

Frequency-Dependent Distortion of Meridional Intensity Changes during Sinusoidal Length Oscillations of Activated Skeletal Muscle

Maria Angela Bagni,* Barbara Colombini,* Heinz Amenitsch,[†] Sigrid Bernstorff,[†] Christopher Charles Ashley,[‡] Gert Rapp,[§] and Peter John Griffiths[‡]

*Dipartimento di Scienze Fisiologiche, Università degli Studi di Firenze, Florence I-50132, Italy; [†]Sincrotrone Trieste S.C.p.A., I-34012 Basovizza TS, Italy; [‡]University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, United Kingdom; and [§]Hasylab at Deutsches Elektronen-Synchrotron, Gebäude 25f, D-22603 Hamburg, Germany

ABSTRACT Bundles of intact, tetanized skeletal muscle fibers from *Rana temporaria* were subjected to sinusoidal length oscillations in the frequency domain 100 Hz to 3 kHz while measuring force and sarcomere length. Simultaneously, intensity of the third-order x-ray reflection of the axial myosin unit cell (I_{M3}) was measured using synchrotron radiation. At oscillation frequencies <1 kHz, I_{M3} was distorted during the shortening phase of the sinusoid (i.e., where bundle length was less than rest length). Otherwise, during the stretch phase of oscillations at all frequencies, during the shortening phase of oscillations above 1 kHz, and for bundles in the rigor state, I_{M3} was approximately sinusoidal in form. Mean I_{M3} during oscillations was reduced by 20% compared to the isometric value, suggesting a possible change in S1 disposition during oscillations. However, the amplitude of length change required to produce distortion (estimated from the phase angle at which distortion was first evident) corresponded to that of a step release sufficient to reach the maximum I_{M3} , indicating a mean S1 disposition during oscillations close to that during an isometric tetanus. The mechanical properties of the bundle during oscillations were also consistent with an unaltered S1 disposition during oscillations.

INTRODUCTION

Tension development by active muscle is thought to arise from distortion of the S1 moiety of the motor protein, myosin, during its interaction with actin. Actin and myosin are arranged as interdigitating, filamentous arrays in skeletal muscle cells, actin-bound S1 forming cross-bridges between the actin and myosin filaments. S1 contains specific actin and nucleotide binding sites (Tokunaga et al., 1987), and exhibits ATPase activity. Recent developments in the study of single molecule interactions between actin and myosin confirm S1 as the site of transduction of the free energy of ATP hydrolysis into mechanical work (Finer et al., 1994; Molloy et al., 1995). S1 crystals show different allosteric states in which its “tail” domain can adopt different dispositions, thought to correspond to various stages in the “power stroke,” the fundamental structural event in the generation of work (Highsmith, 1999). Corresponding changes in tail orientation, detected using bifunctional rhodamine fluorescence, have now been shown from living muscle, showing that S1 structures similar to those observed in in vitro crystallographic studies are likely to be the basis of the power stroke in vivo (Corrie et al., 1999). X-ray diffraction measurements provide a dynamic, non-invasive method of monitoring structural changes in the intact myofibrillar lattice. The meridional reflection corresponding to the third harmonic (M3) of the axial myosin unit cell, which samples the structure amplitude of S1, is one of the stron-

gest reflections in the x-ray pattern from skeletal muscle. The behavior of this reflection has therefore been of great interest in investigation of the relationship between the structural power stroke and the associated axial force response (Huxley et al., 1982; Irving et al., 1992).

A small step-length change applied to an activated skeletal muscle fiber produces an immediate elastic force response, followed by a quick recovery process that restores tension to close to its original level within a few milliseconds (Ford et al., 1977). The quick recovery is thought to be the mechanical manifestation of a synchronized power stroke. Combined x-ray diffraction and mechanical measurements on both whole muscle and single fibers have shown that the quick recovery is accompanied by a reduction in intensity of the meridional x-ray reflection at 14.5 nm (I_{M3}) (Huxley et al., 1983; Irving et al., 1992). Fast time-resolved intensity measurements have shown that I_{M3} decreases instantaneously during the elastic increase of force produced by a stretch, but not during the symmetric elastic fall in tension produced by a small release applied at tetanus plateau (Lombardi et al., 1995). These I_{M3} changes can be simulated by a model in which S1 tilts or bends, both because of the force change resulting from the change in sarcomere length (elastic response) and because of the quick recovery mechanism (Piazzesi et al., 1995). The intensity decrease has been attributed to broadening of the mass projection of the population of actin-bound S1 along the filament axis due to the tilting of S1 away from the perpendicular with the main axis of the myosin filament. The lack of intensity change during the elastic fall in tension accompanying a small release could be explained by assuming that, at the tetanus plateau, S1 tails are oriented at a slightly off-perpendicular angle with respect to the fiber axis, di-

Received for publication 27 October 2000 and in final form 7 March 2001.

Address reprint requests to Dr. P. J. Griffiths, University Laboratory of Physiology, Parks Rd., Oxford OX1 3PT, UK. Tel.: +44-1865-272494; Fax: +44-1865-272469; E-mail: pjg@physiol.ox.ac.uk

© 2001 by the Biophysical Society

0006-3495/01/06/2809/14 \$2.00

rected toward the M line (Piazzesi et al., 1995). During the elastic phase of a release, I_{M3} passes through an intensity maximum at which the tail is perpendicular to the filament axis. I_{M3} does not fall unless the release is sufficiently large to carry the tail past this point.

A sinusoidal length change protocol has the advantage over length steps in that events occurring during a single cycle can be averaged hundreds or even thousands of times in a single tetanus, depending on oscillation frequency. This allows the attainment of very high-resolution time-resolved x-ray data. In addition, the effects of fiber length on x-ray reflections are monitored continuously throughout the period of measurement, rather than just for the chosen length-step amplitude. In some previous studies, the I_{M3} signal during sinusoidal length changes has been reported to show distortion during the release phase of the oscillation period (Wakabayashi et al., 1986; Mitsui et al., 1994). This is consistent with the asymmetry in the I_{M3} changes in response to length steps, i.e., passage through an intensity maximum during a release. However, no distortion of the I_{M3} signal during sinusoidal oscillations was observed by others (Amenitsch et al., 1997a; Griffiths et al., 1998; Dobbie et al., 1998). The absence of distortion in these experiments was explained as being due to a change in mean S1 disposition during oscillations toward a stretched conformation, such that the displacement during the release phase of the oscillation was now insufficient to pass through the I_{M3} maximum. However, it is also possible that distortion is dependent on the frequency of oscillations applied, because at higher frequencies instantaneous, purely elastic distortions of S1 contribute proportionally more to the I_{M3} signal than structural changes related to the power stroke (which occur more slowly with rate constants of the order of 1000 s^{-1}). Here we report I_{M3} changes spanning a range (100 Hz–3 kHz) over which the quick recovery contribution to I_{M3} would be changing appreciably. This protocol also allowed us to calculate the apparent equilibrium orientation of actin-bound S1 during length oscillations. Our results are interpreted in terms of a tilting simulation of S1 structure.

METHODS

Preparation

Single fibers or fiber bundles from the tibialis anterior muscle of the frog (*Rana temporaria*) were mounted horizontally by means of aluminum clips between the arm of a fast-capacitance force transducer (30–60 kHz resonance frequency) and a moving coil motor in a temperature-controlled chamber fitted with a glass floor for laser and normal light illumination. Stimulation was applied by means of two platinum wire electrodes, one mounted on the floor of the chamber, the other mounted on its lid. The experiments were performed at beamline X13 (DESY, Hamburg) and at the SAXS beamline (Elettra, Trieste, see Amenitsch et al., 1997b). The preparation was exposed to an x-ray beam ($\lambda = 0.15 \text{ nm}$, beam dimensions at specimen $0.5 \times 4 \text{ mm}$ at DESY and $0.34 \times 2 \text{ mm}$ at Elettra) during tetanic contractions of ~ 0.6 – 1 s evoked regularly every 3 min. At Hamburg (flux $\sim 10^{11} \text{ ph s}^{-1}$), bundles of ~ 10 – 20 fibers were used, as the multi-wire

detector available at that site coped well with high x-ray reflection intensities; at Trieste, given the higher flux of a wiggler beamline ($5 \times 10^{12} \text{ ph s}^{-1}$), bundles of one to three fibers were used to avoid space-charge distortion of the x-ray spectrum by the one-dimensional delay line detector used.

Experimental protocol

Sinusoidal length oscillations of various amplitudes at 100, 200, 400, and 1000 Hz and 3.12 kHz were applied at the tetanus plateau and maintained for 200–800 ms. Meridional x-ray diffraction patterns were collected in fibers at rest, during the tetanus rise (5 ms sampling time), at the isometric tetanus plateau, and during length oscillations. A sampling time of $1/(20 \text{ or } 25 \text{ times the oscillation frequency})$ during oscillations allowed us to sample one period with 20 or 25 points at all frequencies. Synchronously with x-ray diffraction patterns, we collected time-resolved fiber length, sarcomere length, and force measurements. Sarcomere length was measured by a laser diffractometer using a 3 mW laser diode source at 670 nm wavelength. The position of the first-order laser reflection was measured by a position-sensitive photodiode (SiTek Electro Optics, S-433 30 Partille, Sweden), and converted to sarcomere length in real time using an analog computer circuit. Length changes are expressed as nanometers per half sarcomere (nm/h.s.). All the data collected during tetani for a given bundle and frequency were averaged. No average was made between different bundles. Experiments were performed at a temperature of 2 – 4°C .

