Coiled-Coil Unwinding at the Smooth Muscle Myosin Head-Rod Junction Is Required for Optimal Mechanical Performance

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ABSTRACT Myosin II has two heads that are joined together by an α-helical coiled-coil rod, which can separate in the region adjacent to the head-rod junction (Trybus, K. M. 1994. *J. Biol. Chem.* 269:20819–20822). To test whether this flexibility at the head-rod junction is important for the mechanical performance of myosin, we used the optical trap to measure the unitary displacements of heavy meromyosin constructs in which a stable coiled-coil sequence derived from the leucine zipper was introduced into the myosin rod. The zipper was positioned either immediately after the heads (0-hep zip) or following 15 heptads of native sequence (15-hep zip). The unitary displacement (d) decreased from $d = 9.7 \pm 0.6$ nm for wild-type heavy meromyosin (WT HMM) to $d = 0.1 \pm 0.3$ nm for the 0-hep zip construct (mean $\pm$ SE). Native values were restored in the 15-hep zip construct ($d = 7.5 \pm 0.7$ nm). We conclude that flexibility at the myosin head-rod junction, which is provided by an unstable coiled-coil region, is essential for optimal mechanical performance.

INTRODUCTION

Myosin II, the molecular motor that powers muscle contraction, is a dimeric protein consisting of two heavy chains. The N-terminal half of the heavy chain forms a globular head that hydrolyzes ATP and performs mechanical work as it interacts with actin. C-terminal to the head, the heavy chain sequence contains heptad repeats with hydrophobic residues at positions 1 and 4 of the 7-amino acid repeat, which allows the heavy chains to form a coiled-coil rod (see Fig. 1). Although the role of the head in force and motion production is quite clear, the involvement of the rod is less well established.

One obvious role of the coiled-coil rod is to produce a dimeric motor molecule, a feature that is important both for optimal mechanical function and regulation of motor activity. We have recently demonstrated at the single molecule level that two heads are required for maximal force and motion generation, since double-headed smooth or skeletal muscle myosin generated twice the unitary force and displacement of single-headed myosin (Tyska et al., 1999). Two heads are also required to obtain the inhibited enzymatic state in smooth muscle myosin, which is regulated by phosphorylation of the regulatory light chain (Cremo et al., 1995; Trybus et al., 1997).

One feature of the myosin rod that was only recently recognized is that the region immediately following the heads forms a relatively unstable coiled-coil. Direct evidence for separation at the head-rod junction was first provided by the observation that an expressed smooth muscle heavy meromyosin construct that contained 150 amino acids (25 heptad repeats) beyond the head-rod junction failed to fully dimerize (Trybus, 1994). Immuno-electron microscopic evidence was also used to infer that between 60 and 130 residues of the coiled-coil can separate to allow the heads to move apart (Knight, 1996). Such a separation would explain how both heads of scallop heavy meromyosin (HMM) can bind to adjacent actin monomers in decorated actin filaments observed by electron microscopy (Craig et al., 1980). Similar results were obtained from insect flight muscle fiber studies in rigor using electron tomography (Schmitz et al., 1996).

Here we assessed whether the dynamic nature of the head-rod junction is necessary for myosin's optimal mechanical performance. We inserted a stable 32-amino acid α-helical coiled-coil GCN4 leucine zipper at one of two locations within the rod (Trybus et al., 1997), and then used the optical trap to assess the motion-generating capacity of the expressed mutant constructs. We show that when the stable leucine zipper is present at the head-rod junction, the two heads are prevented from generating their full powerstroke, whereas moving the leucine zipper 15 heptads further toward the C-terminus restores wild-type (WT) mechanical function. These data suggest that rod instability, which exists within the first 100 amino acids, is critical for double-headed myosin to coordinate head interactions so that it expresses its maximal motion generating capacity.

