heads in our heterodimers have steady-state ATPase activities that are unaffected by the other head, both in the presence or absence of actin.

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| Protein | Phos state | Fit | Rate (s$^{-1}$) | $\chi^2$ |
|---------|------------|-----|----------------|---------|
| (−/−) Homodimer | DeP | S | 8.6 ± 0.02 | 0.043 |
| 50:50 Homodimer mixture | DeP | D | 10.2 ± 0.04 | 0.026 |
| (+/−) Heterodimer | DeP | S | 13.6 ± 0.30 | 0.018 |
| 50:50 Homodimer mixture | DeP | S | 43.8 ± 0.3 | 0.021 |
| (+/−) Heterodimer | DeP | S | 37.8 ± 0.2 | 0.096 |

All measurements conducted at 25°C. Raw data were fit with either a single (S) or double exponential (D) equation. Bold values are rate constant(s) (±S.D.) from the better fit, based upon $\chi^2$ values. When only the single fit is listed, its $\chi^2$ value was significantly lower than that of a double exponential. Phos refers to phosphorylated HMM; DeP, dephosphorylated. The value for Phos (+/−) heterodimer is the average of two preparations.
Mechanical Interactions of Myosin Heterodimers

double-headed HMM molecule away from the mass of the actin filament, whereas pyrene fluorescence gives an independent assessment of the rate of ADP release from each head. Because the slower head will dictate the rate at which the entire HMM can move away from actin, a rate dominated by the slower head is expected and was observed.

Motility of the Heterodimers—Both the E470A/WT HMM and the +insert/−insert HMM heterodimers were assayed for their ability to move actin in the in vitro motility assay. Homodimeric E470A HMM does not support motility (Fig. 4A).

Surprisingly, heterodimeric E470A/WT HMM showed an average velocity (cross-hatched bar, 1.50 ± 0.19 μm/s) in 60 mM KCl buffer that was nearly 95% as great as that of WT HMM (black bar, 1.58 ± 0.20 μm/s). These values were not significantly different when assessed by unpaired t tests at a p < 0.001 level of significance. Thus, the presence of a non-cycling, weak-binding cross-bridge head had little impact on actin movement by the WT head.

The heterodimer containing cycling heads with 2-fold different kinetics behaved differently, suggesting head-head interactions. The velocity of the homodimeric (+insert) HMM (black bar, 1.60 ± 0.19 μm/s) was twice that of the −insert HMM (white bar, 0.76 ± 0.10 μm/s), as we previously showed (6) (Fig. 4B). The cumulative average of a number of different 50:50 mixtures of the two homodimeric species assessed on different dates was 1.13 ± 0.13 μm/s (gray bar, Fig. 4B). Six different heterodimeric (+insert/−insert) HMM preparations (cross-hatched bars) moved more slowly on any given date than the 50:50 mixture they were directly compared with and gave velocities ranging from 7% to 26% slower than the cumulative mixture average (Fig. 4B). These differences were in every case significant at the p < 0.001 level. Additionally, the cumulative average actin filament velocity of the six heterodimeric preparations (0.94 ± 0.14 μm/s, n = 454 filaments) was 17% less than that of the 50:50 mixtures (1.13 ± 0.13 μm/s, n = 292 filaments), which was also highly significant (p < 0.0001). These results suggest that the presence of the kinetically slower −insert head within the same molecule exerts a disproportionate slowing effect on the performance of the molecule as a whole as it moves actin.

Because the heterodimers had been exposed to two different types of affinity column and therefore endured more manipulation than either of the homodimer preparations, it could be argued that their decreased velocities were due to damage sustained during preparation. To refute this idea, we isolated a control HMM, which had an N-terminal His tag on one (+insert) heavy chain and a C-terminal FLAG tag on the other (+insert) heavy chain (i.e. homodimeric with respect to HMM sequence and heterodimeric with respect to tags). The control HMM was subjected to the same purification scheme as our experimental heterodimers. An aliquot of HMM eluted from the FLAG column was saved for functional comparison with the elution from the His column, which contained exclusively FLAG/His heterodimers. As shown in Fig. 4C, the velocity of control HMM purified on a single FLAG column (black bar, 1.60 ± 0.19 μm/s) was not significantly different from the material that bound to and was eluted from the FLAG column (white bar, 1.68 ± 0.28 μm/s) or the material that bound to and was eluted from both columns (gray bar, 1.62 ± 0.17 μm/s). This control showed that the decreased velocity exhibited by our +insert/−insert heterodimer is an inherent property of this molecule, not an artifact due to the purification procedure.