A few experiments with length oscillations were also performed on intact bundles under rigor conditions. Rigor was obtained by bathing the preparation with Ringer's solution with 0.4 mM iodoacetic acid added overnight at pH 7.2. Because fibers in rigor became extremely stiff and difficult to manipulate, 50 mM 2,3-butanedione monoxime (BDM) was present during the iodoacetate incubation, and was removed after the bundle was mounted in the experimental chamber. BDM prevents formation of the actomyosin rigor complex, even though ATP levels have declined. After BDM removal, bundles entered the rigor state, as evinced by both their high stiffness and their characteristic rigor equatorial x-ray pattern. Bundles in rigor had a very good laser diffraction pattern, as sarcomere length along the fibers remained uniform. Length oscillations were applied to fibers in rigor after they had been slowly stretched to a length at which tension was similar to P_0 and produced tension changes peak to peak (p-p) similar to P_0 .

To improve the resolution of mechanical measurements, especially that of sarcomere length, experiments were also performed on single frog fibers (*Rana esculenta*) using a striation follower device (Huxley et al., 1981) to measure sarcomere length in a selected fiber segment (1–2 mm long) with greater precision. These experiments were performed to measure y_0 (the p-p amplitude of sinusoidal sarcomere length change necessary to produce a tension change equal to P_0) at various frequencies and to verify the possible alteration of the cross-bridge kinetics induced by the length oscillations.

Data collection and analysis

To ensure that synchrony was maintained between length oscillations and the data collection electronics, sampling of the meridional x-ray pattern was synchronized with length oscillations every two oscillation periods. Force, sarcomere length, and x-ray data were then averaged over all these synchronized periods, and the quality of averaged x-ray data was examined after each tetanus using data averaging and processing with IDL software (Research Systems, Boulder, CO) or the Fortran program OTOKO (Koch and Bendall, 1981). Data collection was abandoned if tetanic tension declined by 15% relative to its value at the start of the experiment, or if deterioration of the x-ray pattern had occurred. Data were then averaged over all tetani, and I_{M3} determined by fitting of the meridional pattern to a Gaussian function plus a polynomial background term, using a Levenberg-

Marquardt fitting algorithm written in Fortran77 (Salford Software Ltd., Salford, UK). An example of the meridional pattern obtained during oscillations is shown in Fig. 1.

Simulation

We have simulated changes in I_{M3} by treating the quick recovery phase of the tension response to a length change as the distortion of a single population of attached cross-bridges (Piazzesi et al., 1995). The structure of S1 was simplified to a cylindrical form, constructed from an assembly of overlapping spheres such that the surface of each sphere was in contact with the center of adjacent spheres at all times (Fig. 2). The number of spheres was determined by the sphere radius (r) and the length of S1 (taken as 17.5 nm). To simulate the power stroke, a fraction of the total number of spheres was selected as the "tail domain" of S1, and allowed to rotate, while the remaining spheres were taken to remain perpendicular to the thin filament axis. Two sources of compliance associated with S1 were permitted; the last sphere of the tail domain was attached to the thick filament by an elastic linkage associated with the S2 moiety (axial stiffness component K_{S2}) to permit subsequent tail rotation after a length step, and the rotation of the tail from a position of zero load was assumed to be associated with a force resisting this rotation from within the S1 moiety (having an axial stiffness component K_{S1}). In addition, a sarcomere axial stiffness component (K_f) was assumed to be in series with both K_{S1} and K_{S2} . The total stiffness of the attached S1 moiety between the z-line and the center of its thick filament is K_s , where $K_s = (1/K_{S1} + 1/K_{S2} + 1/K_f)^{-1}$, which would be the stiffness measured from a T1 curve (Huxley and Simmons, 1971). The total series stiffness outside S1 (K_t) is given by $K_t = (1/K_{S2} + 1/K_f)^{-1}$.

Let P_o be the isometric force per bound S1, and $P(t)$ be the corresponding force after a change in fiber length. Now we define the axial positions of the S1-S2 junction as A. The axial elastic displacement of A along the thin filament axis for a total length change $\Delta y_i(t)$ must be the total displacement minus the length change absorbed by compliance external to S1, i.e.,

$$\Delta y(t) = \Delta y_i(t) - \frac{(P_o - P(t))}{K_t}$$

This displacement is achieved through rotation of the tail element through an angle

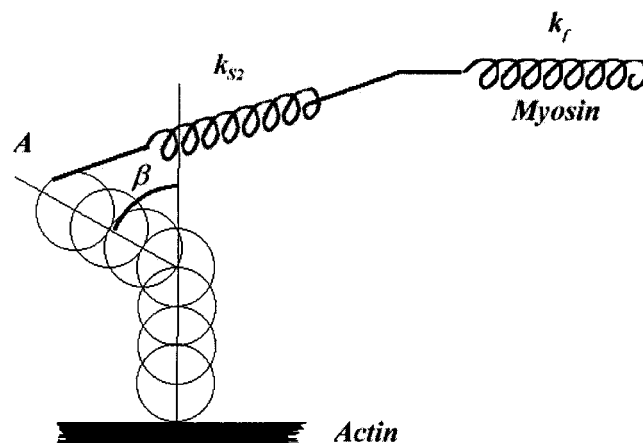


FIGURE 2 Schematic representation of the cylindrical structure of S1, formed from overlapping spheres. K_{S2} is the axial stiffness component of a compliance that is extended or compressed by rotation of the S1 tail during the quick recovery of tension, and is shown as being located in the S2 moiety solely for convenience of representation. Similarly, K_f is shown for convenience in the thick filament, but represents the combined axial stiffness of both thick and thin filaments. K_{S1} (not shown) is the axial component of a stiffness that results from distortion of the S1 tail from its preferred orientation at zero load, and is located inside S1.

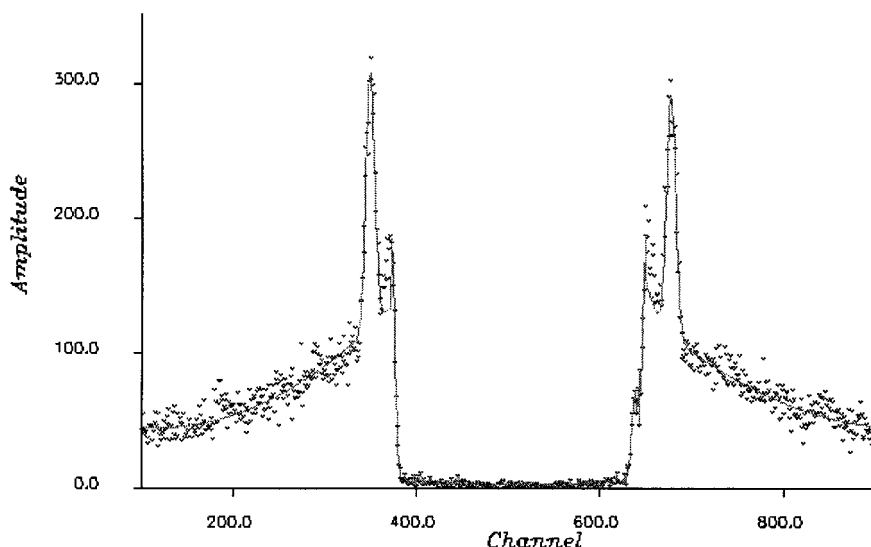
$$\beta(t) = \arcsin\left(\frac{\Delta y(t) + y_b}{l_{tail}}\right)$$

where l_{tail} is the length of the tail domain, $\beta(t)$ is the angle between the tail and a plane perpendicular to the axis of the thin filament, and y_b is the displacement of A at steady-state isometric tension from its position at $\beta = 0$. Fig. 2 shows this arrangement.

The Fourier transform of the axially projected mass of a sphere is given by

$$F(r, R) = \frac{3v(\sin u - u \cos u)}{u^3}$$

FIGURE 1 Meridional x-ray diffraction pattern from a 447- μ m-diameter fiber bundle. Ordinate is the total count of photons in each of 1024 channels of the linear detector (only channels 100–900 are displayed on the abscissa). Data points are the summed counts over a total exposure period of 75.2 ms. This is just one of 40 spectra obtained at 47- μ s sampling points during 1-kHz sinusoidal oscillations. The continuous line is the Levenberg-Marquardt fit to the pattern, from which I_{M3} can be determined.



where $u = 2\pi rR$, $v = 4\pi r^2/3$, and R is the radial vector in reciprocal space. The transform for each sphere was multiplied by a shifting function, required to place it in the appropriate axial position relative to the point of attachment, giving an S1 transform of the form

$$\mathcal{F}[S1] = \sum_{n=1}^m F_n(r, R) e^{2\pi i n r R \sin \beta(t)}$$

where n is the sequence number of each of m spheres composing the tail. Transforms were also calculated for mirror image S1 structures, to allow for interference across the M line, and multiplied by a second shifting function to allow for the phase shift introduced by the M line width. The squared modulus was then calculated for the sum of these transforms to give a signal proportional to intensity. Both the structure and the contribution of the second head of each cross-bridge to I_{M3} are uncertain. In addition, according to Linari et al. (2000), I_{M3} in activated fibers depends mainly on bridges in the myofilament overlap zone, suggesting that detached bridges contribute little to I_{M3} in the activated state. Given these uncertainties, and the possibility that a detached head might contribute little to the activated I_{M3} (and be insensitive to fiber length changes), we have excluded this structure from our simulations.