MATERIALS AND METHODS

Protein preparation

Smooth muscle myosin constructs were expressed in Sf9 cells as previously described (Trybus et al., 1997). The WT HMM construct consisted of amino acids 1–1175 (Fig. 1). The 0-hep zip construct is identical to that used in Trybus et al. (1997). A 32-aa GCN4 leucine zipper sequence (O'Shea et al., 1991) was introduced following Arg-855. After the zipper, a segment of the rod that contains the epitope for antibody S2.2 (Gln-1081–Arg-1175) was included (Fig. 1). The 15-hep zip construct was

Received for publication 18 August 2000 and in final form 3 January 2001.
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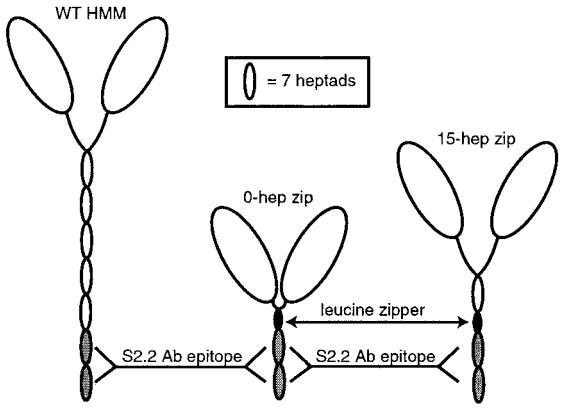

FIGURE 1 Schematic diagram of WT heavy meromyosin and the leucine zipper constructs. WT HMM is depicted as having two heads (*large ovals*) and a coiled-coil rod that is 46 heptads long. Each small white oval represents 7 heptads of rod. In the 0-hep zip, a leucine zipper was placed immediately following the head-rod junction. In the 15-hep zip, the leucine zipper was positioned after 15 heptads of native sequence. The shaded ovals indicate that all three constructs have rod sequence containing the epitope for the S2.2 antibody, which serves as a common attachment site to the motility surface (see Methods for details). For WT HMM and 15-hep zip, the first 7 heptads are illustrated as unwinding, although the extent of rod unwinding that is necessary for optimal motion generation is unknown, but must occur between 0 and 15 heptads based on the data in this study.

modified from that described in Trybus et al. (1997). The 32-amino acid leucine zipper sequence followed 15 heptads of native rod sequence, but in addition the segment of the rod that contains the epitope for antibody S2.2 (Gln-1081–Arg-1175) was added. The S2.2 epitope provided the three constructs with a common attachment site for motility and laser trap studies. All three constructs also contained a FLAG tag at the C-terminus so that the constructs could be purified by affinity chromatography.

The WT HMM and 15-hep zip constructs were thiophosphorylated by the addition of Ca^{2+}, calmodulin, myosin light chain kinase, and MgATPγS. The 15-hep zip had actin filament motility (0.85 ± 0.15 μm/s) that was similar to that of WT HMM (Lauzon et al., 1998). The 0-hep zip did not require phosphorylation for activity because it is only partially regulated (Trybus et al., 1997). Even in the unphosphorylated state, the 0-hep zip construct supports motility (0.18 ± 0.03 μm/s). Previous studies in which partial regulation resulted from a mutation to the actin-binding loop (CABL-HMM construct) showed that the mutant had reduced motility compared to WT HMM but still generated unitary displacements that were identical to its phosphorylated control (Warshaw et al., 2000). Therefore, we believe there is no a priori reason to assume that partial regulation, which is characteristic of the 0-hep zip, should translate into alterations to its inherent mechanical capacity, and thus it was studied in the unphosphorylated state. Actin was purified from chicken pectoralis acetone powder as previously described (Pardee and Spudich, 1982).

Optical trap assay

Detailed instrumentation and protocols for the optical trap assay have been published previously (Dupuis et al., 1997; Guilford et al., 1997; Lauzon et al., 1998). Briefly, an *N*-ethylmaleimide (NEM)-modified myosin-coated microsphere was captured in each of two independent laser traps, and a fluorescently labeled actin filament strung between them. The distance between the microspheres was set such that the actin filament was pretensioned by ~2–4 pN (Dupuis et al., 1997). The actin filament was then moved and centered over a 2-μm silica microsphere, which served as a pedestal to position the myosin off the surface. The constructs were adhered to the surface (4 μg/ml) through the S2.2 antibody that was applied at 100 μg/ml. All experiments were performed at low ionic strength (25 mM KCl), limiting ATP concentration (10 μM), and 25°C, to prolong the unitary event durations. Events were considered to be in the forward direction when the imaged microsphere was pulled in the direction that applied more tension to the filament. If the majority of the events were in the reverse direction, the actin filament polarity was changed by flipping the microsphere-actin-microsphere assembly. Myosin's unitary displacements were recorded under "unloaded" conditions (0.02–0.04 pN/nm/trap).