Average Forces Produced by the +insert and −insert HMM Are the Same—Two methods were used to determine whether the relative amounts of force generated by the homodimeric +insert and −insert HMMs differed, which would allow us to characterize this parameter in the heterodimer. First, the motility velocity of mixtures of varying ratios of the +insert and −insert homodimers was quantified (Fig. 5A). To assess the relative amounts of force generated by the two smooth HMMs, the data were fit using a model for the interaction of two different myosin isoforms on the motility surface (24). The value derived for the ratio of maximum force generated by the −insert to the +insert isoform (P_0(−insert)/P_0(+insert)), was 1.34, showing little difference in average force, as reported previously (25).

The second method used a variation of the motility assay in which α-actinin acts as a load that the HMM works against to create movement (20). The concentration of α-actinin needed to stop movement was assayed as a function of HMM concentration, and data were fit by a simple linear regression (Fig. 5B). The forces produced by the homodimeric +insert and −insert HMMs were indistinguishable by this assay, negating our ability to assess this mechanical property of heterodimeric HMM.

FIG. 3. Light scattering time courses for ADP release experiments. Single- or double-exponential model fits to the data were selected based upon χ² values and are superimposed. The rate constants derived from these fits are listed in each panel. A, raw data and single-exponential fit for the homodimeric (+insert) HMM. B, raw data and single-exponential fit for the homodimeric (+−insert) HMM. C, raw data and double-exponential fit for a (50:50) mixture of the two homodimers. D, raw data and single-exponential fit for the heterodimeric (+−insert/−−insert) HMM.

Discussion

The roles of the two cross-bridge heads of myosin in force and motion production are not fully understood. From an enzymatic perspective, previous work has indicated that two-headed HMM has twice the steady-state ATPase activity of single-headed S1 (1), suggesting that each head operates independently of the other. A direct comparison of equilibrium actin binding by skeletal HMM and S1 showed that HMM bound with both heads only in the absence of nucleotide (26) and suggested that single-headed binding in the presence of the weak binding nucleotide analog AMP-PNP might actually sterically inhibit the binding of additional HMM molecules at adjacent actin sites. More recently, it was proposed from a kinetic comparison of the same two species that strain at the
junction between the two heads cooperatively influences binding to actin (27) and that this mechanism may have a greater impact in smooth muscle than in skeletal muscle (28, 29).

These data all suggest that the interaction between double-headed myosin and actin is more complex than can be accounted for by the simple action of two kinetically independent heads.

Our approach to further addressing the role of myosin’s two heads was to develop an expression and purification scheme to isolate pure populations of heterodimeric double-headed HMM and to compare their performance with molecules containing two identical heads. To ensure that our isolated heterodimer was a homogeneous preparation, we employed separate epitope tags for each type of heavy chain, and used two successive affinity columns, one for each of the tags (Fig. 1). Control experiments showed that protein purified over two columns was functionally equivalent to protein purified by the standard one-column method (Fig. 4C). These results not only refuted the possibility of damage, but also showed that the alternative locations of the two tags at either the amino or carboxyl termini had no impact upon the activity of the molecule. Moreover, quantitative evidence for the homogeneity of the heterodimeric preparation was obtained by showing that the NH$_4$$_2$ATPase activity of a heterodimer with one non-functional head was exactly half that of the homodimer with two functional heads. Western blots of the final species were also consistent with equal mixtures of the two species.

