X-ray diffraction studies of the contractile mechanism in single muscle fibres

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The molecular mechanism of muscle contraction was investigated in intact muscle fibres by X-ray diffraction. Changes in the intensities of the axial X-ray reflections produced by imposing rapid changes in fibre length establish the average conformation of the myosin heads during active isometric contraction, and show that the heads tilt during the elastic response to a change in fibre length and during the elementary force generating process: the working stroke. X-ray interference between the two arrays of myosin heads in each filament allows the axial motions of the heads following a sudden drop in force from the isometric level to be measured *in situ* with unprecedented precision. At low load, the average working stroke is 12 nm, which is consistent with crystallographic studies. The working stroke is smaller and slower at a higher load. The compliance of the actin and myosin filaments was also determined from the change in the axial spacings of the X-ray reflections following a force step, and shown to be responsible for most of the sarcomere compliance. The mechanical properties of the sarcomere depend on both the motor actions of the myosin heads and the compliance of the myosin and actin filaments.

Keywords: skeletal muscle; muscle contraction; myosin; motor proteins; X-ray diffraction

1. INTRODUCTION: ATOMIC MODEL FOR THE WORKING STROKE OF THE MYOSIN MOTOR

Skeletal and cardiac muscles generate force and shortening in well-ordered and parallel arrays of myosin and actin filaments called sarcomeres (figure 1*a*). Each sarcomere contains bipolar myosin-containing 'thick' filaments of length 1.6 μm , overlapping with actin-containing 'thin' filaments of length *ca.* 1.0 μm . The head domains of the myosin molecules, also called 'cross-bridges', project from the surface of the thick filaments and bind to actin monomers in the thin filaments. The myosin heads generate force and shortening by pulling the actin filaments towards the centre of the myosin filament, called the M-line. If the external load on the muscle is low, this results in shortening of the sarcomere (figure 1*a*), with proportional shortening of the muscle. If the length of the sarcomeres is held constant, the myosin heads generate a steady force called the isometric force, T_0 .

The basic mechanism of muscle contraction may be understood in terms of the interaction between only two

proteins, myosin and actin, and one chemical reaction, the hydrolysis of ATP. Actin is thought to play a passive role; it provides a series of binding sites along the muscle thin filaments for the head domain of the myosin motor, and transmits the force to the ends of the sarcomere. The structure of the actin filament was determined by X-ray crystallography and fibre diffraction (Holmes *et al.* 1990; Kabsch *et al.* 1990), and the alpha-carbon backbone of part of the filament containing five actin monomers is shown in figure 1*b* (grey/brown).

Several high-resolution structures of the myosin head domain have been determined by X-ray crystallography (Rayment *et al.* 1993*a,b*; Dominguez *et al.* 1998; Houdusse *et al.* 1999). Two of these structures are shown on the left of figure 1*b*. The lower structure was obtained in the absence of nucleotide (Rayment *et al.* 1993*b*), and is referred to here as the rigor state, by analogy with the state obtained by depleting muscle fibres of ATP. No high-resolution structure of an actin–myosin complex has been described so far, but cryo-electron microscopy of isolated myosin head fragments bound to actin in the absence of ATP established the main structural features of the rigor actin–myosin complex (Rayment *et al.* 1993*a*). The relative position of the rigor myosin head and actin filament in figure 1*b* is taken from those data.

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Figure 1. Structural models for the mechanism of the myosin motor. (a) Arrangement of the actin and myosin molecules in the muscle sarcomere. Grey, actin filament; blue, myosin filament; red, myosin heads. Sarcomere shortening (transition from left to right panel) is associated with tilting of myosin heads so that their mass moves towards the centre of the sarcomere (the M-line). (b) Atomic model for the working stroke in the myosin head. Grey/brown, actin filament (Holmes *et al.* 1990) with filament axis vertical; red, catalytic domain of the myosin heavy chain (residues 1–707); green, converter domain (711–781); blue, long heavy chain helix (781–843); yellow, essential light chain; magenta, regulatory light chain. The lower conformation of the myosin head (light-blue long helix) shows the ATP-free or ‘rigor’ crystal structure of a head fragment bound to the actin filament as determined by cryo-electron microscopy (Rayment *et al.* 1993*a,b*); the upper one (dark-blue long helix; ‘ADP·P_i’) is based on a crystal structure of a smooth muscle myosin head fragment with an ADP·P_i analogue bound at the active site (Dominguez *et al.* 1998), with its catalytic domain superimposed on that of the rigor conformation, and assuming that the light-chain domain of the head moves as a rigid body.

Subsequent crystallographic studies revealed a markedly different conformation of the light-chain domain of the head, containing the long heavy chain helix (blue), the essential light chain (yellow), and the regulatory light chain (magenta), with respect to the catalytic domain, the region that binds actin and ATP (figure 1*b*, red). In muscle, the light-chain domain is connected to the myosin tail domain which forms the backbone of the myosin filament. The upper conformation of the light-chain domain in figure 1*b* (with the darker blue long helix) relative to the catalytic domain is based on a crystal structure of a smooth-muscle myosin head fragment with an analogue of the ATP hydrolysis products ADP and P_i at the active site (Dominguez *et al.* 1998). It is not known whether the catalytic domain of a myosin head with ADP and P_i in the active site would bind to actin in the same way as in the absence of nucleotide but, assuming that it does, the catalytic domains of the two myosin head structures may be superimposed to determine the relative position of the two light-chain domains (Dominguez *et al.* 1998; Geeves & Holmes 1999; figure 1*b*). The result is an atomic model for the motor mechanism in which release of ADP and P_i from the active site of the catalytic domain leads to a tilt of the light-chain domain that moves its tip by *ca.* 11 nm towards the Z-line of the sarcomere (figure 1*a*). If the tip of the light-chain domain were connected to the myosin filament,

as in a muscle fibre, this motion would cause relative sliding of the myosin and actin filaments in the direction required to shorten the muscle sarcomere.