A statistical approach, the mean-variance (MV) analysis (Patlak, 1993) was used for displacement (d) and attachment time (t_{on}) estimation (see Guilford et al., 1997 for details). The MV analysis consists of transforming the data from a time series into a mean-variance histogram, which emphasizes intervals of constant properties within the data. Generation of the MV histogram requires no assumptions about, nor interpretation of the underlying data, and quantitative descriptions of the data are derived from curve fits to the histogram. Thus, MV analysis is less prone to the biases introduced by manual scoring methods, and may be used to estimate the size (i.e., d), distribution, number and duration of events (t_{on}) in the data as described previously (Guilford et al., 1997; Lauzon et al., 1998). Modifications to the analytical algorithms have improved our ability to estimate t_{on}, thus accounting for the slightly lower t_{on} values reported here for WT HMM compared to previous reports (Lauzon et al., 1998).

MV analysis offers high resolving power given the known reduction in the d signal variance that occurs upon myosin attachment to the actin filament (Finer et al., 1994; Molloy et al., 1995; Guilford et al., 1997). This reduction is the result of myosin adding its stiffness to the microsphere-actin filament-microsphere system, thus reducing the Brownian noise. Therefore, d populations are separated from the baseline population in both mean (i.e., d) and variance (see Fig. 2).

RESULTS AND DISCUSSION

To determine whether restricting the flexibility at the head-rod junction has an impact on the mechanical performance of the myosin molecule, we measured the unitary displacement of myosin constructs in which the rod was stabilized by insertion of the leucine zipper sequence at either 0- or 15-heptads beyond the invariant proline (Fig. 1). Five seconds of optical trap displacement time series are shown for all three constructs, with their corresponding MV-histograms, obtained by analyzing the entire data record (~2 min) from which the sample traces were taken (Fig. 2). Unitary displacement events are discernable from the Brownian noise of the microsphere-actin-microsphere assembly and are detected in the MV histograms as a discrete event population (see Methods), having a variance that is below and well separated from that of baseline (labeled "B" in Fig. 2). For the WT HMM and 15-hep zip constructs in Fig. 2, the displacement events, d, are predominantly in the positive direction with the means for the event populations of 8.1 nm and 6.1 nm, respectively. In contrast, displacements for the 0-hep zip construct are smaller and both positive and negative in direction, which is characterized in the MV-histogram as an event population that is distributed about a mean of 0.2 nm for the example shown in Fig. 2.

Similar experiments and analysis of records were performed to estimate d from between 10 and 50 different