RESULTS

Low-frequency oscillations

Fig. 3 shows the result of a typical experiment in which sinusoidal length changes (100 Hz) were applied at the tetanus plateau. The two cycles at the faster time base

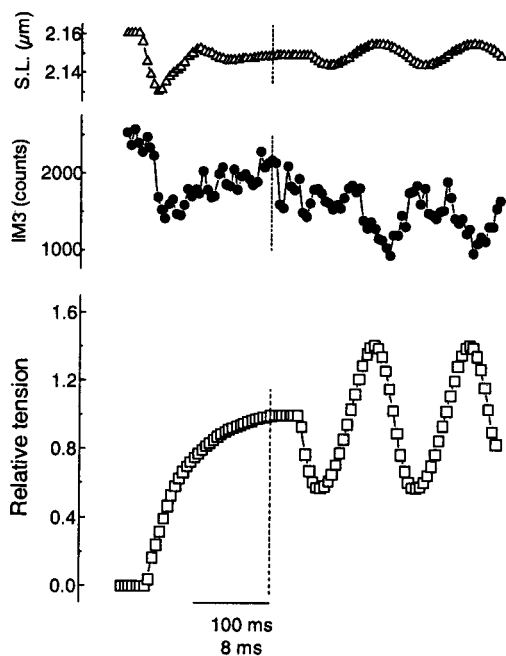
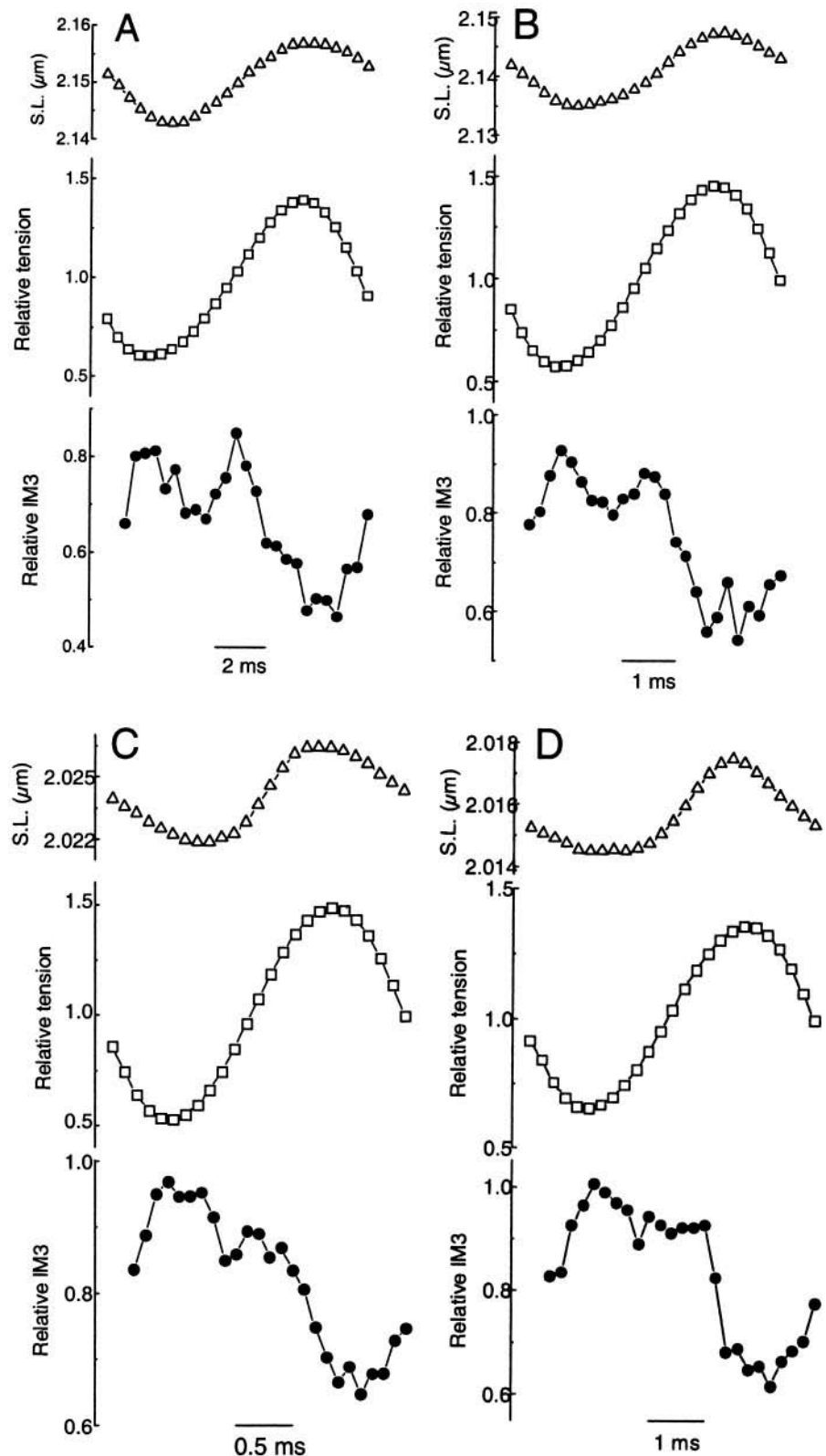


FIGURE 3 Force (squares), M3 intensity (circles), and sarcomere length (triangles) changes during the tetanus rise and at the plateau during sinusoidal length oscillations at 100 Hz and 7 nm/h.s. (p-p) amplitude. Averaged data from 45 tetanic contractions regularly evoked every 3 min in a fiber bundle. The vertical dashed lines indicate the time of the switch from the slow to the fast time base. The two fast-sampled sinusoidal cycles represent the average of all the double cycles (7200 cycles) applied to the fiber.

represent M3 intensity, force, and sarcomere length changes averaged over all the double cycles applied. After stimulation, I_{M3} initially falls, due to the loss of radial alignment of thick filaments, followed by a recovery of intensity as force peaks. The events associated with the length oscillations are better illustrated in Fig. 4 *A*, which shows the data from Fig. 3 averaged over just one cycle. Oscillations (100 Hz frequency and 7 nm/h.s. p-p amplitude) starting with the release phase of a sinusoid, defined as having a starting phase angle of 180° (those beginning with a stretch having a starting phase angle of 0°), caused a p-p force oscillation of $0.85 P_0$. M3 intensity changes were in phase with length changes, increasing during the negative phase of the sinusoid (sarcomere length shorter than isometric, i.e., 180 – 360° phase angle) and decreasing during the positive phase (sarcomere length longer than isometric, i.e., 0 – 180° phase angle). The intensity reduction during the positive phase had an approximately sinusoidal shape, but the intensity increase during the negative phase was strongly distorted, with the appearance of an almost symmetrical double peak. Typically, the double peak was present in all our records at low frequencies (100–400 Hz) when length amplitude applied produced force changes (p-p) close to P_0 , as was found previously at a lower frequency (Wakabayashi et al., 1986; 10 Hz). It can be seen that the well between the two peaks and the minimum intensity are almost in phase with length oscillations, but both length and I_{M3} are delayed with respect to the minimum and maximum force (delay 0.8 ms at maximum shortening, 0.4 ms at maximum stretch). The phase lead of force over length and intensity arises from the quick recovery process which, at 100 Hz, proceeds to a substantial extent during the relatively long cycle, giving the force response a somewhat viscous character. The phase lead is more prominent during the release than during the stretch because of the greater speed of the quick recovery for a release. Fig. 4 *B* shows the response of the same preparation to length oscillations at 200 Hz. Due to the smaller quick recovery occurring at this higher frequency, a length oscillation of 6.25 nm/h.s. p-p now produced a force oscillation of $0.91 P_0$. The double peak on I_{M3} during the negative phase of the length oscillation now appears slightly less pronounced than at 100 Hz, but is still quite evident. As at 100 Hz, the intensity changes are almost in phase with sarcomere length oscillations, with minimum intensity corresponding to maximum stretch, and force leading both intensity and length changes (0.6 ms lead at maximum shortening, 0.2 ms lead at maximum stretch). Fig. 4 *C* shows similar records at 400 Hz frequency. In this case the amplitudes of length changes and of the corresponding force oscillations were ~ 5.5 nm/h.s. and $0.96 P_0$, respectively. The distortion on the intensity during the negative phase is still evident, but the double peak on I_{M3} is very much reduced compared to that at 100

