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Calcium regulates scallop muscle by changing myosin flexibility

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Abstract Muscle myosins are molecular motors that convert the chemical free energy available from ATP hydrolysis into mechanical displacement of actin filaments, bringing about muscle contraction. Myosin cross-bridges exert force on actin filaments during a cycle of attached and detached states that are coupled to each round of ATP hydrolysis. Contraction and ATPase activity of the striated adductor muscle of scallop is controlled by calcium ion binding to myosin. This mechanism of the so-called ''thick filament regulation'' is quite different to vertebrate striated muscle which is switched on and off via ''thin filament regulation'' whereby calcium ions bind to regulatory proteins associated with the actin filaments. We have used an optically based single molecule technique to measure the angular disposition adopted by the two myosin heads whilst bound to actin in the presence and absence of calcium ions. This has allowed us to directly observe the movement of individual myosin heads in aqueous solution at room temperature in real time. We address the issue of how scallop striated muscle myosin might be regulated by calcium and have interpreted our results in terms of the structures of smooth muscle myosin that also exhibit thick filament regulation.

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Introduction

We now know that there are at least 18 different myosin classes (Sellers 2000) (numbered I–XVIII) and they are responsible not only for muscle contraction but also for many aspects of cell motility. Although much attention has turned from studies of muscle myosin IIs to the more recently discovered, non-muscle myosin classes the muscle contractile mechanism remains central to our understanding of force generation and regulation of the acto-myosin motor system (Geeves and Holmes 1999). Comparative studies of diverse muscle types have led to important insights into the general contractile mechanism pertinent both to muscle research and more widely to the role of myosins in other forms of cell motility. Interest in scallop muscle myosin regulation has increased in recent years through the discovery that many of the non-muscle myosins are also controlled through calcium binding to their calmodulin family light chains.

Scallop striated adductor muscle myosins, in common with all muscle myosin IIs are hexameric proteins, comprising two heavy chains and two pairs of dissimilar light chains (Chantler 2005). The N-terminal region of each heavy chain is folded into a globular ''head'' encompassing motor and regulatory domains. The motor domain comprises catalytic and actin binding sites and the regulatory domain is formed by an extended alpha helix that binds two calmodulin family light chains [termed essential and regulatory light chains ELC and RLC, respectively]. The regulatory domain, which is some 8 nm away from the actin-binding interface, is thought to act as a mechanical lever arm to amplify movements of the motor domain and is a common feature of all myosins. The regulatory domain can also control the ATPase activity of the myosin head. The myosin heavy chain then winds into a coiled-coil forming region that allows heavy chain dimerization and enables myosins to pack together to form thick filaments of the muscle sarcomere. The two heads of each myosin molecule project from the surface of the thick filament

and reach out to bind to the surrounding actin thin filaments.

Myosin-linked regulation is the predominant control mechanism in all classes of non-muscle myosin—either by light chain or heavy chain phosphorylation or by calcium binding to the light chains. When regulated, the "on" state is characterized by high ATPase activity accompanied by the production of force or movement in muscle fibers and in vitro motility assays, whilst in the "off" state these activities are inhibited. We know that individual scallop myosin heads produced by limited proteolysis (termed subfragment-1 or S1) are constitutively "on" irrespective of bound calcium (Kalabokis et al. 1996; Szent-Gyorgyi et al. 1973). Consequently, existing crystal structures of scallop S1 (Houdusse et al. 2000) do not reflect the inhibited state, two heads and functional regulatory domains being required for full regulation (Kalabokis and Szent-Gyorgyi 1997). Alternative approaches are therefore necessary to glean information concerning the inhibited state of regulated myosin.

Electron microscopy and analytical ultracentrifugation studies of a two-headed soluble proteolytic fragment (termed heavy meromyosin or HMM) have shown that in the off-state (calcium-free; ADP present), 81% of heads are folded back towards the coiled-coil as compared with only 41% in the presence of calcium (calcium and ADP present) (Stafford et al. 2001). In the latter case, 59% of the heads were shown to be outstretched, perhaps allowing them to bind actin more readily in the presence of calcium (Stafford et al. 2001). One possibility is that, in the absence of calcium, heads are organized in an asymmetric structure akin to the one described for the rapidly sedimenting, 10 S, conformation of smooth muscle myosin previously visualized by cryoelectron microscopy and 3D image reconstruction (Liu et al. 2003), from which they are released upon calcium activation. A recent cryoelectron microscopy study of another thick filament regulated system, tarantula muscle, shows that in the relaxed state (absence of calcium) the myosin heads pack against each other and lie close to the thick filament backbone giving rise to a repeating "J-motif" (Woodhead et al. 2005). Like vertebrate smooth muscle, regulation in tarantula leg muscle is mediated by a calcium-controlled phosphorylation. This is in contrast to scallop myosin in which activation occurs by calcium binding directly to the light chains. Notwithstanding this difference, the structural mechanism for inhibition of myosin ATPase seems to be similar in tarantula and scallop. Woodhead et al. found that there are additional interactions between myosin heads on adjacent crowns of the thick filament longitudinal repeat, similar to earlier findings (Levine et al. 1988). Such intermolecular interactions might contribute extra inhibition but they would not occur in the studies made here using soluble HMM in which the thick filament backbone structure is absent.

Early biochemical studies (Bennett and Bagshaw 1986) showed that the ATPase activity of scallop myosin in the absence of Ca^{2+} is dominated by a small fraction of unregulated myosin heads from which the RLC had become dissociated. Most protein preparations contain a proportion of myosins that have become desensitized in this way (Nyitrai et al. 2002). It was also discovered that loss of one RLC from a pair of myosin heads dramatically reduces mobility of both myosin heads (Wells and Bagshaw 1983) probably through increased intramolecular (the so-called "head–head") interactions. The loss of RLCs can be reduced by including 1 mM free magnesium and by adding free RLC to buffer solutions. In our current study, solutions contained 1 mM free magnesium but did not contain additional RLC suggesting that about 6% of the myosin heads were desensitized to activation by calcium. This means that any changes we observe following calcium activation would be underestimated due to the presence of these rogue, unregulated, molecules.