2. X-RAY FIBRE DIFFRACTION FROM MUSCLE

The atomic model in figure 1 provides a working hypothesis for the molecular mechanism of muscle contraction. X-ray diffraction allows this hypothesis to be tested in an intact muscle fibre. It also reveals the dynamics of the structural change underlying generation of force or shortening, and its coupling to the external load.

X-ray fibre diffraction depends on the alignment of the myosin and actin filaments with the fibre axis, and the regular arrangement of myosin heads and actin monomers within each filament. When a collimated beam of X-rays is incident on a muscle fibre, these periodic structures give rise to X-ray reflections that provide information about the structure and organization of the myosin and actin filaments. Because muscle fibres diffract X-rays very weakly, it is necessary to use a very bright X-ray source, and in practice this means that the experiments must be performed at a synchrotron or electron storage ring.

This article focuses on the myosin-based meridional (or axial) X-ray reflections produced by the regular repeat of myosin heads along the myosin filaments (figure 1*a*). These reflections are sensitive to the axial motions of the

Figure 2. Changes in the intensity of the M3 reflection (I_{M3}) produced by length steps. (a) Changes in force and I_{M3} in response to a shortening step of 6 nm per half-sarcomere (nm hs^{-1}) followed 1 ms later by a re-stretch to the original length. Replotted from figure 3 of Irving *et al.* (2000). (b) Relationship between I_{M3} and the displacement of the myosin head rod junction (Δz), calculated from the structural model in figure 1b. $\Delta z = 0$ corresponds to the ATP-free or rigor structure (circle) (Rayment *et al.* 1993a,b). The square corresponds to the ADP·P_i structure (Dominguez *et al.* 1998).

Figure 3. Origin of the interference fine structure of the M3 X-ray reflection. (a) Sarcomere structure and (b) calculated and observed intensity profiles. Each myosin filament in the sarcomere contains two arrays of myosin heads. A single array would give the diffracted intensity profile shown in red in (b), with a peak near $1/14.5 \text{ nm}^{-1}$. Interference between the two arrays multiplies the diffraction pattern by a sinusoidal function (blue) with spacing corresponding to the interference distance between pairs of myosin heads in the filament. The resulting intensity profile (light green) is similar to that observed from a single muscle fibre during isometric contraction (dark green).

myosin heads associated with force generation. In resting muscle, the myosin heads have a helical arrangement on the surface of the filament, giving rise to a series of X-ray reflections called the M1, M2, M3, etc., which are orders of the *ca.* 43 nm helical repeat of the myosin filament. In actively contracting muscle the helical order is lost, but the M3, M6, M9, etc. reflections remain strong. These

correspond to orders of the 14.5 nm axial repeat of the myosin heads along the filaments (figure 1a).

Each of these X-ray reflections provides three independent parameters that yield useful information about muscle ultrastructure: the *intensity* (or amplitude) of a reflection on the conformation of the myosin heads; the *interference fine structure* on axial motions of the heads; and

the *spacing* on the periodicity and compliance of the myosin filament.

3. THE STRUCTURAL CHANGES IN MYOSIN HEADS THAT DRIVE MUSCLE CONTRACTION

(a) *Determining myosin head conformations from axial X-ray intensities*

The intensities of the axial X-ray reflections depend on the projection of the mass distribution of the array of myosin heads onto the filament axis, and therefore on their orientation and orientational dispersion. Because the relative phases of the reflections are unknown, it is not possible to calculate these orientation parameters directly from the measured intensities. However, if changes in intensities are recorded under conditions in which the conformation of the myosin heads changes without any change in their number or dispersion, a unique interpretation in terms of head conformation is possible. These conditions may be achieved by imposing a rapid change in sarcomere length on actively contracting muscle, so that all the myosin heads that are attached to actin are distorted by the same amount. The intensity change must be recorded on a time-scale faster than that of detachment of the heads from actin, i.e. on the millisecond time-scale or faster.

(b) *Early studies using whole muscles*

The pioneering experiments of this type were performed on intact muscles of the frog by H. E. Huxley, R. M. Simmons and their co-workers (Huxley *et al.* 1983), who showed that the intensity of the M3 axial reflection, from the 14.5 nm axial repeat of the myosin heads along the filaments, was greatly reduced when the muscle was either released or stretched by *ca.* 1% in 1 ms during isometric contraction. These results suggested that the length steps induced large changes in the conformations of the myosin heads attached to actin. The structural response to a release, but not that to a stretch, was delayed by *ca.* 0.5 ms behind the length change. This delay suggested that the conformational change in the heads might not be related to the length step itself, but rather to the rapid recovery of force that follows such a step. This rapid force recovery is thought to be associated with the working stroke in the myosin head (Huxley & Simmons 1971). However, this process cannot be temporally resolved in whole muscle preparations.