FIGURE 4 M3 intensity (filled circles), force (open squares), and sarcomere length changes (open triangles) during sinusoidal oscillations at various frequencies. (A) Same data as Fig. 3. The fast-sampled cycle reported represents the average of all the cycles applied to the preparation (14,400). M3 intensity increases during the negative part of the length sinusoids and decreases during the positive part. The decrease is roughly sinusoidal, but the increase is strongly distorted, with formation of an almost symmetrical double peak. Note that at this low frequency there is a phase lead of force over sarcomere length, as expected from the quick force recovery. M3 intensity changes lag sarcomere length. I_{M3} and tension are expressed relative to the isometric plateau values. (B) Oscillations at 200 Hz and 6.25 nm/h.s. (p-p) amplitude. As at 100 Hz, a clear double peak is present during the negative phase of length oscillations. Force leads sarcomere length and intensity lags sarcomere length. Responses averaged over all the 14,400 cycles applied to the bundle during 60 tetanic contractions. (C) Oscillations at 400 Hz frequency and 5.5 nm/h.s. (p-p) amplitude. Because of the smaller amplitude and the higher frequency, which reduce the effect of the quick recovery, I_{M3} changes are smaller than at 100 and 200 Hz and the double peak is much reduced. Data averaged from 90 contractions and 10,800 cycles. (D) Oscillations at 200 Hz as in (B) but with a smaller amplitude (5.6 nm/h.s.). As expected from the tilting head model, reducing the length changes reduces the distortion on the intensity increase during the release phase. The double peak is now much smaller and the upper half of the sinusoid approximates to a square wave. The distortion on the sarcomere length signal is artificial and is due to condensation on the upper glass surface trough, through which must pass the laser beam of the diffractometer. Data averaged from 60 tetanic contractions and 14,400 cycles.



and 200 Hz. The phase lead of force over length and intensity is still present, but reduced compared to that observed at 100 and 200 Hz (0.2 ms at maximum short-

ening, 0.1 ms at maximum lengthening). Fig. 4 D shows the effects of length oscillations at 200 Hz again, but with a reduced amplitude. The principal difference from Fig. 4 B is

the very much reduced well depth during the release phase. These results show that the amplitude of the double peak depends on both the frequency and the amplitude of sinusoidal length changes imposed.

High-frequency oscillations

We also measured the intensity changes produced by length oscillations at higher frequencies (1 kHz and 3.12 kHz). At 3.12 kHz the amount of the quick recovery is small and the myosin head movement can be considered exclusively elastic. In this case, length oscillations that produce a p-p force equal to P_0 would correspond to a peak movement of the myosin head (assuming 50% compliance in the head and y_0 of 4 nm) of only ~ 1 nm. Fig. 5 shows a response to a length oscillation of ~ 3.6 nm/h.s. p-p amplitude. The M3 intensity signal is noisier than at lower frequencies, but it seems clear that a double peak is not present, in agreement with previous data on single fibers (Dobbie et al., 1998). This result shows that 0.9 nm peak elastic movement of the head is not sufficient to generate a double peak on I_{M3} . The situation is slightly different at 1 kHz, where some quick recovery is expected. The record in Fig. 6 shows the intensity changes

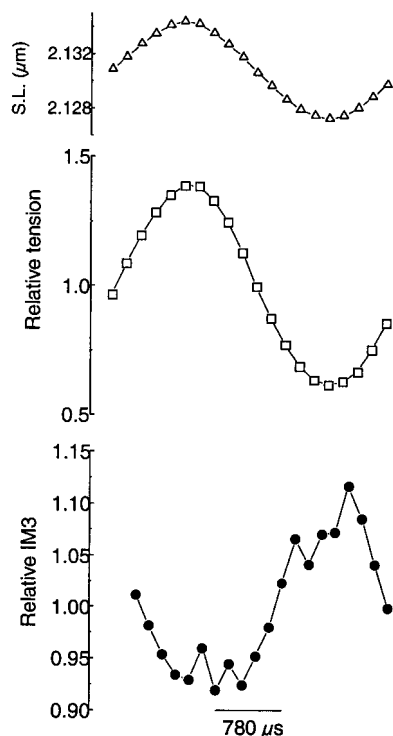


FIGURE 5 M3 intensity, force, and sarcomere length during sinusoidal length oscillations at 3.12 kHz, 3.6 nm/h.s. p-p amplitude. Data averaged from 26 tetanic contractions, 15,600 cycles. The intensity changes are now roughly sinusoidal. M3 intensity is almost perfectly in phase with force changes and sarcomere length. The M3 intensity (*circles*) and tension (*squares*) values are expressed as a fraction of the mean value during the oscillations.

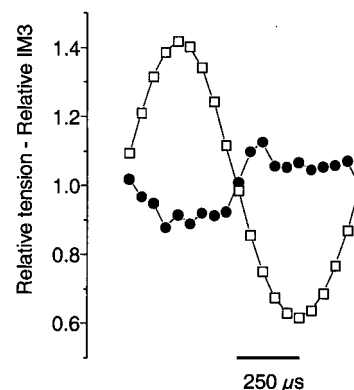


FIGURE 6 Response to length oscillations at 1 kHz frequency. As at low frequency, the intensity increase during the release is strongly distorted, although it is not clear if a double peak is present or not. Sarcomere length not recorded. Fiber length change was 0.885% fiber length. On the basis of the mean values of y_0 reported in Table 1 and the p-p force change measured, sarcomere length change would correspond to 3.8 nm/h.s.; 3100 cycles in 31 tetani. Symbols and M3 intensity and units as in Fig. 5. Experiment on small bundle (3 fibers).

at 1 kHz during a length change of 3.8 nm p-p. The double peak is not apparent on the M3 signal, but the increase during the negative phase is strongly distorted and similar to data at 200 Hz with small length amplitude (Fig. 4 D).

Rigor experiments

If the movement of the myosin head during the quick recovery affects I_{M3} signals during oscillations, these effects should disappear under rigor conditions. The experiment shown in Fig. 7 indicates that this is actually the case. We recorded intensity and force changes produced by length oscillations at 400 Hz frequency applied to a bundle in rigor.

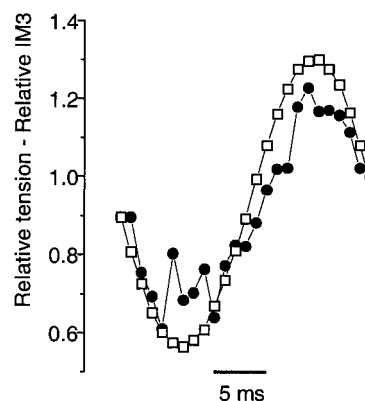


FIGURE 7 Force and M3 intensity changes during sinusoidal oscillations at 400 Hz frequency in a fiber bundle in rigor. Before applying length oscillations, the preparation was slowly stretched to a tension similar to P_0 . Sarcomere length change not recorded. The amplitude of the length oscillations was adjusted to produce force oscillations of $\sim 0.8 P_0$ p-p. The I_{M3} signal is now reversed, increasing during the stretch and in phase with the tension.

The intensity changes are reversed with respect to those measured in tetanized fibers, an effect that has been attributed to a different equilibrium tilt of the myosin heads in rigor (Dobbie et al., 1998). The phase lead of force has disappeared, and both force and intensity sinusoids are in phase. The I_{M3} signal was undistorted. This result, which was found throughout the range of frequencies tested (300 Hz–1.8 kHz), shows the myosin heads in rigor are compliant and move upon force application.

Frequency dependence of I_{M3} amplitude

Fig. 8 shows the normalized p-p amplitude of I_{M3} during oscillations as a function of frequency of the length oscillations applied. It can be seen that the p-p intensity increases as the frequency decreases. This is consistent with myosin head movement being contributed to substantially by the movement associated with the quick recovery. This is negligible at frequencies above 1 kHz, where myosin structural changes are mainly elastic, but becomes progressively more important as the frequency decreases. This figure can be compared with Fig. 10, showing the dependence of y_0 on frequency.

Mean M3 intensity during oscillations decreased compared to the value at the isometric steady state. In 10 experiments at frequencies between 100 and 3.12 kHz (mean oscillation amplitude 5.47 nm/h.s. p-p), we found that the mean intensity decreased to 79% of the steady-state isometric intensity.

Mechanics

To calculate the equilibrium tilt it is necessary to know the value of y_0 at a frequency at which no significant quick

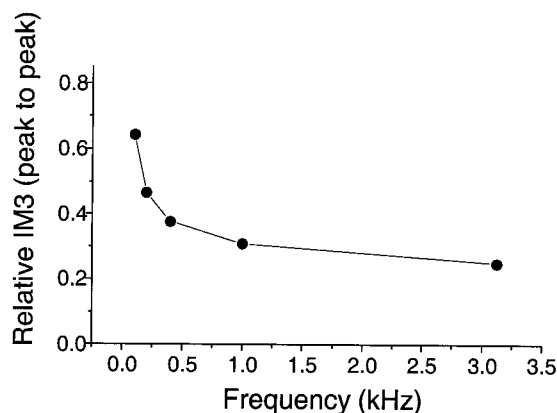


FIGURE 8 I_{M3} changes (p-p) during oscillations as a function of oscillation frequency. The intensity plotted was first normalized for the mean intensity during the oscillations, and then for the ratio of force changes (p-p) over P_0 at each frequency. In this way they represent the changes obtainable for a length change equal to y_0 at each frequency. Intensity change increased at low frequencies.

recovery occurs. For this purpose, we performed a series of purely mechanical experiments on single fibers in which we determined the value of y_0 at all the frequencies used. To improve resolution of sarcomere length changes, sarcomere length was measured using a striation follower device (Huxley et al., 1981). As in our x-ray experiments, the amplitude of the sarcomere length oscillations applied to the fibers was adjusted to produce force oscillations (p-p) close to P_0 at all frequencies investigated. An example of the response of a single fiber to oscillations at frequency of 3.4 kHz, applied at tetanus plateau, is shown in Fig. 9 A. It can be seen that force and sarcomere length signals are almost perfectly in phase. This indicates that the amount of quick recovery is small, and therefore the value of y_0 at 3.4 kHz has been taken as the purely elastic value. At 200 Hz frequency (Fig. 9 B), a clear phase lead of force over sarcomere length is now present on the records (0.2 ms at peak stretch, 0.5 ms at peak shortening). In addition, the force response is slightly distorted due to the asymmetric speed of the quick recovery during stretch and release. The mean value of y_0 at 3.4 kHz frequency on seven experiments was 3.98 ± 0.14 nm, in agreement with previous data on single fibers at the same temperature (Ford et al., 1977; Piazzesi et al., 1992; Bagni et al., 1999). The dependency of y_0 on frequency is shown in Fig. 10 and in Table 1. Because of the progressive increase in force truncation due to the quick recovery, y_0 increased noticeably at low frequencies, going from ~ 4 nm at 3.4 kHz to ~ 8 nm at 100 Hz. The increase of y_0 was accompanied by an increase of the phase lead of force over sarcomere length.

