X-Ray Diffraction Measurements of the Extensibility of Actin and Myosin Filaments in Contracting Muscle

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ABSTRACT We have used a small angle scattering system assembled on the high flux multipole wiggler beam line at CHESS (Cornell) to make very accurate spacing measurements of certain meridional and layer-line reflections from contracting muscles. During isometric contraction, the actin 27.3 Å reflection increases in spacing from its resting value by approximately 0.3%, and other actin reflections, including the 59 and 51 Å off-meridional reflections, show corresponding changes in spacing. When tension is augmented or diminished by applying moderate speed length changes to a contracting muscle, changes in spacing in the range of 0.19–0.24% (when scaled to full isometric tension) can be seen. The larger difference between the resting and isometric spacings suggests either nonlinearity at low tension levels or the presence of a component related to activation itself. Myosin filaments also show similar increases in axial period during slow stretch, in addition to the well known larger change associated with activation. An actin spacing change of 0.25–0.3% can also be measured during a 2 ms time frame immediately after a quick release, showing that the elastic behavior is rapid. These observations of filament extensions totaling 2–3 nm per half-sarcomere may necessitate some significant revision of the interpretation of a number of mechanical experiments in muscle, in which it has usually been assumed that virtually all of the elasticity resides in the cross-bridges.

INTRODUCTION

Earlier x-ray diffraction observation of the repeating axial periodicities in the actin and myosin filaments in striated muscle showed that these remained approximately constant during passive length changes and during contraction (Huxley, 1953; Elliott et al., 1965; Huxley et al., 1965) and provided strong evidence that filament shortening could not provide the mechanism for the substantial sarcomere shortening that could take place. However, the accuracy of the measurements was of the order of 1%, sufficient to exclude large scale length changes, but not adequate to exclude any length change whatsoever. Subsequently, very careful measurements of the myosin 14.3 nm cross-bridge repeat showed that this underwent a length *increase* of approximately 1% between rest and isometric contraction (Huxley and Brown, 1967; Haselgrove, 1975) but that this change seemed to be associated with cross-bridge attachment rather than tension generation (Huxley, 1979). Measurements of the actin axial periodicities (Huxley and Brown, 1967) indicated that they remained constant to within about 0.5%, but could not exclude the occurrence of smaller changes associated with activation or with tension development.

This matter has assumed considerable relevance to the detailed mechanism of force production in muscle in recent years. The immediate elastic response to a step length change seen in single active muscle fibers (Huxley and Simmons, 1971; Ford et al., 1977) represents a length change per half-sarcomere of approximately 4 nm, or 0.4% of muscle length, when tension falls to zero from its isometric value. This elastic element, which has been thought to be almost entirely within the cross-bridges themselves (Ford et al., 1981), is re-extended during the rapid tension recovery phase after a quick release, and its actual location is therefore of some importance to the detailed mechanics of that process. Indeed, measurements of muscle stiffness made by applying small and very rapid length oscillations to an active muscle have been widely used to estimate the number of cross-bridges attached under various conditions, on the assumption that any extensibility in the filaments is negligibly small.

The possibility that significant extensibility is present in the actin filaments has been raised recently by Yanagida and his colleagues on the basis of direct mechanical measurements of an individual isolated actin filament (Kojima et al., 1994), and also by Oosawa (Oosawa, 1980, 1983) from earlier observations of the Brownian motion of fluorescently labeled single actin filaments. Filament extensibility will also cause spacing changes in the axial periods of the x-ray reflections from muscle. The actin monomers along each strand of the two-stranded filaments have a repeat of 54.6 Å, and the repeats in the strands are half staggered, so that the whole filament has an axial repeat of 27.3 Å. The diffraction pattern therefore has a meridional reflection at 27.3 Å, and at higher orders of that spacing (e.g., 13.65 Å, 9.1 Å). Other strong actin reflections are generated by the helical character of the actin structure and occur off the meridian at axial spacings of approximately 51 and 59 Å. These correspond to the pitches of the right-handed and left-handed one-start helices on which all the monomers lie, and they too will be affected by any stretching of the actin filament. The myosin filaments have an axial repeat of 143 Å, which arises from the groups of cross-bridges that repeat with this axial spacing

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but in different azimuths along the length of the thick filaments, giving the helical repeat of 429 Å. The myosin diffraction pattern therefore has meridional reflections at 143 Å and at higher orders of that repeat (e.g., 5th order at 28.6 Å). Both the actin and myosin meridional reflections are very sharp in an axial direction, because the structures repeat precisely along the whole length of the filaments. Recent developments in x-ray technology have facilitated much more accurate measurements of the axial periodicities in the diffraction patterns from contracting muscles, and we have taken advantage of these possibilities to study the small changes in the actin and myosin axial repeats associated with isometric tension development and with changes in tension in active muscles during steady lengthening or shortening, and after a quick release. Our results indicate that at least one-half, possibly as much as three-quarters, of the compliance of an active muscle resides in the actin and myosin filaments themselves.