In the current study, we used a single molecule technique to measure the orientation of myosin heads bound to actin in the absence of nucleotide (rigor) and deduced their geometry with respect to the thick filament axis. Observation of fluorescently labeled actin filaments that had been cross-linked by a single, two-headed, HMM molecule, has enabled us to measure both the mean and standard deviation of resting angles between the filaments. Assays that were conducted in the presence and absence of calcium give evidence in favor of an increased flexibility (i.e., reduced stiffness) in the presence of calcium ions. Prior to this study, little or no calcium sensitivity has been found for interaction between scallop myosin and actin in the absence of nucleotide in terms of binding or unbinding kinetics (Nyitrai et al. 2003; Wells and Bagshaw 1984). These new data suggest that calcium binding (even in the absence of nucleotide) leads to an increased independence of head action by splaying the myosin heads apart. In the absence of calcium, the regulatory region might stiffen leading to intramolecular interactions that inhibit ATPase activity and motor domain binding to actin.

Materials and methods

Protein preparations and ATPase measurements

Myofibrils and myosin from scallop (*Pecten maximus*) striated adductor muscles were prepared by standard procedures (Chantler and Szent-Gyorgyi 1980; Patel et al. 2000). Highly regulated HMM, which unlike the intact molecule does not form filaments, was prepared as described (Kalabokis and Szent-Gyorgyi 1997; Stafford et al. 2001). Minor variations of the original protocol were determined through pilot studies on the particular batch of trypsin from our supplier (Sigma-Aldrich Co.). Briefly, 1 g of myosin (10 mg/ml) was digested with trypsin (2.5 U/mg myosin) in 10 mM P_i, 0.5 M NaCl, 10 mM Mops, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM DTT, *p*H 6.8, for 1 min at 23°C. Digestion was

terminated through the addition of soybean trypsin inhibitor at 10 mg/mg trypsin then the mixture was dialyzed at 4°C against 10 mM P_i, 40 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 3 mM NaN_3, 0.5 mM DTT, pH 7.0. Following clarification by high speed centrifugation (30 min at 100,000*g*), diadenosine pentaphosphate was added to a concentration of 5 μM prior to HMM precipitation in 70% ammonium sulfate, 0.1 mM EGTA, 5 mM NaCl, 2.5 mM $MgCl_2$, 0.25 mM ADP, 0.7 mM DTT, 3.0 mM NaN_3, pH 6.8. Subsequent steps followed the published protocol (8) although column fractionation into shorter (141 kDa) and longer (156 kDa) components was not attempted. SDS gel (12.5%) electrophoresis of a typical HMM preparation is shown in Fig. 1 and appears similar to that described previously (see Fig. 1d of Stafford et al. 2001). It consists of a heavy chain doublet (156 and 141 kDa) together with breakdown products at about 90 and 70 kDa [clearly visible at this same location in the earlier paper (Stafford et al. 2001) yet referred to as having molecular weights of 70 and 50 kDa].

ATP turnover number was measured using a colorimetric technique described previously (Chantler et al. 1981; Patel et al. 2000). Briefly, the reactions were performed in 3.0 ml volumes containing 1.2 mg HMM, 2 mM Mg.ATP, 20 mM NaCl, 20 mM Tris pH 7.5, in the absence (0.2 mM EGTA) and presence (0.2 mM EGTA, 0.25 mM $CaCl_2$) at room temperature. Over a 10-min period, aliquots (0.2 ml) of the reaction mix were removed at 150-s intervals and quenched by acid precipitation prior to phosphate determination using a tungstate-based colorimetric assay (Patel et al. 2000).

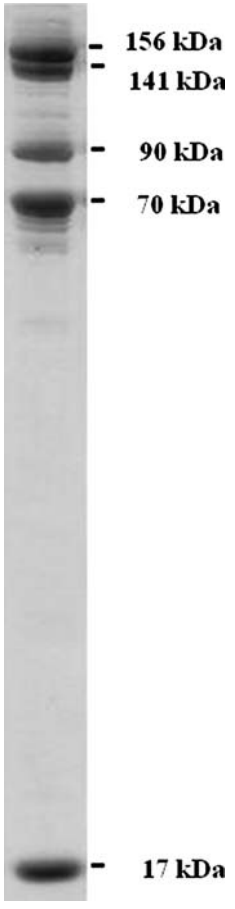

Fig. 1 Polyacrylamide (12.5%) SDS gel electrophoresis of a typical HMM preparation used in this study. Molecular weight assignments were made from measurements using standards run on a sister lane of the same gel (not shown) (see the text for details)

In vitro motility assays

The in vitro sliding velocity of actin filaments moving on purified scallop HMM was measured according to standard methods (Kron et al. 1991). Actin filaments, labeled with rhodamine–phalloidin were observed moving on myosin coated microscope coverslips using an image intensified camera system attached to a fluorescence microscope (Molloy et al. 1995). Images were recorded directly to computer hard-disc using a frame grabber card and actin filament sliding velocity was determined using proprietary image analysis software. Briefly, coverslips (22×22 mm^2) were coated with 0.1% nitro-cellulose in amyl acetate and then built into a flow cell arrangement by gluing (using UV curing glue) two thin strips of coverslip to opposite edges and then fixing these to a microscope slide; this gave a flow-cell volume of about 50 μl volume. Scallop HMM (50 μg/ml in AB−) was infused into the flow cell, and then the surface was washed with buffer (AB−) and blocked with BSA (0.5 mg/ml) containing solution. Finally, labeled actin filaments were introduced into the flow cell and this was then viewed using a fluorescence microscope. Experimental solutions were based on those described earlier (Kron et al. 1991); AB− contained: 25 mM KCl, 25 mM imidazole–HCl, 4 mM $MgCl_2$, 1 mM EGTA, *p*H 7.4; AB+ was identical to AB− but supplemented with 2 mM ATP. A series of calcium containing solutions were made by adding 1.1 mM $CaCl_2$ to AB+ and mixing this in varying proportions to calcium-free AB+. The concentration of free calcium was calculated using a computer program, SOLCON (written by D.C.S. White based on Perrin and Sayce 1967). On the day of the experiment, all solutions were degassed and an anti-photobleaching cocktail (consisting of 20 mM DTT, 0.5 mg/ml glucose oxidase, 5 mg/ml glucose, 0.2 mg/ml catalase) was added before use. Solutions were stored in disposable hypodermic syringes (with needles fitted) so as to reduce the rate of oxygen diffusion into the buffers during the experimental procedures. All motility assays were performed at 23°C.