Because of the asymmetry in the speed of the quick recovery and the consequent force truncation between stretches and releases, we expected to find an asymmetry on the sinusoidal force response, with the stretch phase greater than the release phase. This effect would produce a shift in mean tension during oscillations to values greater than the isometric tetanus plateau. However, as shown in Fig. 9 A, this was not the case in our experiments. In fact, the shift we found was negligible: at 3.4 kHz, the mean shift in five experiments (15 measurements) was an increase of 0.0100 ± 0.0064 SEM P_0 . The largest increase in mean tension during oscillations was only $0.035 P_0$. The same result, i.e., no significant change in the mean tension during the oscillations as compared to P_0 , was also found at all the frequencies examined (100, 200, 400 Hz and 1 kHz), irrespective of whether the starting phase of the oscillations was 0° (length change starting with the stretch phase) or 180° (starting with the release phase). A significant increase of mean tension during oscillations was found when oscillations started with a phase of 270° (i.e., length changes started with a stretch from the bottom of the sinusoid and sarcomere length was always higher than at isometric plateau) as shown in Fig. 11. In general, the force response resembled a superposition of the oscillation force response and the transient accompanying a step stretch of one-half of

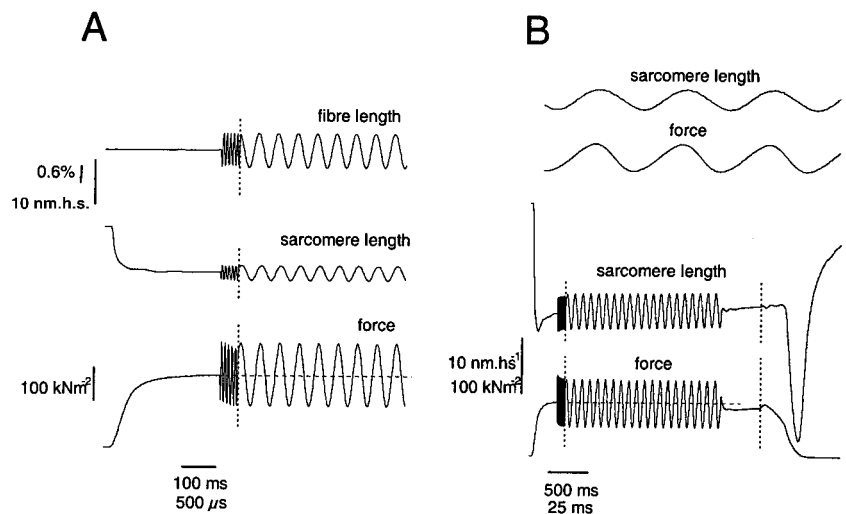


FIGURE 9 (A) Force and sarcomere length responses during fiber length oscillations at 3.4 kHz frequency and 3.1 nm/h.s. p-p amplitude applied at tetanus plateau in a single fiber. The vertical and the horizontal dashed lines indicate the change from the slow to the fast time and the isometric tension, respectively. The oscillations to the left of the dashed line are due to the aliasing phenomena occurring because the sampling time (2 ms/point) was too slow to sample correctly the 3.4 kHz oscillation frequency. After an initial transient period (not visible in figure because of the slow sampling time), which lasted for a few cycles, the force response reached a steady state, during which it was almost perfectly sinusoidal, in phase with sarcomere length oscillations and symmetric. The mean tension during oscillations was not significantly different from P_0 . Starting phase angle of length oscillations was 0° . Sarcomere signal measured with the striation follower. (B) As in (A), but at 200 Hz frequency and 6.64 nm/h.s. p-p amplitude. The faster time base refers to the portion of the records between the two dashed vertical lines. The two upper traces are an expanded portion (~ 3 cycles) of the sarcomere and tension traces below. As at 3.4 kHz, it can be seen that the mean tension during oscillations is not significantly different from the isometric level. However, now a clear phase lead of force over length is evident on the records. In addition, the force response is not a perfect sinusoid, but it is slightly distorted due to the different speed of the quick recovery between stretch and release. Starting phase of length oscillations, 0° .

the p-p oscillation amplitude. In four experiments of this kind (12 measurements) the mean tension was $1.054 \pm 0.0043 P_0$. Opposite and almost symmetrical results were obtained in the same experiments when oscillations started with 90° phase (i.e., sarcomere length during oscillations always lower than at plateau). The mean tension during oscillations decreased to $0.96 \pm 0.0084 P_0$.

According to the dependence of the quick recovery half-time on the amplitude and direction of an imposed length change (Ford et al., 1977), a shift of the myosin head position during length oscillations, as that postulated by

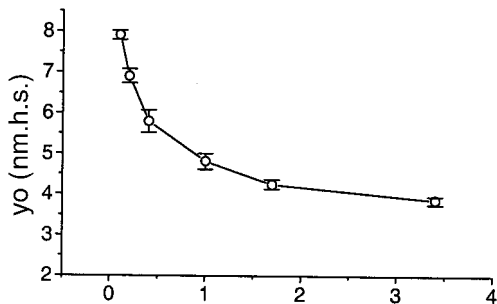


FIGURE 10 y_0 as function of the oscillation frequency. Values at each frequency were obtained by multiplying sarcomere length change for P_0 /force change ratio. The amplitude of the length changes applied was adjusted at each frequency to give a p-p force response of $0.8-1 P_0$. As expected from the force truncation by the quick recovery, y_0 greatly increases at low frequencies. Data of Table 1.

Dobbie et al. (1998), should also affect the kinetics of the quick force recovery following a step length change. To investigate this point, we applied quick stretches or releases at the tetanus plateau with and without length oscillations superimposed. By subtraction, we obtained the step force transient free from oscillations, which was then compared with that obtained at the isometric plateau. A typical sample record from these experiments is shown in Fig. 12 A. Fig. 12 B shows the comparison of the force responses to a step release applied with and without length oscillations (at 3.4 kHz) superimposed. Despite the procedure of subtraction, some residual force oscillations are still present on the subtracted record during the quick recovery. It is clear, however, that the quick recovery kinetics are not significantly influenced by the length oscillations. This was true at all the frequencies examined for both releases and stretches. Since we found that the mean tension during length oscil-

TABLE 1 y_0 values (mean plus standard error) at various frequencies			
Freq. (Hz)	y_0 (nm/h.s.)	SE	n
100	7.91	0.11	6
200	6.90	0.17	7
400	5.79	0.28	6
1000	4.62	0.2	5
1700	4.25	0.12	3
3400	3.98	0.14	7

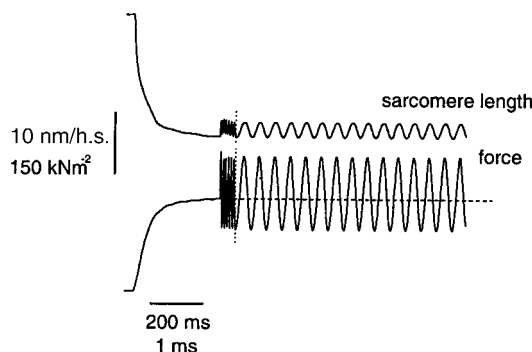


FIGURE 11 Force and sarcomere length changes during sinusoidal oscillations at 3.4 kHz starting with a 270° phase angle. Vertical and horizontal dashed lines as in Fig. 9. Sarcomere length oscillations are now completely asymmetric and shifted upward by half p-p amplitude compared to the isometric sarcomere length. As in Fig. 9, the force response has an initial transient phase (not visible on the figure because of the slow sampling), which lasts for several cycles after which the response attains a steady-state condition. The horizontal dashed line on the force trace shows that the force oscillations are not symmetrical about the isometric level, but are shifted upward by $0.05 P_0$.

lations was the same as at the isometric tetanus plateau, our mechanical results show no evidence of a change in cross-bridge kinetics during oscillations, and suggest that myosin head conformation is not modified by the presence of oscillations.