(c) *Single muscle fibres: temporal resolution of the working stroke*

In an isolated intact muscle fibre it is possible to apply a length change of a few nanometres in each sarcomere that is complete in *ca.* 0.1 ms. In principle, this allows the structural changes associated with the length step to be temporally resolved from those associated with the *ca.* 1000 s⁻¹ force recovery that follows it, the putative working stroke. However single muscle fibres diffract X-rays very weakly, and measurements of the intensity of the M3 reflection from a single fibre with the required time resolution only became feasible as a result of two further developments. Brighter synchrotron X-ray beams became available, and the discovery of the rapid repriming of the working stroke during a series of length steps (Lombardi *et al.* 1992) allowed extensive signal averaging of the X-ray

responses (Irving *et al.* 1992, 2000; Lombardi *et al.* 1995; Dobbie *et al.* 1998).

Figure 2a shows an example of such an experiment, replotted from the data of Irving *et al.* (2000), which shows the main features of the changes in the intensity (I_{M3}) of the M3 reflection in response to rapid length steps. At the start of the trace the muscle fibre was producing its isometric force (T_0). At zero on the time axis the length of the fibre was decreased in *ca.* 150 μ s, so that each sarcomere shortened by *ca.* 12 nm. Because the sarcomere is symmetrical about its centre and each set of myosin heads would have experienced local filament sliding of half the sarcomere shortening (figure 1a), the extent of shortening is conventionally expressed in units of nanometres per half-sarcomere (nm hs⁻¹). In this example the shortening was *ca.* 6 nm hs⁻¹. During the shortening step the force in the muscle fibre fell to about one-quarter of T_0 . This reflects the undamped elasticity of the sarcomere ('phase 1'; Huxley & Simmons 1971), which is partly a property of the myosin heads themselves, and partly a property of the actin and myosin filaments, as discussed in § 5 below. In the 1 ms period after the shortening step, the force partly recovers. This is the 'phase 2' process first described by Huxley & Simmons, and proposed to be a result of the working stroke in the myosin heads.

The intensity of the M3 X-ray reflection (I_{M3}) increases slightly during the elastic phase 1 of the force transient, then decreases substantially during the rapid force recovery, phase 2 (Irving *et al.* 1992; figure 2a). This decrease shows that a large change in the conformation of the myosin heads accompanies the rapid force recovery, as expected from the working stroke model (figure 1).

The fibre was restretched to its original length 1 ms after the shortening step. The force response to the stretch is the opposite of that to a release; the force increases in phase 1 and decreases in phase 2, during the recovery of the working stroke (Huxley & Simmons 1971). The M3 reflection showed markedly asymmetric behaviour for shortening and stretch. I_{M3} increased during the stretch, and showed little further change during the subsequent phase 2 recovery after a stretch.

These distinct I_{M3} responses to shortening and stretch are not the result of a difference in the kinetics of the underlying structural changes. If a similar stretch is applied from the isometric force level, I_{M3} decreases during both phase 1 and phase 2 (Lombardi *et al.* 1995). If a small (2.5 nm hs⁻¹) shortening step is followed 1 ms later by another shortening step, I_{M3} shows almost no change in response to the first step, but decreases during both the phase 1 and phase 2 response after the second step (Irving *et al.* 2000).

(d) *Quantitative interpretation in terms of myosin head conformations*

All the results described in § 3c can be quantitatively explained by the structural model in figure 1. The catalytic domain of the myosin heads is assumed to remain bound to actin in the conformation in figure 1b. A general conformation of the light-chain domain is described by the axial displacement (Δz) of the distal end of the light-chain domain (i.e. the junction between the myosin head and myosin rod) with respect to the catalytic domain. Δz is taken to be zero for the rigor conformation (Rayment *et al.*

1993a,b; lower conformation in figure 1b; circle in figure 2b), and is then +10.6 nm for the ADP·Pi conformation (Dominguez *et al.* 1998; upper conformation in figure 1b; square in figure 2b). The M3 reflection is relatively weak in both these conformations because the axial mass projection of the light-chain domain is displaced from that of the catalytic domain (figure 1b); the maximum value of I_{M3} is obtained for an intermediate tilt of the light-chain domain, $\Delta z = 5$ nm (figure 2b), when both domains have the same axial position.

When a length step is applied, we postulate that the light-chain domain of the myosin heads tilts during both the elastic phase 1 response and the active phase 2 response. I_{M3} is calculated from the structural model (figure 1b), for the general case of any bend between the catalytic and light-chain domains of the myosin head, as the $1/14.5 \text{ nm}^{-1}$ component of the Fourier transform of the axial mass distribution of the myosin heads (figure 2b). The changes in I_{M3} produced by shortening and stretch and summarized in § 3c are reproduced if Δz during isometric contraction is +7 nm (Dobbie *et al.* 1998; Irving *et al.* 2000; figure 2b, triangle). I_{M3} is almost constant during phase 1 of a large shortening step (Irving *et al.* 1992, 2000) or during either phase 1 or 2 of a smaller shortening (Irving *et al.* 2000), because Δz is decreasing from 7 nm, across the plateau of the I_{M3} versus Δz relation (figure 2b). I_{M3} decreases during phase 2 after a large shortening step (figure 2a), or during phase 1 of a second shortening step 1 ms after a conditioning release (Irving *et al.* 2000), because Δz has been shifted to the steep ascending limb of the I_{M3} versus Δz relation. Application of a stretch from the isometric state increases Δz from 7 nm, on the steep descending limb of the I_{M3} versus Δz relation, so I_{M3} decreases during both the phase 1 and phase 2 response to a stretch from the isometric state (Lombardi *et al.* 1995). If the stretch is imposed 1 ms after a 6 nm release (figure 2a), phase 1 of the stretch response is on the steep ascending limb of the I_{M3} versus Δz relation, so I_{M3} increases in phase 1, but there is little change in phase 2 because the final part of the change in Δz occurs in the plateau region.