Single molecule studies of scallop regulatory domain flexibility

To measure the flexibility and relative disposition of the two myosin heads we bound HMM to fluorescent actin filaments so that, on average there was a myosin head available to bind to each F-actin filament present. We mixed varying ratios of scallop HMM and rhodamine–

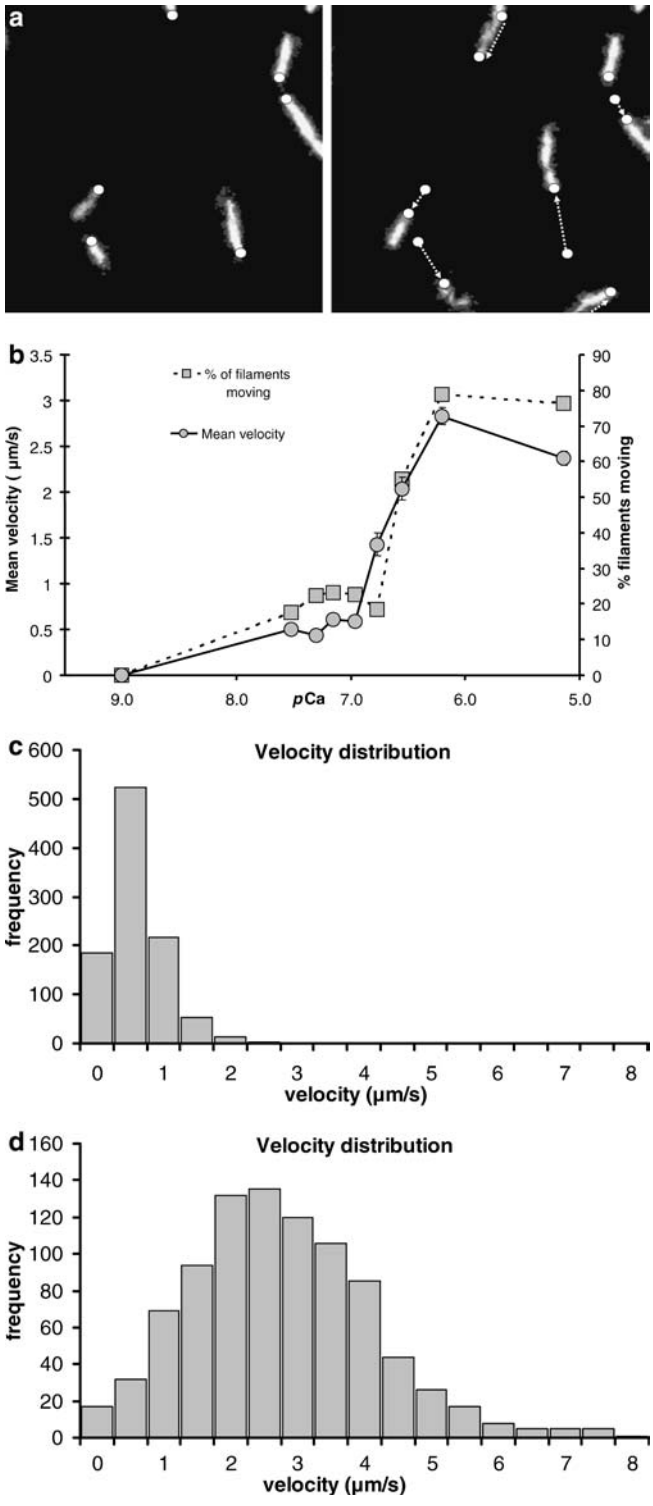

Fig. 2 In vitro motility assays, in which individual rabbit actin filaments stabilized with rhodamine–phalloidin are visualized using fluorescence light microscopy. **a** Shows two captured video images separated in time by 2 s in which the positions of fluorescent actin filaments, which appear white, are identified by *white dots*. Careful measurement of the distance moved by the filaments between sequential video images allows their sliding velocity to be calculated. The width of the image is 25 µm. **b** Shows the distribution of actin filament sliding velocities measured at 2 mM Mg.ATP, $p\mathrm{Ca} > 8.0$. **c** Shows the velocity distribution measured under the same conditions but at much higher free calcium ion concentration, $p\mathrm{Ca}$ 5.1. **d** Shows how the average sliding velocity and proportion of moving filaments depends upon the free calcium ion concentration (all experiments were performed at 23°C)

phalloidin labeled actin under rigor conditions and then viewed them by fluorescence light microscopy. Apyrase (Sigma-Aldrich Co.) was used to catalyze conversion of contaminant ATP $\rightarrow$ ADP + Pi $\rightarrow$ AMP + Pi (where 1 U enzyme activity liberates 1 µmol of Pi/min at 30°C).