Simulation of I_{M3} signals during oscillations

Because the results from the previous section suggest that the apparent disposition of S1 during oscillations appears to be close to that at constant length, it was of interest to examine what the predicted changes in I_{M3} would be in this case. Fig. 13 *A* shows the data from Fig. 4, with the output of the spherical simulation of S1 superimposed. The value of β_0 has been chosen to set the tip of the tail to -0.5 nm (i.e., tail deflected by 0.5 nm toward the M line), in consideration of the calculations performed in the Discussion. The form of the simulated response resembles the actual data closely, but is much smaller. This is because the small value of β means that the tail element is so close to vertical that $\mathcal{F}[S1]$ is rather insensitive to the deflections produced by the oscillations. If the change in intensity of the simulation data is increased by a factor of 1.8 , the match between the simulation and the experimental data is much closer (Fig. 13 *B*). This suggests that the DC intensity level predicted by the simulation is too large in comparison to the actual I_{M3} level. When the simulations are performed using β_0 displacement of -2.1 nm, as proposed in Dobbie et al. (1998), the amplitude of the experimental intensity changes is now well accounted for by the simulations, but the double peak is absent from the release part of the transient. Our simulations showed that the only way to obtain a double peak with an equilibrium position of -2.1 nm, was to

increase the movement of the myosin head. A possible way to obtain such an increase is to reduce the filament compliance to a negligible level, which seems highly unlikely. In addition, the intensity changes resulting would be much higher than those found experimentally. Therefore, the behavior of our simulations suggests that y_0 is approximately the same, both at constant length and during oscillations.

DISCUSSION

Relation of I_{M3} changes to structural events

The data presented here show that the 14.5 -nm meridional reflection intensity decreases during the stretch phase of a sinusoidal length change and increases during the release phase, as previously reported (Wakabayashi et al., 1986; Mitsui et al., 1994; Dobbie et al., 1998; Griffiths et al., 1998). At 3.12 kHz, the intensity changes shown here are, within the limits of the noise, sinusoidal and in phase with length and force oscillations. However, at lower frequencies (100 – 400 Hz), I_{M3} changes during the negative part of the sinusoids are not sinusoidal, but are strongly distorted, with the formation of a double peak. According to a recently proposed model, I_{M3} changes accompanying length steps or oscillations are due to tilting and/or bending of S1, which would occur as a consequence of both the force resulting from the length oscillations (elastic effect) and the head movement associated with the quick force recovery (Piazzesi et al., 1995). Intensity changes would be produced by the spreading of the mass projection of the myosin head onto the fiber axis as the myosin head tilts or bends away from the perpendicular. To account for the observation that I_{M3} changes accompanied the elastic portion of the force response to step stretches, but not to step releases (Lombardi et al., 1995), it was assumed that, at the tetanus plateau, actin-bound S1 tail domains are oriented such that movement in the release direction of ~ 1 nm brings the head into perpendicular alignment with the filament axis, at which orientation maximum M3 intensity occurs.

Our results with 100 – 400 -Hz oscillations can be easily accommodated within the framework of this model. If one assumes that heads are tilted slightly away from the perpendicular (toward the M line, see Fig. 2) with respect to the filament axis at the isometric steady state, then during the release, heads will approach the perpendicular and intensity will increase (Irving and Piazzesi, 1997). However, any further movement in the same direction will cause a reduction of intensity, as the tilt of the heads increases again on the other side of the perpendicular. During the stretch part of the negative phase of the length oscillation, these changes will be reversed. As shown by the simulation, this effect will tend to produce a double peak on the intensity changes during the negative phase of the oscillations. M3 intensity at the well between the two peaks and the intensity difference between well and peak will depend on the amount of head

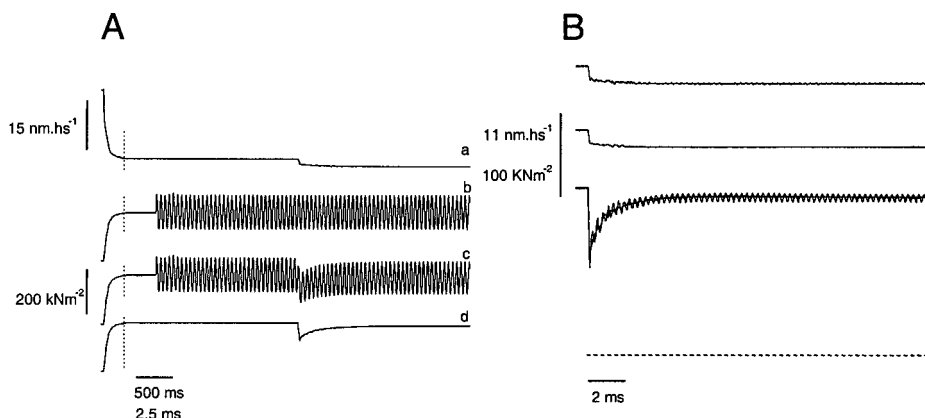


FIGURE 12 (A) Force and sarcomere length traces during step and oscillation length changes applied at tetanus plateau. *a*: sarcomere length signal during a step release (1.67 nm/h.s. amplitude, 160 μ s duration); *b*: force during length oscillations at 3.4 kHz frequency, 2.38 nm/h.s. amplitude; *c*: force during length oscillations as in *b*, but with the step release as in *a* superimposed; *d*: force response during the step release. (B) Subtracted traces. Trace *a* represents the step release during the oscillations and is obtained by subtracting the sarcomere oscillations from the trace with oscillations and step release superimposed. *b* is the step release without oscillations. The thick line in *c* represents the force response to the step release only while the thin line represents the force response to the step in presence of length oscillations, and it is obtained by subtracting the force response to oscillations only from the force response to oscillations plus step. The subtraction effectively eliminated the oscillations on the force trace at plateau; however, a significant residue of oscillations is present after the step during the quick recovery. This is attributable to a small drop of the sarcomere stiffness after the step release. It is seems clear that the time course of the recovery is not influenced by the oscillation presence. The dashed line at the bottom is the zero tension level. Oscillations frequency, 3.4 kHz; amplitude, 3.26 nm/h.s.; step amplitude, 2.06 nm/h.s.; step duration, 120 μ s.

movement beyond the perpendicular. For example, in Fig. 4 *D* the frequency of the length oscillations was 200 Hz, as in Fig. 4 *B*, but the length oscillations were of a smaller amplitude (5 nm/h.s.⁻¹ p-p instead of 6.25 nm/h.s.). Comparison of the two figures makes it clear that the reduction of length changes caused the double peak to almost disappear. The contribution of the quick recovery to the head movement is evinced by the phase shift between force (due to the length change of the elastic component) and intensity (due to head movement), and also by the absence of this shift in rigor bundles, where the quick recovery is absent. The observation that the size of the well between the double peaks also depends on the frequency of the length change being in agreement with this explanation.

In the scheme described above it is possible to calculate the equilibrium tilt of the myosin head during length oscillations from the position of the double peak in the cycle. To calculate this tilt, it is necessary to know 1) the relative amount of head and series compliance, and 2) the apparent head movement caused by the quick recovery. To have a clear double peak on the M3 intensity changes, it is necessary for the head to move a substantial distance past the perpendicular during the negative phase of the length oscillations. If, at the equilibrium position, the S1 tail is assumed to be tilted by $\sim 8^\circ$ toward the M line (i.e., the tip of S1 displaced by ~ 1 nm from the perpendicular toward the M line, as in the isometric state), a movement of the head > 2 nm p-p is required to make the double peak evident. Assuming that the compliance associated with the head ($1/K_{S2} + 1/K_{S1}$) is 50% of the total ($1/K_S$; Huxley et al., 1994; Wakabayashi et al., 1994), and assuming no quick recovery,

this means that the length change applied to the preparation at 3.4 kHz must be > 4 nm/h.s. p-p. In our experiments, the mean value of y_0 was ~ 4 nm/h.s.; therefore, such a length change will produce a force oscillation of p-p amplitude greater than P_0 . At low-frequency oscillation (100–400 Hz) there is a substantial myosin head movement due to the quick recovery which adds to the elastic movement (Piazzesi et al., 1995). It is therefore possible to reach a greater excursion of the myosin head with a relatively small force oscillations.

Calculation of the equilibrium tilt

If the origin of the double peak is as described above, the bottom of the well during the release phase represents the I_{M3} associated with the myosin head position at maximum excursion past the perpendicular, i.e., at the peak of the sarcomere shortening during oscillations (assuming no phase shift between length and I_{M3}). In Fig. 4 *B*, if we go back from the bottom of the well by six time intervals (i.e., 90°), we find the intensity corresponding to the position of the head at the midpoint of its oscillations (i.e., the third data point). Peak intensity was reached at the fourth data point; therefore the peak is reached at $\leq 15^\circ$ (1 time interval) beyond the equilibrium point. The excursion of the end of the S1 tail needed to reach the perpendicular is therefore less than the peak excursion multiplied by $\sin 15^\circ$. For our simulation at low frequency, the peak amplitude of the head movement does not simply correspond to the sarcomere length changes applied minus the portion absorbed by the