The I_{M3} versus Δz relation in figure 2b was calculated assuming that all the myosin heads in the fibre have the same orientation, but the relationship is almost identical for a constant Δz dispersion of a few nanometres (Irving *et al.* 2000). Thus the critical assumption is that the dispersion does not change during the length step, i.e. that the myosin heads that were attached to actin before the length step was applied remain attached during these rapid mechanical responses. There is good evidence that myosin heads remain attached during phase 1 of the response (Ford *et al.* 1977), but there is a small amount of detachment during phase 2 (Lombardi *et al.* 1992), and this is likely to be responsible for the incomplete recovery of I_{M3} after the stretch in figure 2a (Irving *et al.* 2000). This phenomenon does not affect the main conclusions of this series of experiments on the changes in I_{M3} induced by length steps: that myosin heads tilt during both the elastic response and during the working stroke and that, in terms of the *in vitro* structural model for the working stroke (figure 1a), the conformation of the light-chain domain of the myosin head is tilted up by 7 nm from that observed in the rigor (ATP-free) complex.

4. AXIAL MOTIONS OF MYOSIN HEADS IN THE SARCOMERE: X-RAY INTERFERENCE

(a) X-ray interference from the muscle sarcomere

The intensity profiles of the myosin-based axial X-ray reflections from skeletal muscle do not have the simple shape expected for diffraction from a uniformly spaced array of myosin heads, but are split into two or more sub-peaks (Huxley & Brown 1967; Haselgrove 1975). This effect is a result of X-ray interference between the two arrays of myosin heads in each myosin filament (figure 3). Each half-filament has 49 levels of myosin heads with a regular axial periodicity of *ca.* 14.5 nm. The calculated intensity distribution in the M3 region of the axial diffraction pattern corresponding to spacings *ca.* 14.5 nm is shown as the red curve in figure 3b. However, for every level of heads in one half of each myosin filament there is another level in the other half *ca.* 860 nm further along the filament. Interference between the diffracted X-rays from the two arrays has the effect of multiplying the diffraction pattern from a single array (red curve) by a finely spaced sinusoidal function (blue curve), with a periodicity corresponding to the *ca.* 860 nm interference distance. The resulting M3 reflection (light green curve) is split into two main peaks, with smaller satellite peaks on each side. The M3 profile observed during isometric contraction of a single muscle fibre (dark green curve) is similar to the calculated one. The differences between the two curves, the slightly broader axial profile and the higher central minimum of the observed profile, are a result of the limited spatial resolution of the X-ray camera and detector.

This explanation of the fine structure of the M3 reflection was tested by stretching muscle fibres to different sarcomere lengths before stimulating them (Linari *et al.* 2000). As the sarcomere length is increased, the actin filaments are pulled out from the myosin filament array, so that the region of overlap between the myosin and actin filaments is shorter, and the myosin heads near the centre of the myosin filaments can no longer bind to actin. One consequence is that the active force generated by the fibre is reduced, in proportion to the degree of overlap between the filaments (Gordon *et al.* 1966). The intensity of the M3 reflection during active contraction also decreased in proportion to filament overlap, suggesting that the heads that cannot bind to actin during active contraction are disordered, and make little contribution to the M3 reflection (Linari *et al.* 2000). Consistent with this explanation, the interference distance, measured from the separation between the two main peaks of the M3 reflection, increased with sarcomere length as expected from the increased distance between the two arrays of actin-attached myosin heads. The relative intensity of the two main peaks of the M3 reflection was independent of sarcomere length; at larger sarcomere lengths the interference distance is greater by an integer number of repeats of the 14.5 nm spacing, so the phase relation between the diffraction patterns from the two arrays of heads in each filament (figure 3) is preserved. Each level of myosin heads maintains a precise distance from the centre of the filament, presumably set by the atomic packing of the myosin tails into the filament core. These experiments provide strong support for the explanation of the fine structure of the M3 reflection in terms of interference between myosin heads in the two halves of the myosin filament (figure 3).

Figure 4. Change in the interference fine structure of the M3 reflection following a length step. (a) Length change in nanometres per half-sarcomere (nm hs⁻¹) and force transient normalized by the isometric force before the step (T_0). (b) Axial intensity distribution in the region of the M3 X-ray reflection recorded during isometric contraction (T_0 , green), at the end of phase 1 (T_1 , red), and at the end of phase 2 (T_2 , blue). Based on figure 1 of Piazzesi *et al.* (2002b).