Rhodamine–phalloidin labeled actin (stock concentration 2.5 µM in AB^{+}) was diluted to give 5 nM final concentration in AB−. Serial dilutions of scallop HMM (stock concentration 10 µM in AB−) were created over the range 1 nM to 10 pM by dilution in AB−. The actin and HMM containing solutions were then mixed in equal volumes (100 µl total volume including 1 U apyrase) and incubated for 30 min. We found that a 1:100, HMM:actin molar ratio (2.5 nM actin and 25 pM HMM) yielded a reasonable number of actin filaments that were cross-linked at a single point and rather few that were bundled. To perform the observations, the microscope slide and coverslip were first pre-blocked with BSA (0.5 mg/ml in AB−), to prevent non-specific adsorption of actin to the surfaces. We added a small number of 5 µm diameter glass beads (Bangs Laboratories) to the actin–myosin containing solutions (prepared as above) and applied a 10 µl droplet of the mixture to the slide surface. The coverslip was placed on top of the droplet and moderate pressure applied so that the space between coverslip and slide was limited by the presence of the 5-µm beads. This procedure ensured that the cross-linked actin filaments remained in one plane of view during observation. The coverslip was fixed in place using clear nail varnish. Images were recorded by fluorescence light microscopy (using a rhodamine excitation/emission filter set) and observations were recorded using an image-intensified camera (Photon ICCD, EEV, Chelmsford, UK) attached to a frame–grabber card (Picolo, Euresys). Video data was saved directly to computer hard disc. Images were later analyzed using ImageJ software (http://www.rsb.info.nih.gov/ij/). The angle between crossed actin filaments was measured from individual video frames by fitting lines to each of the filaments by eye; these angles were tabulated and further statistical analysis was performed using Microsoft Excel. It was not possible to view the same filaments in the presence and absence of calcium due to difficulties in introducing new buffer solutions without disturbing the field of view.

Molecular structure modeling

Acto-myosin complexes were modeled using Insight II (MSI Inc.). Since there is no atomic structure for the

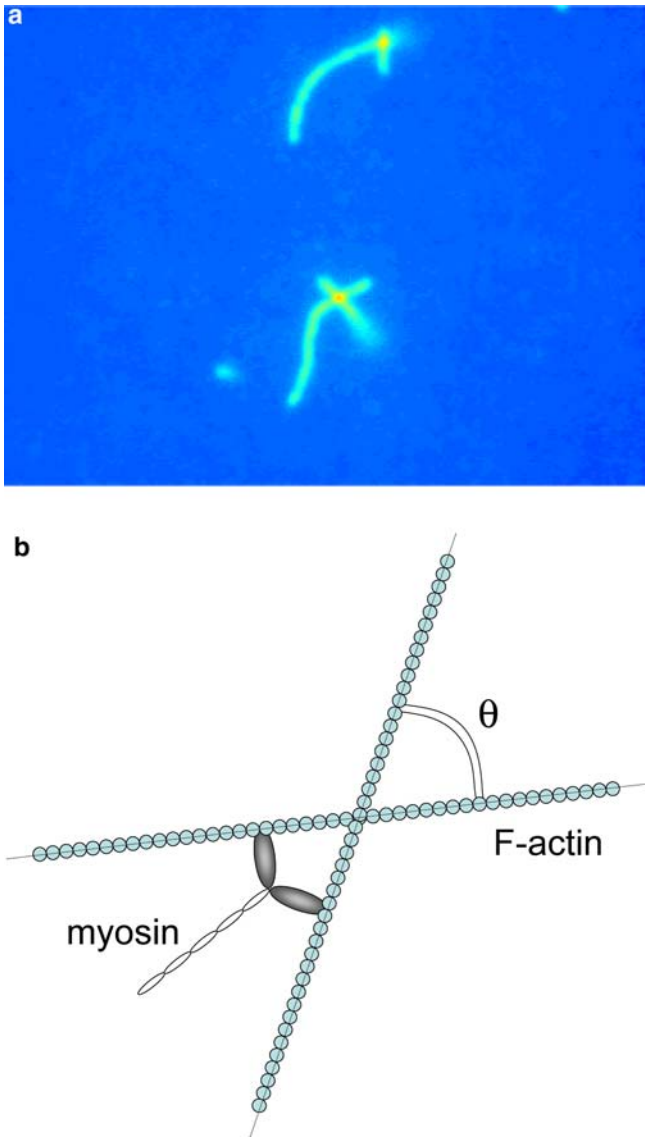

Fig. 3 **a** When actin filaments were mixed with low concentrations of scallop adductor heavy mero-myosin (at a ratio of 100 actin monomers to 1 HMM molecule) in the absence of ATP (rigor conditions) characteristic cross-linking of the filaments occurred. The angular disposition of the filaments was measured (see cartoon, **b**) from stored video images. Cross-linked filaments were observed at low and high free calcium ion concentrations

acto-myosin complex, our starting point was to use acto-myosin atomic coordinates derived from the fitted X-ray crystallographic structures of actin and myosin II to the published insect flight muscle tomographic electron micrograph reconstructions (Taylor et al. 1999) (accession number: IM8Q.pdb). We superimposed the chicken skeletal muscle myosin structure (Rayment et al. 1993) (2MYS.pdb which contains C_α-carbon coordinates only). These were then further superimposed upon the modeled structure for scallop myosin HMM neck region ($+Ca^{2+}$) (Offer and Knight 1996) (PDB file courtesy of Dr. Gerald Offer) and also the structure for smooth muscle myosin ($-Ca^{2+}$) (Wendt et al. 2001) (1I84.pdb).

Results

To perform our studies it was essential to use a two-headed, regulated preparation of scallop myosin that was soluble under physiological salt conditions. We have therefore taken advantage of a recently developed preparation of regulated scallop HMM (Kalabokis and Szent-Gyorgyi 1997; Stafford et al. 2001). Preparations were purified by chromatography and routinely checked by gel electrophoresis (Fig. 1) to ensure that the correct fragments were isolated. We did not, however, make any attempt to fractionate the two heavy chain components (Stafford et al. 2001). ATPase activity, in vitro motility and single molecule flexibility studies were performed on this regulated HMM.