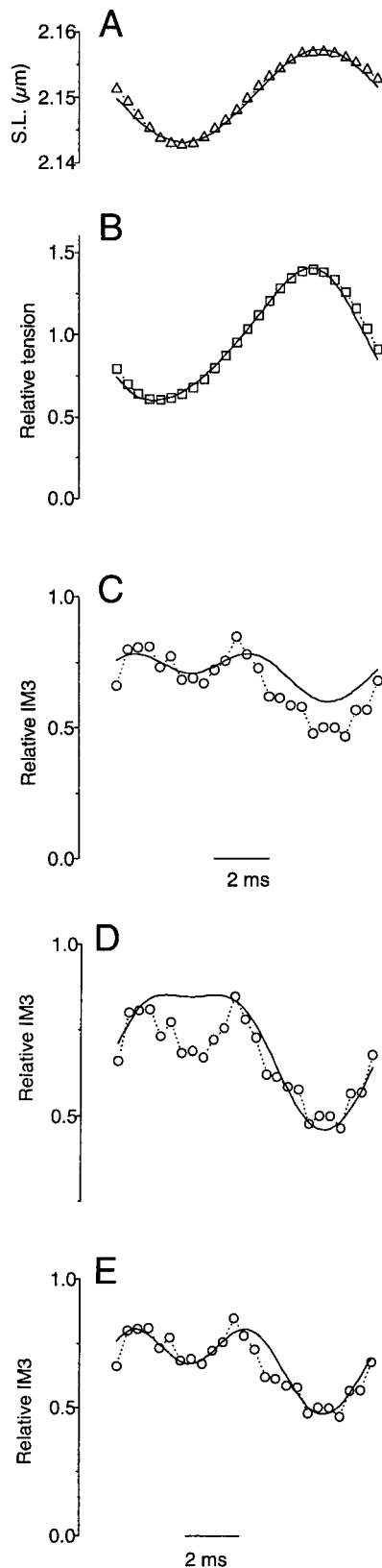


FIGURE 13 Comparison between experimental data and model response to length oscillations at 100 Hz frequency and 7 nm p-p amplitude. The myosin head was assumed to be composed of six spheres, of which four are rotating during elastic changes in force and during the quick recovery (see

myofilament compliance, because apparent movement associated with the quick recovery should also be considered. It can be calculated as follows. The length change applied to the preparation in Fig. 4 B was 6.25 nm and y_0 at high frequency (i.e., with a negligible quick recovery) was ~ 4 nm (see below). If we assume that the compliance ($1/K_{S1} + 1/K_{S2}$) is 50% of $1/K_s$, the p-p extension of the series compliance for a force oscillation of $0.91 P_0$ is $2 \text{ nm}/P_0$ (half sarcomere) $\times 0.91 = 1.82 \text{ nm/h.s.}$ This means that of the applied length change, 1.82 nm are taken up by the series elastic component K_F , while the remainder (4.43 nm p-p, (6.25–1.82)) is taken up by the compliance ($1/K_{S1} + 1/K_{S2}$). Taking the whole of this remainder to be the displacement of the S1 tail, the peak movement is possible is $4.43 \text{ nm}/2 = 2.21 \text{ nm}$. The product $2.21 \times \sin 15^\circ$ is 0.57 nm. This means that, at the midpoint of the oscillations, the end of the head is located not $>0.57 \text{ nm}$ away from the perpendicular. A release moving the head by this distance or less is then necessary to reach the maximum of I_{M3} .

The greatest uncertainty in this estimation is probably due to the difficulties in precisely locating the bottom of the well on the I_{M3} record in Fig. 4 B, because of the relatively long sampling time and noise. For instance, if the bottom is located halfway between points 8 and 9, the starting point will be located halfway between points 2 and 3 at an angle of $15^\circ \times 1.5 = 22.5^\circ$. The mean position will then be $2.21 \times \sin 22.5^\circ = 0.85 \text{ nm}$.

We have also implicitly assumed that the quick recovery and the elastic movement are giving the same I_{M3} change for the same head movement. This is true only if tilting or elastic bending are occurring for the same domain of S1. It should be pointed out, however, that the intensity changes simulated with a simple model seems to be relatively independent on which part of the myosin head is assumed to bend or tilt (Dobbie et al., 1998). Another assumption we made is that the myosin head movement is sinusoidal. This is true at higher frequencies, but at 100–400 Hz some distortion must be present in the head movement, because the force response is distorted (see Fig. 4 A, for example).

Fig. 2). y_0 was assumed to be 4 nm/h.s. and total compliance was assumed equally distributed between myosin and myofilaments. Symbols are experimental results; lines are results of the simulation (A) equilibrium position of the myosin head, -0.5 nm (as calculated from the double peak). It can be seen that sarcomere length (A) and force response (B) are well-simulated by the model. Simulation of I_{M3} (C) reproduces the double peak well during the release part of the sinusoid; however, the intensity changes are clearly smaller than the experimental ones. (D) Parameters of the simulation as in (C) except that the equilibrium position was -2.1 (as suggested by Dobbie et al., 1998). Now the amplitude of the intensity changes is simulated accurately; however, the double peak is not present on the simulated signal and is substituted by a flat region. (E) Simulation parameters as in (C), but the intensity signal has been multiplied by a factor of 1.8 and shifted appropriately. The form of the simulated I_{M3} signal is in good agreement with the experimentally observed changes in I_{M3} during length oscillations.

This is due to the asymmetry of the speed of the quick recovery for stretches and releases. However, the degree of distortion is relatively small and the error associated with it is very likely smaller than that associated with the location of the well and peak on the I_{M3} record. Finally, we also assumed that the relative amount of compliance in the myofilaments amounted to 50% of $1/K_s$. Different values, however, would only slightly change the results. For instance, if filament compliance is taken as 40% of the total, the filament elasticity elongation would be 2.18 nm ($4 \times 0.91 \times 0.6$). The peak head movement would be $(6.25 - 2.18)/2 = 2.03$ nm, therefore the displacement of the head from the perpendicular would be $2.03 \text{ nm} \times \sin 15^\circ = 0.53$ nm.

The calculation above can be repeated for the response at 100 Hz shown in Fig. 4 A. Here the bottom of the well is likely to be at point 8, and the equilibrium point is therefore at point 2. The peak is at point 3, 15° after the start. Because the p-p force amplitude is $0.85 P_0$, the length change of the series elasticity is $2 \times 0.85 = 1.7$ nm. Therefore, of the 7 nm applied, 5.3 nm constitutes the head movement. The amplitude of the peak length change is then equal to $5.3/2 \times \sin 15^\circ = 0.69$ nm, not too different from the value calculated previously at 200 Hz.

Similar calculations have been made for all the I_{M3} records showing a clear double peak. In no case was the calculated position of the end of myosin head shifted >0.9 nm away from the perpendicular.

To quantitatively account for both the M3 intensity changes and the drop of mean intensity during length oscillations, Dobbie et al. (1998) proposed that, during high-frequency oscillations, the equilibrium head position would move away from the perpendicular by 1.2 nm ($\sim 10^\circ$) with respect to its position assumed at the plateau of an isometric tetanus. Our results do not confirm this shift at lower frequency. The equilibrium position calculated from our data resulted at <1 nm away from the perpendicular, close to the head position assumed at plateau in absence of oscillations. It is possible that this discrepancy is due to the lower frequency at which we made our measurements, perhaps because of some cross-bridge cycling during the relatively slow-length cycles. It should be pointed out, however, that we found no mechanical evidence for a shift of the head equilibrium position caused by oscillations even at high frequencies. In fact, the presence of length oscillations did not change the mean tetanic tension as well as the time course of the quick recovery following both releases or stretches, suggesting no significant displacement of the S1 tail domain from its position in the isometric state.

At <1 kHz oscillation frequency, we find a marked distortion of the I_{M3} signal in the phase angle region of the sinusoid between 180 and 360° . In contrast, at >1 kHz we find no clear sign of double peak on the I_{M3} signal. This agrees with the tilting head model, because in this frequency domain there is no significant contribution of the quick

recovery to the head movement and the maximum length change we applied (3.6 nm/h.s. p-p) was not great enough to bring the heads beyond the perpendicular.

I_{M3} during high-frequency length oscillations is lower than at constant bundle length

Mean intensity during oscillations was reduced by 21% compared to I_{M3} on the tetanus plateau at constant length. If this reduction in intensity were due to a further 10° rotation of the S1 tail domain as compared to its orientation at constant length (Dobbie et al., 1998), such an increased tilt would correspond to the state reached after a stretch sufficient to displace the tip of the S1 tail domain by 1 nm toward the M line. This should cause an increase in mean tension and a change in the kinetics of the quick force recovery process (Huxley and Simmons, 1971; Ford et al., 1977). Taking 50% of sarcomere compliance to lie in the myofilaments, such a displacement would be expected from a sarcomere stretch of 2 nm/h.s. , which is half of y_0 , and therefore would be expected to produce an increase in mean tension during oscillations of $\sim 0.5 P_0$. In fact, Dobbie et al. (1998) and Piazzesi et al. (1997) found only a $0.1\text{--}0.15 P_0$ increase in mean tension during oscillations. In our mechanical experiments we observed very much smaller increases in tension during sinusoidal length changes. The starting phase of length oscillations is not mentioned explicitly in Dobbie et al. (1998), but inspection of their Fig. 1 A (*upper trace*) shows that sarcomere length oscillations are shifted upward with respect to sarcomere length before and after oscillations, similar to our records in Fig. 11, suggesting a possible starting phase of 270° . If this were the case, comparisons should be made with our mechanical results at 270° , in which the mean tension during oscillations was raised to $1.05 P_0$, which is not too different from their result. However, this level of mean tension remains inconsistent with their proposed increase in tilt of the S1 tail domain during oscillations, which should have increased mean force to $1.5 P_0$. In addition, the mean disposition of S1 might be displaced toward that adopted after the quick recovery from a stretch, because the mechanical response of fibers to oscillations beginning with a phase angle of 270° resembled the superposition of the oscillation force response with that to a step stretch. A reduction in S1 binding to actin during the oscillations could accommodate both the low force and the increased tilt during oscillations. However, the observations that y_0 measured in our experiments with oscillations (4 nm) is the same as that measured with steps (Ford et al., 1977; Piazzesi et al., 1992) and that the mechanical response to a length step is not affected by the oscillation presence makes this hypothesis very unlikely. Instead, it seems likely that the tilt of S1 remains similar to that at the isometric plateau even at high

frequencies of oscillation, and I_{M3} is reduced for other reasons.