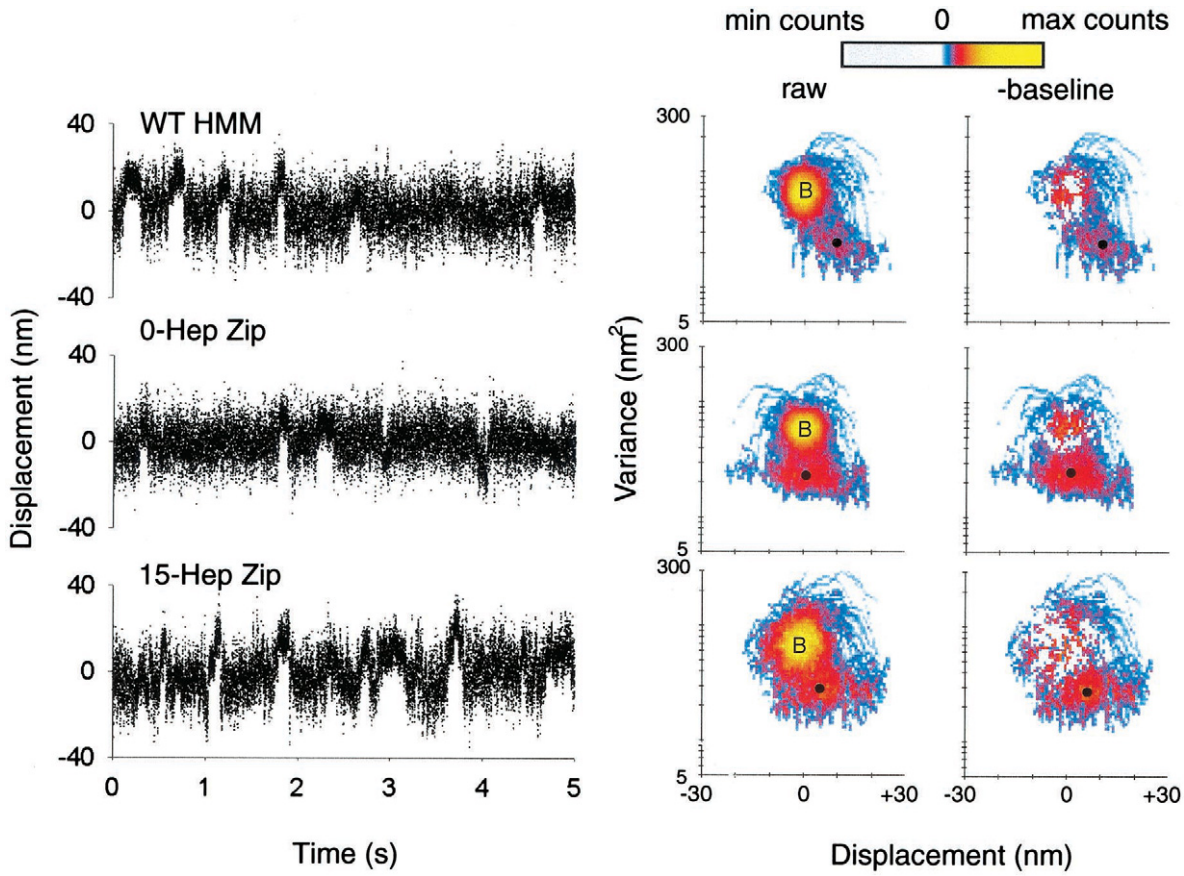

FIGURE 2 Unitary displacement time series and corresponding MV histograms for WT HMM and the zipper constructs. *Left:* five seconds out of ≈2 min of each data set are shown. *Right:* the MV histograms are shown in their raw format and with their baseline subtracted (Guilford et al., 1997; Lauzon et al., 1998). B denotes the baseline population and the center of the dominant fitted event population is shown by a black dot. The color bar indicates log-counts with maximum and minimum counts of 3524 and −568 for WT HMM, 5092 and −380 for the 0-hep zip, and 1277 and −301 for the 15-hep zip. Note that after baseline subtraction negative counts are shown in gray, revealing that the baseline subtraction results in both randomly distributed negative and positive residual counts remaining in the region of the baseline. The WT HMM data set was obtained from Warshaw et al. (1998).

single myosin molecules for each construct (Fig. 3). The unitary displacements, d, of WT HMM (9.7 ± 2.8 nm (SD)) and the 15-hep zip construct (7.5 ± 2.2 nm) are statistically indistinguishable (Fig. 3). In striking contrast, the 0-hep zip construct generates on average, considerably smaller displacements of <1 nm (Fig. 3). However, individual molecules of the 0-hep zip construct did have the ability to generate displacements that ranged between +5 nm and −5 nm (Fig. 3). Estimates of the length of time the head stays attached to actin following the powerstroke, t_{on}, were also determined. The event durations for the three constructs were similar: WT HMM, 97 ± 6 ms; 15-hep zip construct, 105 ± 10 ms; 0-hep zip, 101 ± 8 ms (Fig. 3, inset).

These results suggest that the presence of a stable coiled-coil at the head-rod junction significantly impairs the mechanical performance of the myosin molecule. The position of the stable coiled-coil sequence is critical because moving the leucine zipper 15 heptads away from the head-rod junction restores normal mechanical function. We conclude that a separation of the coiled-coil near the head-rod junction is required for myosin to express its optimal mechanical performance, and that fewer than 105 amino acids (15 heptads) are involved in this process. The 0-hep zip construct was also impaired with respect to its degree of regulation by light chain phosphorylation, while the 15-hep zip construct was the minimal length construct that showed a degree of regulation similar to that of WT HMM (Trybus et al., 1997).

The flexibility at the head-rod junction of the native molecule is likely due to a separation of the coiled-coil (Trybus, 1994; Knight, 1996). The extent of unwinding may extend to the first 25 heptads, given that an expressed short HMM of this length forms an equilibrium between monomers and dimers (Trybus, 1994). Why are the first 25 heptads of the rod unstable? Recent studies have shown that a 13-residue trigger sequence is required for the assembly of a stable coiled-coil that exists within the *Dictyostelium* actin-bundling protein, cortexillin I, and the yeast transcriptional activator GCN4 (Frank et al., 2000; Kammerer et al., 1998; Steinmetz et al., 1998). In fact, Kammerer et al.