ATPase data

ATPase activities were measured under the conditions described in Materials and methods at *p*Ca 4.0 and 7.5 to establish the degree of regulation. We found the specific site activity to be 2.56 ± 0.28 mol ATP mol head^{-1} s^{-1} (*p*Ca 4.0) and 0.156 ± 0.09 mol ATP mol head^{-1} s^{-1} (*p*Ca 7.5), at 20°C providing, on average, a 16-fold increase in activity upon addition of calcium. Earlier work (Wells and Bagshaw 1985) indicates that fully sensitized scallop myosin would theoretically give 300-fold activation by calcium. Our results show that the calcium sensitivity of our protein preparation [(ATPase^{+Ca} – ATPase^{-Ca})/ATPase^{+Ca+}] is 94% (Wells et al. 1982) or, in other words, that the proportion of desensitized myosins was about 6%. This compares favorably with values obtained by other workers.

In vitro motility assays

We measured the in vitro sliding velocity of rabbit skeletal muscle actin filaments, stabilized with rhodamine–phalloidin and moving on regulated scallop HMM, by fluorescence light microscopy (Fig. 2a–c). This type of measurement has not previously been performed using a highly regulated preparation of scallop HMM, but the profile is broadly consistent with an earlier in vitro assay that used scallop myosin-coated beads moving on *Nitella* actin filament cables (Vale et al. 1984). We found that at low calcium concentrations (*p*Ca > 7.0) less than 15% of the filaments, within any single field of view, moved; of the filaments that did move, the average velocity was 0.5 μm/s (Fig. 2b). Both the proportion of moving filaments and their average velocity increased dramatically as the calcium concentration was raised above *p*Ca 6.5. The Hill coefficient and the $pCa_{½}$ were found to be ~4.0 and 6.6, respectively. At saturating calcium level, the maximum sliding velocity was 2.8 μm/s with over 75% of filaments moving (Fig. 2c, d).

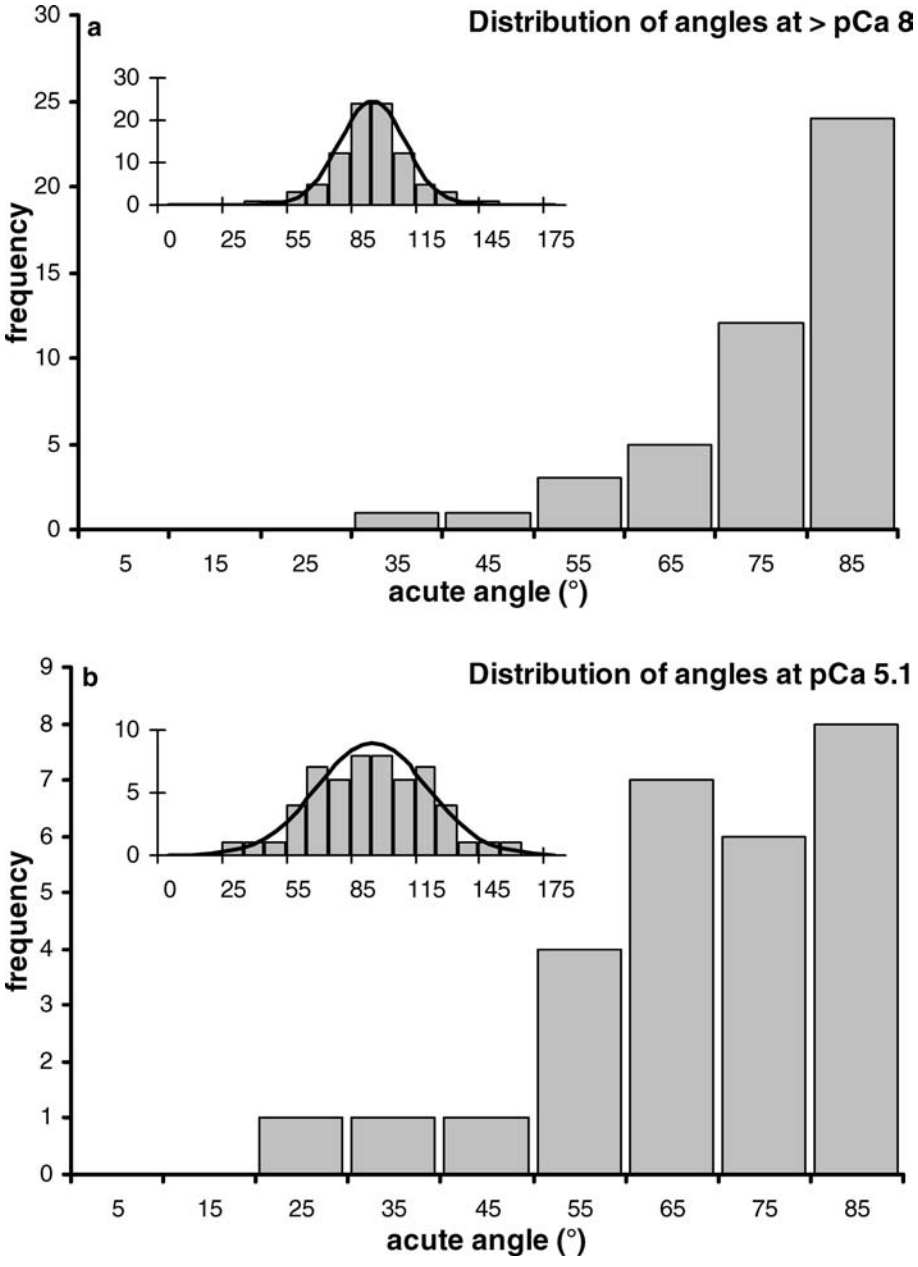

Fig. 4 The distribution of angles measured at the cross-over between actin filaments was measured at **a** low free calcium > *p*Ca 8 and **b** high free calcium *p*Ca 5.1. The *inset graphs* show the same data reflected about the orthogonal axis as we were unable to determine the polarity of the actin filaments so the angle is ambiguous. The fitted lines assume that angular dispersion is due to thermal vibration (see Eq. 2 and main text for details) which gives an estimate of the torsional rigidity of the myosin cross-links, the fits are: 0.52 pN nm/rad at low calcium conditions and 0.17 pN nm/rad at high calcium