MATERIALS AND METHODS

Techniques

Because the changes that we wish to study represent differences in spacing of at most a few parts per thousand, conventional methods involving either the comparison of patterns on separate films or the use of electronic position-sensitive detectors, seemed unlikely to give the required degree of accuracy and reproducibility. Instead, we have used an imaging plate (Amemiya et al., 1988) as a detector and a camera system in which the patterns to be compared are recorded side-by-side on the same plate from the same muscle during a single experimental series. The imaging plates provide the desired combination of high spatial resolution and reproducibility and high sensitivity. The patterns are read out with a pixel size of 100 μ × 100 μ, and the reflections we are studying each extend over about 20 pixels axially. After a few seconds exposure, each reflection will contain from ten thousand to hundreds of thousands of individual photons within the central 10 pixels of the peak, on top of a background of approximately equal intensity (i.e., the signal/background ratio is 1) and, in principle, we should be able to determine the position of the peak with an accuracy of $\frac{1}{10}$ pixel or better. Analyses of simulated patterns with appropriate noise levels confirm that this is the case. The pairs of reflections that we are measuring are between 600 and 1300 pixels apart under the conditions of our experiments, and so the system should give the required accuracy, better than 0.05%.

In one set of experiments, the imaging plate was held stationary and a blanking-off plate was moved to cover alternate halves of the pattern during alternate exposures; the muscle was alternated between rest or isometric contraction or, in other experiments, between isometric contraction and steady lengthening or steady shortening. In another set of experiments, the imaging plate was displaced sideways in alternate directions between successive exposures, so that a larger fraction of each of the two patterns, including a complete meridional coverage, was recorded for the two conditions, but at the expense of possible artifacts caused by changes of axial position or tilt of the plate. However, no evidence for such movements was found from fiduciary marks on the patterns, and the spacing changes observed in the muscle patterns from experiments with the two systems were very similar. In each case, the spacing of the reflections in the two patterns could be compared with great accuracy and convenience because they lay adjacent to each other in the same image. A recovery period of 3 min was provided between successive contractions, and average tensions during a series were of the order of 80–90% or more of the starting value. In some experiments, the muscle was tilted through the appropriate angle (approximately 2° at the wavelength used (0.9 Å)) to bring the reciprocal lattice point corresponding to the 27.3 Å meridional reflection accurately into the sphere of reflection, and so avoid artifacts arising from changes in the shape of the layer line. However, no effect was observed in the measurements, possibly because the angular separation from the meridian lies within the natural dispersion of orientations of the fibers and fibrils in the muscle.

Other technical considerations

A possible disadvantage of using imaging plates or film as the recording medium, rather than some type of electronic device, is that the detector remains sensitive to incoming photons for the whole period of the experiment, which may be several hours, whereas the total exposure of the muscle pattern may be only a few hundred milliseconds, in the case of experiments involving 2 ms time slices. This makes it necessary to take extreme precautions to prevent hard radiation from leaking through the camera between the desired exposures. This was done by providing heavy lead shielding at all vulnerable parts, and by installing secondary shutters, the thickest one about 2 cm thick, which were open only the minimum time necessary to bracket the opening of the rapid shutter that defined the state of the muscle being recorded.

In the case of our shortest exposures, the very short open time (2 ms) was achieved using a rotating lead disc about 1 m in circumference with a 2-mm-wide slot near the periphery. The disc was rotated at about 60 rpm by a constant speed motor and synchronized to the contraction and quick release of the muscle by electronic means. The secondary shutters already described ensured that only a single exposure would occur during each contractile cycle and that the effect of any leakage of hard radiation through the lead disc (about 1 mm in thickness) was minimized.