The interference fine structure of the M3 reflection provides an extremely sensitive measure of the motion of the myosin heads towards the M-line of the sarcomere. A relatively small axial motion of the heads, producing a small change in the interference distance, leads to a large and easily measurable change in the relative intensity of the two main peaks of the M3 reflection. A spatial resolution for axial head motions of *ca.* 1 Å is achievable in practice (Piazzesi *et al.* 2002b; Reconditi *et al.* 2003). It is convenient to define the ratio R_{M3} as the relative intensity of the higher and lower angle peaks of the M3 (the higher angle peak is the upper peak in figure 3). The structural information obtained from R_{M3} is independent of that from the total intensity of the reflection, I_{M3} . To a good approximation R_{M3} depends only on the axial displacement of the centre of mass of the myosin heads from the M-line and, for a given centre of mass position, it does not depend on the conformation of the myosin heads. Moreover R_{M3} is also independent of changes in the disorder of the myosin heads, again in contrast with I_{M3} . These properties mean that changes in R_{M3} can generally be interpreted without a detailed structural model, increasing the generality of the conclusions.

(b) Changes in X-ray interference produced by imposing a length step

We used the X-ray interference technique to measure the axial motions of myosin heads produced by applying a rapid shortening step during active contraction (Piazzesi *et al.* 2002b; figure 4). We recorded the interference fine structure of the M3 reflection during isometric contraction (green, T_0), at the end of the 150 µs shortening step and the accompanying fall of force associated with the instantaneous elasticity of the sarcomere, phase 1 (red, T_1), and after the rapid recovery of force associated with the working stroke in the myosin heads, phase 2 (blue, T_2). The total intensity of the M3 reflection, I_{M3} , was almost constant during the shortening step, but decreased substantially during the rapid force recovery, phase 2, as shown previously (figure 2a). To emphasize the changes in the interference fine structure of the reflection, the M3 intensity profiles in figure 4b have been normalized by the intensity of the lower angle (left) peak. There was a large

change in the interference fine structure of the M3 reflection during phase 1 (figure 4b, green to red). The ratio of the intensity of the higher angle peak of the M3 reflection (on the right in figure 4b) to that of the lower angle peak, R_{M3} , decreased during phase 1. A decrease in R_{M3} is expected for a decrease in the interference distance (figure 3) as myosin heads attached to actin filaments move towards the M-line as the sarcomere shortens. The observed decrease in R_{M3} was quantitatively reproduced by a model in which about half of the myosin heads that contribute to the M3 reflection move in response to the length step (Piazzesi *et al.* 2002b). By contrast, there was little further change in the interference fine structure of the M3 reflection during the active force recovery, phase 2 (red to blue). Thus there is very little axial motion of the centres of mass of the myosin heads towards the M-line during the working stroke.

The absence of axial motion of the myosin heads during the working stroke appears to contradict our previous interpretation of the decrease in I_{M3} as a tilting of the light-chain domain of the heads (figure 2). If the junction between the myosin head and the myosin rod is fixed on the 14.5 nm axial periodicity of the myosin filament backbone, why does tilting of the light-chain domain not displace the mass of the actin-bound catalytic domain towards the M-line, as shown in figure 1a? The answer is related to the compliance of the myosin and actin filaments (Huxley *et al.* 1994; Wakabayashi *et al.* 1994; Dobbie *et al.* 1998; Linari *et al.* 1998). This is considered in more detail below, but here we note that tilting of the light-chain domain of the myosin heads could drive phase 2 force recovery at constant sarcomere length if the resulting axial displacement of the head were taken up by elastic extension of the filaments. Tilting of the light-chain domain would then displace the head-rod junction away from the M-line and the catalytic domain towards the M-line, with the result that there was little net movement of the centre of mass of the heads with respect to the M-line. The measured values of filament compliance, discussed below, can in fact provide a quantitative explanation for the almost constant interference fine structure of the M3 reflection during the phase 2 recovery after a shortening step, provided that only about

Figure 5. Change in the interference fine structure of the M3 reflection following a load step, and interpretation in terms of the axial motions of the myosin heads. (a) Force step normalized by the isometric force (T_0), and length change in nanometres per half-sarcomere (nm hs^{-1}). (b) Axial intensity distribution in the region of the M3 X-ray reflection at the periods corresponding to the X-ray exposure times shown in (a). (c)–(f) Motions of the myosin heads at the four stages of the velocity transient shown in (b): (c) isometric contraction (L_0 , brown); (d) start of phase 2 (L_{2s} , orange); (e) end of phase 2 (L_{2e} , pink); (f) end of phase 3 (L_3 , blue). Replotted from figure 1 of Reconditi *et al.* (2004).

half of the myosin heads move in response to the length step (Piazzesi *et al.* 2002b).

Figure 6. Load dependence of the axial motions of myosin heads. (a) Ratio R_{M3} of the intensities of the higher and lower angle peaks of the M3 reflection plotted against time for three different loads. Blue, 0.75 T_0 ; red, 0.50 T_0 ; green, 0.25 T_0 . Open triangles, phase 2; filled circles, phase 3. (b) Relationship between R_{M3} and the extent of filament sliding in nanometres per half-sarcomere (nm hs^{-1}), from the same experiments as (a). Replotted from figure 2 of Reconditi *et al.* (2004).

(c) Load-clamp experiments

Because the working stroke cannot be observed directly using X-ray interference experiments with length steps, we turned to a different type of protocol in which a rapid change in load is imposed on the fibre. The elastic or phase 1 response to a load step is the same as that to a length step, but the later phases are distinct. In particular, phase 2 of the response to a 150 μs load step occurs at constant load, and therefore at constant filament strain. Thus, with a load-step protocol, the structural basis of the working stroke can be investigated by X-ray interference in the absence of the effects of filament compliance.