Single molecule measurements of myosin head angles and flexibility

We were able to infer the angle between the two myosin heads of the HMM molecule by measuring the angle between two actin filaments cross-linked by a single molecule. Care was taken to ensure that a single molecule was responsible for this cross-linking by performing a dilution series in which the ratio between HMM and actin was systematically varied such that cross-linked filaments were only rarely observed within a single field of view. We found that a molar ratio of one HMM to ~100 actin monomers was suitable for these studies. At higher molar ratios we observed bundling of actin in which, we presume, multiple myosins bind along the filaments causing them to pack in parallel fashion to form very bright and rigid structures. Individual cross-linked actins were observed by fluorescence light microscopy (Fig. 3a) and the angle of intersection, θ, was measured for many individual filaments (Fig. 3b). The distribution of angles measured at low calcium (*p*Ca > 8.0) and high calcium (*p*Ca 5.1) were plotted as histograms (Fig. 4a, b). Current methods to polarity-mark actin filaments resulted in only a small population of filaments being marked, making it impossible for us to resolve ambiguity in the angle, θ, with respect to actin filament polarity. Consequently, our interpretation of the distribution of angles from the histograms has inherent ambiguity (see Discussion) but shows, nonetheless, that the distribution of observed angle is broader at high free calcium concentrations. The variance in our observed angles, $<\theta^2>$, is due mainly to thermal

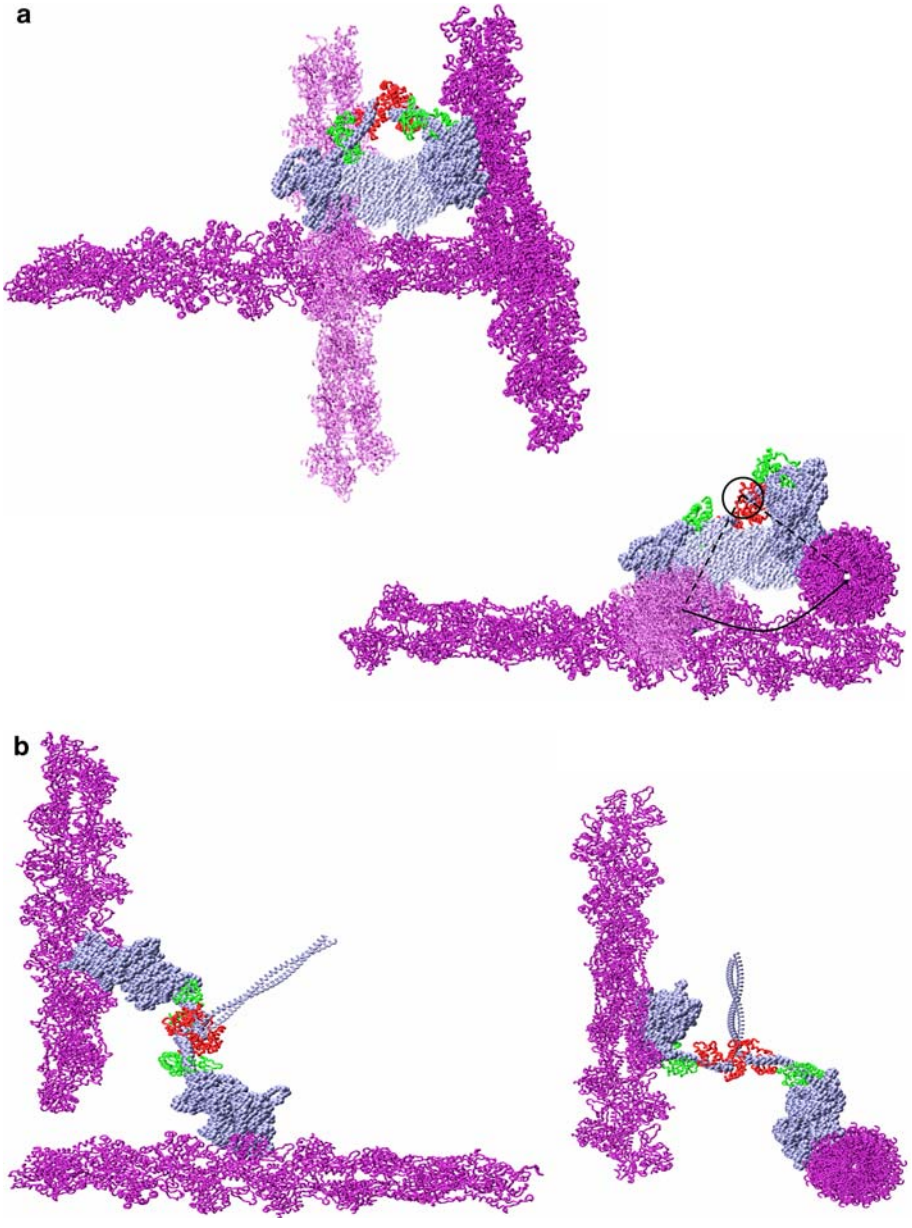

Fig. 5 Molecular models of the two-headed scallop acto-myosin cross-linked structure were created using insect tomographic reconstructions of the acto-myosin rigor state (Liu et al. 2003) combined with the strained, asymmetric, smooth muscle (low calcium, ''off'' state) (**a**) and the modeled symmetric two-head conformation proposed for smooth-muscle myosin rod backbone structure (high calcium, ''on'' state) (**b**). The pairs of images (*left and right*) represent two views that are related by a 90° rotation about the horizontal plane in (**a**) and the vertical plane in (**b**). In (**a**), there is a steric clash, such that the actin filaments interfere with one another. This clash was resolved by rotating one of the myosin heads about the head–head junction. The initial structure that we built shows how one of the actin filaments (light magenta) clashes with the other actin filament. The dark magenta color is used to represent the resolved structures. The axis of rotation is shown in the *lower, right figure*. Both the **a** and **b** structures show actin binding sites related by an axis of symmetry such that bound actins intersect at close to 90°. The structure in (**a**) is more compact than (**b**) and consistent with the stiffer (i.e., less variable) cross-linking observed at low calcium (see Fig. 4a). The models are also consistent with the rigor myosin heads binding to actin at an angle of 45° to the thick filament backbone. Color coding: myosin is *silver*, regulatory light chain is *red*, essential light chain is *green*, actin monomers are *magenta*