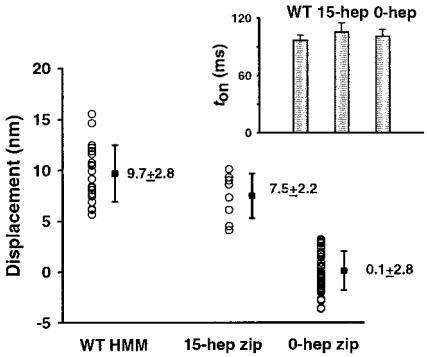

FIGURE 3 Unitary displacements and attachment time. Unitary displacements, d, and t_{on} (*inset*) for WT HMM, 15-hep zip and 0-hep zip. The scatter plots represent the distribution of d estimates obtained from 20, 10, and 51 individual MV histograms (i.e., individual myosin molecules) of WT HMM, 0-hep zip, and 15-hep zip, respectively. The mean and standard deviation for a group is plotted next to the respective distribution. In the inset are the corresponding t_{on} and standard errors from 10, 10, and 46 individual MV histograms (i.e., individual myosin molecules) of WT HMM, 15-hep zip, and 0-hep zip, respectively.

(1998) identified such a trigger sequence between heptads 28 and 29 of the smooth muscle myosin heavy chain, which could explain the lack of stability of the dimeric 25-heptad construct, and the stability of dimeric WT-HMM, which has 46 heptads (Trybus, 1994).

We propose that by eliminating the inherent flexibility of the rod in the 0-hep zip construct, this double-headed construct is limited in its displacement-generating capacity. Motion generation in the native molecule probably involves some level of coordination between the two heads because double-headed smooth or skeletal myosin produces twice the step-size (≈10 nm) of the comparable single-headed constructs (≈5 nm) (Tyska et al., 1999). Either both heads attach sequentially and contribute equally to displacement, or one head serves to tether and guide the second head so that it generates its maximum displacement (i.e., ~10 nm). With the 0-hep zip this coordination may be lost, as this construct produces average displacements of <1 nm. If both heads of 0-hep zip can attach simultaneously, then neither head can generate a complete powerstroke due to hindrance caused by the neighboring head. Alternatively, only one head attaches and generates the observed displacements. For this case, either the second head blocks the motion-generating head from undergoing its maximal displacement due to its close proximity, or the single attached head behaves like a single-headed myosin, generating up to 5-nm displacements (Tyska et al., 1999). Based on the histogram in Fig. 3, the latter interpretation seems plausible because individual molecules were capable of generating 5-nm displacements, albeit in both the positive and negative directions. This result might suggest that the 0-hep zip construct has lost its ability to bias its displacements in the normally positive direction. Regardless of the mechanism, by rigidly stabilizing the head-rod junction, the required coordination of two heads has been compromised, resulting in less than optimal mechanical performance.

The profound effect of stabilizing the head-rod junction on single myosin molecule mechanics clearly focuses attention to this region as a critical component in myosin's ability to generate a powerstroke. In fact, based on tension transient measurements in single smooth muscle cells, Warshaw and Fay (1983a, b) proposed that smooth muscle myosin's significantly greater compliance compared to skeletal muscle myosin might allow the myosin head to interact with a greater number of actin sites. This could contribute to the smooth muscle's high force-generating capacity (VanBuren et al., 1994). It is possible that the flexibility at the head-rod junction of smooth muscle myosin contributes to the compliance measured in the tension transient studies.

It remains to be determined whether separation of the coiled-coil is a feature specific to smooth muscle myosin, or other double-headed conventional and unconventional myosin species also need this feature to attain their optimal mechanical performance. The rod region of smooth muscle myosin can form a folded conformation (Trybus et al., 1982), and thus it is already known that it has properties that are not shared with other myosins. Nevertheless, the ability to optimally attach both heads of one myosin molecule to actin would appear to be a feature that would confer mechanical advantages to any myosin species.

We thank Yelena Freyzon, Terri Messier, Eric Hayes, and Janet Vose for expert technical assistance, and Art Rovner for helpful comments.

This work was supported by funds from the National Institutes of Health (HL54568 to K.M.T. and D.M.W.) and the Totman Fund for Cerebrovascular Research (to D.M.W.). A.-M. L. is a Parker B. Francis fellow.

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