Load step perturbations were first used to study the contractile mechanism more than 40 years ago (Podolsky 1960), but subsequently received little attention until recent improvements in mechanical techniques allowed the phase 2 component of a response to a load step to be resolved for the first time (Piazzesi *et al.* 2002a). An example of such an experiment (Reconditi *et al.* 2004) is shown in figure 5a. The fibre was initially generating the isometric force T_0 at a fibre length L_0 . The force was reduced to 0.5 T_0 in 150 μs , then it was clamped at this level for the remainder of the response. Each half sarcomere shortened by *ca.* 3 nm during the load step; this is the same phase 1 elastic response seen with a length step. The sarcomeres continued to shorten rapidly for a few

milliseconds after the load step; this is phase 2 of the velocity transient, associated with the working stroke in the myosin heads. The shortening velocity then transiently decreased (phase 3), before increasing once more to the value associated with the steady state force–velocity relationship of the fibre (phase 4; Piazzesi *et al.* 2002a).

The interference fine structure of the M3 reflection (figure 5b) was recorded during isometric contraction (L_0 , brown), at the start and end of phase 2 (L_{2s} , orange and L_{2e} , pink, respectively), and at the end of phase 3 (L_3 , blue). The higher angle peak of the reflection decreased during phase 1 (brown to orange), as seen previously with the length step protocol (figure 4). However, in contrast with the length step protocol, the higher angle peak of the M3 reflection continued to decrease during phase 2 of the velocity transient (figure 5b, orange to pink), before recovering in phase 3 (pink to blue). These results show that myosin heads remain attached to actin and move towards the M-line of the sarcomere during both phase 1 (figure 5c to d) and phase 2 (figure 5d to e) of the response to a load step. Because the load and the strain in the filaments are constant during phase 2 of the velocity transient, the axial motions of the myosin heads in this period provide a direct measure of the structural change associated with the working stroke. The recovery of the interference fine structure of the M3 reflection during phase 3 of the velocity transient shows that myosin heads detach from actin in this period and reattach to new actin monomers further from the M-line (figure 5e to f).

The ratio of the intensities of the higher and lower angle peaks of the M3 reflection (R_{M3}) during the velocity transient gives a direct measurement of the working stroke in the myosin heads. The experiments were made at three different loads (Reconditi *et al.* 2004; figure 6). At each load R_{M3} decreased during phase 2 of the velocity transient, the working stroke (figure 6a, open triangles), then recovered during phase 3, the detachment from actin at the end of the working stroke (filled circles). Both the working stroke and the subsequent detachment from actin were markedly slower at higher loads.

The size of the working stroke is most conveniently determined by plotting R_{M3} against the extent of filament sliding in each half sarcomere (figure 6b). In this plot the data for phase 2 of the velocity transient (open triangles) fall on the same line, independent of the load. This part of the relationship corresponds to myosin heads moving with actin towards the centre of the sarcomere during phase 2; the extent of such motion depends only on the filament sliding and not on the load. The recovery of R_{M3} during phase 3 of the velocity transient (filled circles) is revealed as a deviation from the phase 2 relationship when the heads start to detach from actin. This deviation occurs after different extents of filament sliding at different loads. Thus, at the lowest load studied ($0.25 T_0$), there was *ca.* 12 nm of filament sliding before R_{M3} recovery, but at the highest load ($0.75 T_0$), there was only *ca.* 6 nm of sliding before R_{M3} recovered. These results show directly that the working stroke of the myosin motor is smaller at a higher load. At a low load the working stroke is *ca.* 12 nm, a value that is consistent with the atomic model for the working stroke derived from X-ray crystallography (figure 1b), but more than double that observed in some recent *in vitro* studies of isolated myosin II molecules at low load (Molloy

et al. 1995; Mehta *et al.* 1999). The latter comparison suggests that those *in vitro* assays underestimate the performance of the motor under physiological conditions, presumably because they do not preserve the native structural and geometrical relationship between the myosin and actin filaments.

At higher loads the myosin head detaches from actin before reaching the structural limit that defines the low-load stroke. The load-dependence of the size and the speed of the working stroke are likely to be two of the basic molecular parameters that determine the macroscopic performance and efficiency of muscle contraction.

5. COMPLIANCE OF THE MYOSIN AND ACTIN FILAMENTS

(a) *Part of the sarcomere compliance is a result of filament compliance*

The compliance of the myosin and actin filaments plays a fundamental role in the molecular mechanism of muscle contraction, as already mentioned in § 4b. Before 1994 it was generally assumed that filament compliance made a negligible contribution to the overall compliance of the sarcomere, i.e. that the origin of the sarcomere compliance was in the myosin cross-bridges. In this view each myosin cross-bridge could tilt and generate force by stretching its own local spring, which could have been located in the myosin head domain, the subfragment-2 coiled-coil domain that links the head to the myosin filament, or the actin monomer to which the head was bound. Such a local spring would allow each myosin cross-bridge to function relatively independently of the others along the same filament.