The I_{M3} changes of $\sim 20\%$ p-p at 3.12 kHz for a length change of 3.6 nm/hs^{-1} p-p is in agreement with the value shown previously (Dobbie et al., 1998). This is a relatively great intensity change and can be accounted for by the tilting model only if the equilibrium position of the head during the oscillations moves further away from the perpendicular by something like 1.2 nm. If, as suggested above, the myosin head position does not shift in orientation during oscillations, then the intensity changes calculated with the tilting model should be much smaller (about one-half) than those found experimentally. This suggests that 1) the tilting model considering only one actin-bound S1 state is too simplified; 2) other factor(s) may contribute to the intensity changes during length oscillations in addition to myosin tilting (for example, the second S1 of each bound cross-bridge); or 3) destructive interference may occur between x-rays scattered by actin-bound S1 and those scattered by other components of the myofilament lattice having a similar axial period (for example, the apparently large population of unbound S1 (Corrie et al., 1999)), causing the fractional change in I_{M3} to become larger.

In conclusion, the distortion of the I_{M3} signal, observed during the release phase of sinusoidal length oscillations at very low frequency, can also be detected at frequencies up to 1 kHz. The frequency domain over which the amplitude of this distortion changes rapidly corresponds to that over which the contribution of the force quick recovery to force transients is also rapidly increasing. In rigor, where the force quick recovery is almost absent, no I_{M3} signal distortion was observed. The shape of the distortion allows prediction of the length at which the maximum I_{M3} signal would be reached during oscillations. In contrast to the findings of earlier studies, this length corresponds to that found using length steps instead of high-frequency oscillations. The mean I_{M3} signal was reduced at all oscillation frequencies examined without corresponding changes in either mean tension or force transient kinetics.

The authors express their thanks to the EMBL Outstation and Elettra for experimental facilities, to B. H. Kunst for technical assistance, and to the Wellcome Trust, the CNR, Telethon Italia, and the EU Large Installation Programme (CHGE-CT93-0040) for financial support.

REFERENCES

- Amenitsch, H., C. C. Ashley, M. A. Bagni, S. Bernstorff, G. Cecchi, B. Colombini, and P. J. Griffiths. 1997a. Meridional x-ray diffraction intensity changes during sinusoidal length oscillations in skeletal muscle fibres of *Rana temporaria*. *J. Physiol.* 505:88.
- Amenitsch, H., S. Bernstorff, M. Kriechbaum, D. Lombardo, H. Mio, M. Rappolt, and P. Laggner. 1997b. Performance and first results of the ELETTRA high-flux beamline for small-angle x-ray scattering. *J. Appl. Crystallogr.* 30:872–876.
- Bagni, M. A., G. Cecchi, B. Colombini, and F. Colomo. 1999. Sarcomere tension-stiffness relation during the tetanus rise in single frog muscle fibres. *J. Muscle Res. Cell Motil.* 20:469–476.
- Corrie, J. E. T., B. D. Brandmeier, R. E. Ferguson, D. R. Trentham, J. Kendrick-Jones, S. C. Hopkins, U. A. van der Heide, Y. E. Goldman, C. Sabido-David, R. E. Dale, S. Criddle, and M. Irving. 1999. Dynamic measurement of myosin light-chain-domain tilt and twist in muscle contraction. *Nature*. 400:425–430.
- Dobbie, I., M. Linari, G. Piazzesi, M. Reconditi, N. Koubassova, M. A. Ferenczi, V. Lombardi, and M. Irving. 1998. Elastic bending and active tilting of myosin heads during muscle contraction. *Nature*. 396:383–387.
- Finer, J. T., R. M. Simmons, and J. A. Spudis. 1994. Single myosin molecule mechanics: piconewton forces and nanometre steps. *Nature*. 368:113–119.
- Ford, L. E., A. F. Huxley, and R. M. Simmons. 1977. Tension responses to sudden length changes in stimulated frog muscle fibres near slack length. *J. Physiol.* 269:441–515.
- Griffiths, P. J., H. Amenitsch, C. C. Ashley, M. A. Bagni, S. Bernstorff, G. Cecchi, B. Colombini, and G. Rapp. 1998. Studies on the 14.5 nm meridional x-ray reflection during length changes of intact frog muscle fibers. *Adv. Exp. Med. Biol.* 453:247–257.
- Highsmith, S. 1999. Lever arm model of force generation by actin-myosin-ATP. *Biochemistry*. 38:9791–9797.
- Huxley, H. E., A. R. Faruqi, M. Kress, J. Bordas, and M. H. Koch. 1982. Time-resolved x-ray diffraction studies of the myosin layer-line reflections during muscle contraction. *J. Mol. Biol.* 158:637–684.
- Huxley, A. F., V. Lombardi, and L. D. Peachey. 1981. A system for fast recording of longitudinal displacement of a striated muscle fiber. *J. Physiol.* 317:12–13.
- Huxley, A. F., and R. M. Simmons. 1971. Proposed mechanism of force generation in striated muscle. *Nature*. 233:533–538.
- Huxley, H. E., R. M. Simmons, A. R. Faruqi, M. Kress, J. Bordas, and M. H. J. Koch. 1983. Changes in the x-ray reflections from contracting muscle during rapid mechanical transients and their structural implications. *J. Mol. Biol.* 169:469–506.
- Huxley, H. E., A. Stewart, H. Sosa, and T. Irving. 1994. X-ray diffraction measurements of the extensibility of actin and myosin filaments in contracting muscle. *Biophys. J.* 67:2411–2421.
- Irving, M., V. Lombardi, G. Piazzesi, and M. A. Ferenczi. 1992. Myosin head movements are synchronous with the elementary force generating process in muscle. *Nature*. 357:156–158.
- Irving, M., and G. Piazzesi. 1997. Motion of myosin heads that drives muscle contraction. *N. I. P. S.* 12:249–254.
- Koch, M., and P. Bendall. 1981. Proc. Digital Equipment Computer Users Soc. DECUS (UK).13–16.
- Linari, M., G. Piazzesi, I. Dobie, N. Koubassova, M. Reconditi, T. Narayanan, O. Diat, M. Irving, and V. Lombardi. 2000. Interference fine structure and sarcomere length dependence of the axial x-ray pattern from active single muscle fibers. *Proc. Natl. Acad. Sci. USA*. 97:7226–7231.
- Lombardi, V., G. Piazzesi, M. A. Ferenczi, H. Thirlwell, I. Dobbie, and M. Irving. 1995. Elastic distortion of myosin heads and repriming of the working stroke in muscle. *Nature*. 374:553–555.
- Mitsui, T., K. Wakabayashi, En-Z. Wang, H. Iwamoto, H. Tanaka, T. Kobayashi, T. Hamaoka, Y. Amemiya, H. Sugi, H. Ohshima, and W. Hiraoka. 1994. Frequency dependence of the variation of the x-ray diffraction pattern from tetanized frog skeletal muscle during sinusoidal length changes. In *Synchrotron Radiation in the Biosciences*. B. Chance, editor. Clarendon Press, Oxford. 460–472.
- Molloy, J. E., J. E. Burns, J. Kendrick-Jones, R. T. Tregear, and D. C. White. 1995. Movement and force produced by a single myosin head. *Nature*. 378:209–212.
- Piazzesi, G., F. Francini, M. Linari, and V. Lombardi. 1992. Tension transient during steady lengthening of tetanized muscle fibres of the frog. *J. Physiol.* 445:659–711.
- Piazzesi, G., N. Koubassova, M. Reconditi, I. Dobbie, M. A. Ferenczi, V. Lombardi, and M. Irving. 1997. Cross-bridge and filament compliance

- measured by x-ray diffraction in single frog muscle fibres. *Pflügers Arch.* 434:57.
- Piazzesi, G., V. Lombardi, M. A. Ferenczi, H. Thirlwell, I. Dobbie, and M. Irving. 1995. Changes in the x-ray diffraction pattern from single, intact muscle fibers produced by rapid shortening and stretch. *Biophys. J.* 68:92S–98S.
- Tokunaga, M., K. Sutoh, C. Toyoshima, and T. Wakabayashi. 1987. Localisation of the ATPase site of myosin determined by three-dimensional electron microscopy. *Nature.* 329:635–638.
- Wakabayashi, K., Y. Sugimoto, H. Tanaka-H, Y. Ueno, Y. Takezawa, and Y. Amemiya. 1994. X-ray diffraction evidence for the extensibility of actin and myosin filaments during muscle contraction. *Biophys. J.* 67: 2422–2435.
- Wakabayashi, K., H. Tanaka, T. Kobayashi, Y. Amemiya, T. Hamanaka, S. Nishizawa, H. Sugi, and T. Mitsui. 1986. Time-resolved x-ray study of effect of sinusoidal length change on tetanized frog muscle. *Biophys. J.* 49:581–584.