vibration and we expect a Gaussian distribution given by the equipartition principle (Eqs. 1, 2):

$$<\theta^2> = k_B T/\kappa, \quad (1)$$

$$N_{obs} = A \times \exp(-\kappa(\theta - \theta_0)^2/k_B T, \quad (2)$$

where κ is the torsional stiffness of the myosin heads, k_B is Boltzman's constant and T is the absolute temperature and the observed distribution of angles, N_{obs}, will be centered about the mean angle, θ_0. By this analysis, we estimate the torsional stiffness to be 0.52 pN nm/rad at

low calcium conditions and 0.17 pN nm/rad at high calcium (Fig. 4a, b). These values are 100-fold lower than our earlier estimates (Tyreman et al. 2003) of myosin torsional stiffness made using skeletal muscle myosin subfragment-1 bound non-specifically to a nitrocellulose surface, but similar to values obtained when the proteins were bound via a biotin–streptavidin linkage (MJA Tyreman, PhD thesis, University of York, UK).

We observed how the angle made between individual cross-linked actin filaments changed with time by replaying video sequences. We found that there were slow fluctuations in angle with a relaxation time on the order of several seconds. However, our data sets were not sufficiently good to establish whether the angular fluctuations in time were truly Lorentzian (Hunt and Howard 1993) and therefore strictly consistent with the model of a torsional spring (i.e., the myosin heads) plus viscous damping (i.e., actin filaments moving in aqueous solution). To confirm this model we would have needed to observe individual cross-linked filaments for hundreds of seconds. We found translational drift in x, y and z planes prevented us from doing this. Given that the length, L, of our actin filaments was between 2 and 5 μm, and F-actin has a radius, r, of 2.5 nm the drag, β, for unbounded rotational diffusion about the filament mid-point (see Eq. 3) (Howard 2001) would be 5 pN nm rad^{-1} s.

$$\beta = \frac{1}{3[\ln(L/2r) - 0.66]}\pi\eta L^3. \quad (3)$$

This means that we expect to see mechanical relaxation on a timescale, β/κ, of around 10 s which is consistent with our observations (above) that there was slow diffusional motion about the resting angle.

Molecular modeling

We docked the all-atoms structure of chicken skeletal myosin S1 (2MYS.pdb) onto the structure of the acto-myosin complex derived from tomographic reconstructions of rigor insect flight muscle (1M8Q.pdb) in order to establish the relative angle between the actin filaments axis and the myosin S1 head (currently, this is the only set of acto-myosin coordinates in the public domain). Since the myosin II head structure is highly conserved across scallop, chicken and insect striated muscles, it is likely that the gross angle derived in this way will be satisfactory. Taking this as our all-atom acto-myosin rigor structure we docked the neck region of the myosin molecule onto the neck region of the asymmetric model proposed for the inhibited state of smooth muscle myosin (1I84.pdb) (Wendt et al. 2001) and also onto the symmetrical model (Offer and Knight 1996) of scallop myosin (Fig. 5a, b). Figure 5a, which uses the asymmetric head structure (“off” state) results in a compact spatial relationship, with an approximately orthogonal intersection of bound actin filaments but with a severe steric clash between actin filament structures. We resolved this clash by rotating one myosin head about an axis located at the head–head junction. Figure 5b indicates that the symmetric myosin head structure (“on” state) again leads to an approximately orthogonal relationship of the bound actin filaments but the structure is now much more open and there is no steric clash.

Discussion

Bivalve mollusks, such as the scallop, *P. maximus*, have a powerful striated adductor muscle that is used to bring together its two outer shells during swimming. The muscle works against a rubbery material called abductin present in the hinge region of the shell that is squeezed out of shape when the shell is closed and so produces the necessary force to open the shell when the adductor muscle is relaxed. This muscle has been the subject of considerable interest since myosin-linked regulation was first discovered in these animals (Lehman and Szent-Gyorgyi 1975; Szent-Gyorgyi et al. 1973), and it has served as a model system for myosin-linked regulation, which is an underlying theme of acto-myosin regulation in smooth muscle and non-muscle myosin classes. The motivation for the current study was to examine how scallop myosin is regulated by calcium via its light chain region and clarify whether this might be via a mechanical change that allows myosin to bind more readily to actin. In the current study, we have employed a novel, single molecule, technique to measure the disposition and flexibility of scallop myosin heads in the presence and absence of calcium.

Although vertebrate smooth muscle regulation is activated through phosphorylation of Ser19 on the RLC (Pfitzer 2001), many invertebrate muscle myosins, such as those from the striated adductor muscle found in scallops, are activated directly through a mechanism involving calcium binding to calcium-specific sites located singly on either ELC (Szent-Gyorgyi and Chantler 1994). Several crystal structures are now known corresponding to scallop myosin head fragments (S1) trapped in different states through the use of various nucleotide analogues (Houdusse et al. 2000); these states differ from each other in the disposition of the lever arm with respect to the motor domain.