Two X-ray diffraction studies of whole muscle published in 1994 forced a revision of this view (Huxley *et al.* 1994; Wakabayashi *et al.* 1994). These studies were based on very accurate measurements of the axial spacings of myosin- and actin-based reflections, and suggested that both filaments had a compliance corresponding to an extension of *ca.* 0.25% for an increase in force equal to the isometric force T_0 . Because the total compliance of the half-sarcomere, measured by applying rapid length changes to isolated single muscle fibres, is *ca.* 0.5%/ T_0 (Ford *et al.* 1977; Dobbie *et al.* 1998), these results provided strong evidence that a substantial fraction of the half-sarcomere compliance is in the myosin and actin filaments.

(b) *Changes in the axial periodicity of the myosin filament*

To determine the relative contribution of the cross-bridges and filaments to the sarcomere compliance more precisely, it is necessary to make the mechanical and structural measurements in the same muscle preparation, because the isometric force T_0 depends on the muscle, the species and the temperature of the experiments. A further complication is that the myosin filament (and possibly also the actin filament as discussed below) exhibits spacing changes that are not related to mechanical compliance. When muscle is activated to produce an isometric contraction (force T_0), the spacings of the myosin-based axial X-ray reflections increase by 1.5% (Huxley & Brown 1967; Haselgrove 1975; Piazzesi *et al.* 1999; Linari *et al.* 2000). Although the time course of this spacing change during muscle activation is similar to that of the rise of force (Huxley *et al.* 1982; Piazzesi *et al.* 1999),

several lines of evidence show that it is not a manifestation of filament compliance. It is much larger than the sarcomere compliance measured with rapid length steps. Moreover, changes in the spacings of the myosin-based axial X-ray reflections of the order of 1% have been observed when the tension in the filament is constant, for example during rapid shortening of active muscle (Faruqi *et al.* 1991; Piazzesi *et al.* 1999), and on changing the temperature of resting mammalian muscle (Xu *et al.* 1999). These results suggest that the myosin filament can exist in two structural states with different axial periodicities. The structural transition appears to take place in the backbone of the myosin filament, as it is seen in the higher-order axial reflections which mainly arise from the filament backbone (Huxley *et al.* 1983, 2003; Linari *et al.* 2000). By contrast with the M3 reflection, the M6 and higher order reflections are relatively insensitive to the axial motions of the myosin heads (Huxley *et al.* 1983; Reconditi *et al.* 2004).

The structural transition in the myosin filament that is responsible for the 1.5% increase in filament periodicity on muscle activation is relatively slow, and is unlikely to contribute to the change in filament periodicity produced by rapid length steps, which should therefore provide the most reliable protocol for measuring the myosin filament compliance. Such measurements are technically demanding, and were initially made using the most intense axial reflection, the M3. The spacing of the M3 reflection, S_{M3} , changes with the force by $0.12 \pm 0.01\%/T_0$ during 3 kHz sinusoidal length changes (Dobbie *et al.* 1998), and by $0.14 \pm 0.03\%/T_0$ during a 150 μ s length step (Piazzesi *et al.* 2002b). However, the spacing of the M3 reflection does not act as a simple compliance in these experiments. The change in S_{M3} during the rapid force recovery (phase 2) after such a length step was $0.34 \pm 0.05\%/T_0$, much larger than that during phase 1. The difference is not because of the structural transition in the filament backbone during phase 2, because the change in the spacing of the M6 reflection, S_{M6} , is the same, $0.26 \pm 0.01\%/T_0$ during phase 1 and phase 2 (Reconditi *et al.* 2004). This value is therefore likely to reflect the true compliance of the myosin filament. The more complex behaviour of the spacing of the M3 reflection is probably a result of its being dominated by myosin heads that are cross-linking the myosin and actin filaments, so it does not yield a reliable measurement of the compliance of the myosin filament.

(c) Changes in the axial periodicity of the actin filament

The compliance of the actin filament has been estimated from the axial periodicities of the actin-based layer line reflections from the actin helix, from the 2.7 nm axial reflection from the actin monomer repeat, and by mechanical measurements (Huxley *et al.* 1994; Wakabayashi *et al.* 1994; Dobbie *et al.* 1998; Linari *et al.* 1998; Bordas *et al.* 1999). The spacing of the 2.7 nm axial reflection, $S_{2.7}$, is 0.25–0.3% larger during isometric contraction than at rest in whole muscles (Huxley *et al.* 1994; Wakabayashi *et al.* 1994; Bordas *et al.* 1999). Similar axial spacing changes were observed for the 5.1 and 5.9 nm layer line reflections from the actin helix. When 3 kHz oscillations were imposed on active single muscle fibres, the spacing of the 5.9 nm layer line, $S_{5.9}$, changed by a similar amount, $0.30 \pm 0.13\%/T_0$ (Dobbie *et al.* 1998), suggesting that the increase in the

periodicity of the actin filament on activation is due to an instantaneous filament compliance.

Actin filament compliance has been estimated independently from the dependence of sarcomere stiffness on sarcomere length in isolated intact muscle fibres in the region from 2.00 to 2.15 μ m, in which the length of actin filament that is in series with the myosin cross-bridges in the filament overlap zone is changing. The result corresponded to an average filament strain of $0.15 \pm 0.02\%/T_0$ (Linari *et al.* 1998), rather less than that observed in the X-ray experiments. However, the mechanical measurements were made in *Rana esculenta* fibres, which generate a lower isometric force than the *Rana temporaria* fibres used for the measurements of $S_{5.9}$ described above, and those of S_{M3} and S_{M6} described in § 5b. Correcting for the force difference, the *in situ* mechanical measurement of actin filament compliance would correspond to $0.22\%/T_0$ in *R. temporaria*, closer to the $0.30\%/T_0$ inferred from $S_{5.9}$ in this preparation.