The Two Heads of HMM Are Enzymatically Independent—As expected, the actin-activated ATPase activity of the heterodimeric (+insert/−insert) HMM was the same as the activity of an equal mixture of the two homodimeric HMMs, indicating that the two heads hydrolyze ATP independently. This conclusion was strengthened by the results from the NH$_4$$_2$ATPase comparison of the heterodimeric E470A/WT HMM with its two cognate homodimers. Our measurements of ADP release obtained by pyrene fluorescence were also consistent with the idea of independent heads, as the heterodimer revealed distinct fast and slow rates that were quite similar to those of the homodimers (Table II). In contrast, when light scattering was used to measure the rate of ADP release, there was a bias toward the rate of the slower head. This result suggests that the light scattering signal remains the same whether one or two heads are bound and only changes when both heads are dissociated from actin. The rate at which the whole HMM molecule dissociates from actin could only occur as fast as the slowest head comes off.

Contribution of the Two Heads to Actin Movement—Smooth muscle S1 (21) and single-headed smooth muscle HMM (30) move actin at least 2-fold more slowly than WT HMM. Even a construct lacking only the motor domain on one of the two heads, but retaining both neck regions, showed lower rates of motility than WT HMM (30). Moreover, a recent laser trap study showed that the unitary step size of double-headed myosin (−10 nm) was twice that of single-headed myosin (−6 nm), although the length of time that the myosin was attached to actin following the power stroke was the same for both species (4). Here we show that a second weak binding, non-cycling head can restore motility to WT HMM levels, whereas concurrent single molecule studies showed that the E470A/WT HMM heterodimer can achieve the same working stroke of −10 nm as WT HMM (21). One interpretation of these data is that the ability of the mutant head to interact weakly with actin allows the stroking head to achieve maximal performance. Thus, a reasonable model is that only one head of HMM moves actin, while the second head optimally orients the working head with respect to the actin filament. Alternatively, a second head may be necessary simply to maintain the structural integrity of the head-rod junction and/or to minimize flexibility and unfavorable orientations of the working head. In this case, the ability of the second head to bind actin is not essential. The current experiments cannot distinguish between these two mechanisms, but it is clear that the presence of the second head is necessary for optimal mechanical performance.

In contrast to the E470A/WT HMM heterodimer, the +insert/−insert HMM was always slower than the comparable mixture of homodimers, by an average of 17%. This heterodimer differs from the one described above in that both heads are cycling. If only one head of the pair is involved in motion generation at any given time, the simplest interpretation of these data is that kinetic differences between the two heads account for most of the difference in motility.
species cause the −insert head to interact with actin slightly more frequently than the +insert head. This assumption is reasonable, because it is known that changes at the active site can be propagated to the actin-binding interface and vice versa. The most obvious example of communication between the nucleotide and actin-binding sites is the dissociation of myosin subfragment 2 (36), suggesting that structural differences in regions of the molecule other than loop 1 are necessary to obtain different levels of force generation.

Implications for Heterodimeric Myosins in Disease States—Here we showed that, when the two heads of myosin cycle at different rates, one head can exert a disproportionate effect on the mechanical properties of the molecule. Although this situation might occur in mammalian hearts in the context of the V2 isoform, it could have even more profound effects in diseases such as familial hypertrophic cardiomyopathy and dilated cardiomyopathy, where in many cases mutations in the β-cardiac myosin gene are thought to be the genetic basis for the disease (37). Because the mutation occurs most often in a single allele, heterodimeric myosins with one WT and one mutant head should be prevalent, and there is considerable evidence that some of these mutations enhance the kinetics of the mutant myosin molecule (38, 39). Thus, it is reasonable to suggest that the mutant head would mechanically predominate in these molecules, altering the contractile kinetics of the heart and exacerbating the phenotype. Our ability to isolate heterodimeric HMMs will position us to more closely examine the processes that give rise to the disease.

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Fig. 5. Average forces of +insert and −insert homodimeric HMMs measured in vitro by two methods. A, actin filament velocities of mixtures of WT and −insert homodimers in the in vitro motility assay. The superimposed curve is the best fit of the interaction model of Warshaw’s group (24). B, concentrations of α-actinin required to stop actin filament movement at different concentrations of the two HMM types. The lines are best-fit linear regressions.
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