Nucleotides bind to either intact scallop myosin or to its regulated two-headed HMM subfragment through a cooperative mechanism in the absence of calcium (Kalabokis and Szent-Gyorgyi 1997; Nyitrai et al. 2002); introduction of calcium to the system reduces nucleotide affinity and abolishes their cooperative binding, allowing independent behavior of each head. Calcium binding to myosin is also cooperative in the presence of nucleotides at physiological ionic strength (Kalabokis and Szent-Gyorgyi 1997; Nyitrai et al. 2002), but not in the absence of nucleotide or in the presence of Mg.ATP at low ionic strength (Chantler et al. 1981; Kalabokis and Szent-Gyorgyi 1997).

In the absence of nucleotide, both myosin heads bind very tightly to actin irrespective of the calcium ion concentration (Nyitrai et al. 2003). The two heads can bind either to the same actin filament or, as we have found here, can also bind across two different actin filaments so as to cross-link the two actin filaments. Because filamentous actin exhibits considerable torsional (Yasuda et al. 1996) and flexural (Gittes et al. 1993; Isambert et al. 1995) rigidity on the micrometer length scale, we considered the possibility of probing the disposition of myosin heads in the presence and absence of calcium by measuring the angle, θ, between actin filaments cross-linked by the two heads of a single HMM molecule. The fact that very low concentrations of myosin produce characteristic actin "crosses" shows that the actin binding sites on the myosin heads must have an orthogonal relationship so they could not simultaneously be "ideally configured" to bind to actin in the linear filament lattice of the intact muscle sarcomere. We know that the two heads of rabbit skeletal muscle myosin can bind to the same actin filament, but that this requires considerable intramolecular strain (Conibear and Geeves 1998). This hints at a possible steric constraint in terms of matching the orientation of the actin filament and preferred myosin head orientation within the muscle sarcomere. At higher calcium concentrations we found that the spread of measured angles increases and this means that the rigidity is lower (allowing greater angular dispersion). This effect might relieve the steric constraint in terms of allowing myosin to bind to actin within the sarcomere lattice. Angular motion of the regulatory domain of vertebrate skeletal muscle myosin II relative to its motor domain (in the absence of nucleotide and actin) has been measured previously by negative stain electron microscopy (Burgess et al. 1997). The range of angular motion observed in that study is of the same order (approximately 45° peak-to-peak) as the range of movement found here and is consistent with our finding that scallop muscle myosin heads make large angular excursions about their head–head junction in the presence of calcium.

Consistent with an asymmetric myosin head structure, only one ADP is observed bound to scallop HMM in the absence of calcium (Nyitrai et al. 2002). Furthermore, engineered scallop myosin molecules possessing mutant RLCs (Colegrave et al. 2003) have negated a proposed symmetrical structure for the off-state of scallop myosin, which had been formulated previously through molecular modeling (Offer and Knight 1996). Electron microscopy and sedimentation studies (Stafford et al. 2001) show that in the off state (calcium-free, ADP present), 81% of heads are folded towards the coiled-coil rod (heads down). In the on state (calcium and ADP presence), only 41% of heads were in the down position whilst 59% were in the heads up position. These authors proposed that in the heads down position, geometry of the two heads is asymmetric, and the actin-binding interface of one head is in contact with the converter region of the other, thus inhibiting ATPase activity by preventing domain motion necessary for phosphate release. The two myosin heads might interact not only with each other but also with the coiled-coil rod region. The effect of calcium is to disrupt communication between the two heads allowing them to assume a more symmetric structure.

Our current study also shows that the HMM proteolytic fragment (Kalabokis and Szent-Gyorgyi 1997; Stafford et al. 2001) derived from scallop adductor muscle exhibits calcium activation of both ATPase and in vitro motility. Our in vitro motility assays showed that both the velocity of movement and the number of moving filaments depended upon calcium concentration and that activation was highly cooperative (Hill coefficient of 4) consistent with earlier studies (Kalabokis and Szent-Gyorgyi 1997). Also, we observed that at very low calcium, actin remained bound to the motility assay surface. This implies that either actin must bind to myosin that is in the "off" state, or is held in place (but not moved) by the small number of desensitized, constitutively "on" myosins, which we estimate to be about 6% of the total. The gradation in sliding velocity can be explained by two possible mechanisms. (1) The average speed might be determined by the balance of active (i.e., "on") and inactive (i.e., "off") myosins interacting with the actin filament. Since active myosins would produce a pulling force whereas inactive (but bound) myosins might produce a resistive drag force, velocity would then depend upon the balance of forces given by the ratio of active to inactive myosins and the relative forces that they produce. (2) Alternatively, since actively cycling myosins spend only a brief part of their total catalytic cycle time bound to actin [known as a low duty-cycle ratio (Howard 1997)] the movement produced by individual heads summates in time. We know that a single myosin head produces about 5 nm of movement (Molloy et al. 1995) for each ATP turnover and we have measured the maximum turnover number for scallop HMM to be 2.5/s. This means that a single active myosin head would move actin at just 12.5 nm/s which is 200 times slower than our maximal in vitro actin filament sliding velocity of 2.5 μm/s. As calcium levels are raised more and more myosin heads become "switched on" and their individual, asynchronous, movements would summate to produce an increased velocity. The fact that the number of moving filaments also depends upon calcium implies that there is another level of complexity involved and that at intermediate levels of activation some filaments become completely stalled. This effect is not readily explained by either of the above arguments and requires some form of cooperative mechanism that acts between myosins bound to an individual actin filament. Similar behavior was discovered in a study of thin filament based regulation (Fraser and Marston 1995) but it is perhaps easier to

explain in that case where one can envision an entire thin filament being cooperatively switched on or off.

We conclude that when the scallop, striated adductor muscle is activated by calcium there is a structural rearrangement of the myosin heads that allows them greater freedom of movement. Myosin head–head interactions are weakened and myosin–actin interactions made more favorable. This process would facilitate a high actin-activated ATPase activity, accelerate the in vitro motility and bring about muscle contraction.

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