We take the average of these two estimates, $0.26\%/T_0$, as the best estimate of actin filament compliance in *R. temporaria* at 4 °C. This value is numerically equal to the myosin filament compliance estimated from S_{M6} in these conditions. The estimate of actin filament compliance is less precise than that of myosin filament compliance. Moreover there is evidence for changes in the periodicity of the actin filament related to activation *per se* (Wakabayashi *et al.* 1994), and for larger changes in actin filament periodicity during the transition between isometric contraction and rapid shortening (Bordas *et al.* 1999). Thus it remains possible that the actin filament, like the myosin filament, can undergo structural transitions associated with changes in axial periodicities that are distinct from an instantaneous compliance. These effects may have other functional consequences, and merit further study.

(d) Compliance of the myosin heads

Measurements of the compliance of the myosin and actin filaments using sub-millisecond length changes to isolate the instantaneous elastic properties of the filaments suggest that the instantaneous compliance of the myosin and actin filaments in *R. temporaria* at 4 °C is $0.26\%/T_0$ in both cases. The compliance of the half-sarcomere in these conditions is 5.1 nm/ T_0 (Dobbie *et al.* 1998). The relative contribution of the myosin cross-bridges and the myosin and actin filaments to the overall half-sarcomere compliance can be calculated from these values by taking into account the dimensions of the filaments and the distribution of strain along the half-sarcomere (Ford *et al.* 1981; Linari *et al.* 1998). These calculations show that the actin filaments, myosin filaments and myosin cross-bridges contribute 2.1, 1.6 and 1.4 nm/ T_0 respectively to the total compliance of the half sarcomere.

The 1.4 nm/ T_0 compliance of the myosin cross-bridges could in principle be located in the coiled-coil subfragment-2 region linking the heads to the myosin filament, in the light-chain domain or catalytic domain of the head, at the actin-binding interface, or within the actin monomer to which the myosin head is bound. The cross-bridge compliance is frequently drawn in subfragment 2 for graphical convenience, but this location is inconsistent with the intensity changes of the M3 reflection produced by imposing length steps (§ 3), which show that all or part of the head must tilt during the elastic response. Polarized

fluorescence from probes on the light-chain domain of the head provide independent evidence for such tilting (Irving *et al.* 1995; Corrie *et al.* 1999), whereas the extent of tilting of probes in the catalytic domain is at least an order of magnitude smaller (Berger *et al.* 1996). These results provide strong evidence that most of the cross-bridge compliance is either within the light-chain domain or at the junction between the light-chain domain and the catalytic domain.

The conclusion that the myosin cross-bridges contribute only 1.4/5.1, or about one-quarter of the compliance of the half sarcomere, has further implications. The working stroke at low load (§ 4c) is almost an order of magnitude larger than the elastic strain in the myosin head. In models of the type introduced by Huxley & Simmons (1971), the working stroke generates force by stretching a distinct elastic element in the myosin cross-bridge. This type of model becomes less plausible, at least in its simplest form, when the working stroke is much larger than the compliance. A related problem is that the energy stored by stretching the compliance is related to the square of its strain, so there would be a large energy barrier for such a transition. These problems can be overcome by splitting the working stroke into a series of sub-steps, and this possibility was already raised in the original model (Huxley & Simmons 1971). Sub-steps in the working stroke suggest a series of structural transitions within the myosin head, rather than the all-or-none switch between two structural states that is implied by the X-ray crystallographic data (figure 1*b*). At present there is no high-resolution structural evidence for such intermediate transitions.

6. CONCLUSIONS AND PERSPECTIVES

X-ray diffraction and interference are powerful methods for studying the mechanism of muscle contraction at the molecular level in an intact cell. These methods are complementary to *in vitro* techniques, with greater structural resolution, because they can provide a dynamic characterization of the native protein complexes, and relate structural changes directly to their functional consequences.

This article has focused almost exclusively on studies of the axial X-ray reflections in intact muscle fibres. These studies established the average conformation of the myosin heads during isometric contraction, and showed that the heads tilt during the working stroke and during the elastic response of the head to a change in force.

X-ray interference provides precise and independent information about the axial motions of the myosin heads in the sarcomere. This technique allowed the size of the working stroke to be measured directly in a working muscle fibre, and showed that both the size and speed of the working stroke depend on the external load. Interpretation of the X-ray interference data emphasized the need for the compliance of the actin and myosin filaments to be fully characterized. Fortunately the X-ray method provides these data with high precision, and showed that the filaments are responsible for most of the compliance of the sarcomere. In the resulting view of the mechanism of muscle contraction, the basic function unit is the half-sarcomere, and the functional properties of muscle should be understood in terms of the coupled mechanical properties of the myosin heads and the filaments.

Many questions remain about the motor mechanism. Some of these can be addressed with the experimental tools described above, others will require complementary data from other techniques. The technology for X-ray diffraction studies of muscle continues to advance rapidly, and the availability of brighter X-ray beams and better X-ray detectors will allow the weaker parts of the diffraction diagram to be investigated.

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GLOSSARY

ADP: adenosine diphosphate
 ATP: adenosine triphosphate
 P_i: phosphate