The very high x-ray flux (around 10^{13} photons per s) provided by the F-1 beam-line at CHESS and the small angle camera described by Irving and Huxley (1994) was necessary to be able to make satisfactory recordings of the two-dimensional x-ray patterns, out to spacings of 13 Å and beyond, during the short time intervals required. At these flux levels, radiation damage can be detected if a muscle is left stationary in the x-ray beam (which is about 0.5 mm in vertical width, and 5-mm-long at the specimen) for longer than 10 s. Accordingly, the specimen chamber was scanned up and down so that the x-ray beam was distributed over an area of muscle 5 mm in height. Because a very heavily exposed pattern could be obtained with a total exposure of only 1 or 2 s (and quite informative ones with 100 ms exposures), and because in these experiments we are essentially recording only two time frames instead of the much larger number usually recorded with electronic detectors, radiation damage should not be a problem at present and we never detected any signs of it except when the specimen scanning device was inoperative.

Accurate measurement of changes of spacing

The changes in spacing were anticipated to be in the range of 0.1–0.4% of initial length. In the case of the first meridional actin reflection at a spacing of approximately 27.3 Å, the distance on the imaging plate detector from the center of the pattern, with a specimen-to-detector distance of approximately 2 m and using radiation of wavelength ~0.9 Å, is approximately 66 mm. Thus, the actual shift in position is likely to be in the range of 0.06–0.26 mm and it would be desirable to measure the shift with an accuracy of at least one-fifth of this amount, i.e., 0.01 mm or one-tenth of a pixel with the current scanning system.

We investigated two principal methods of determining the change in position of the reflections. In the first, a computer fitting program was used to give the best match between Gaussian peaks of appropriate widths and spacings and the experimental results in the form of sections through the two-dimensional intensity data close to and parallel to the meridian projected onto a meridional line and with the background intensity stripped. This technique was particularly useful in the case of measurements on partially overlapping peaks. We also used a centroid method for determining peak position, i.e., we determined the center of gravity of the peak, usually using the top half of the peak only, to reduce the effect of background noise at larger distances from the center. This latter method gave results that were reproducible to about 0.1 pixels.

The muscles were mounted so that any changes in their tilt or orientation between different experimental conditions were minimized. In most cases, no evidence was detected in the patterns for any significant changes of this kind, and where such changes were seen (e.g., significant changes in the apparent center of the pattern), the results were discarded.

Other experimental details

Sartorius and semitendinous muscles were dissected from medium sized bull frogs (4–5 inches long, Connecticut Valley Biological Supply Co., Inc., Southampton, MA) and mounted as described previously (Huxley et al., 1982) with the long axis vertical. The procedure was standardized to give a sarcomere length of approximately 2.3 μ with the sartorius muscles, which gave tensions in the range 2–3 kg/cm^2. Some experiments were carried out at 10°C, but during the summer months more reliable performance of the muscles was obtained at 14°C. The muscles were stimulated tetanically with supramaximal current pulses of 1 ms width and alternating polarity with a repetition frequency of 50 Hz, for periods of between 300 and 600 ms with intervals of 2–4 min between successive tetani to allow recovery. Average tensions during an experiment (which usually consisted of about 20–50 contractions) remained within 80–90% of the starting value.

Muscle lengths were controlled by a rapid acting solenoid system described previously (Huxley et al., 1983), so that shortening or lengthening ramps or very rapid length changes could be imposed on the muscle at the time of the x-ray exposure by appropriately synchronized wave form generators and servomechanisms. Muscle tensions were measured by strain gauges in the same apparatus, and all of the experimental parameters were recorded throughout the experiments and stored in a minicomputer.

RESULTS

Spacing changes in isometric contraction

When the pattern from a resting muscle was compared with that from the same specimen during isometric contraction, the changes in spacing of the first actin meridional reflection at 27.3 Å were sufficiently large to be detected by eye in the two-dimensional patterns taken off the imaging plate (Figs. 1 and 2). The reflections move inward, toward the center of the pattern, by approximately 2 pixels, i.e., by one-quarter of their full width at half-maximum height, so the change in position is very clear in the intensity plots (Figs. 3 and 4) and can be measured quite accurately (Table 1). The values determined by centroid measurements range in value between 0.25 and 0.39% with an overall average of 0.31 ± 0.03% (average error).

The actual values of the change may be slightly higher than this, for the following reason. In the resting pattern, in addition to the prominent myosin reflection at a spacing of 28.6 Å (15th order of 429 Å), there is a weak myosin meridional reflection (16th order of 429 Å) at 26.8 Å, i.e., about 12 pixels away from the center of the actin reflection, sufficiently far so that the two reflections do not overlap significantly (Fig. 4 *B*). During contraction, the myosin meridional reflections all undergo quite a large change in spacing, probably in large part associated with activation itself, which we will discuss later. The myosin periodicity increases by approximately 1.3%, so that a 16th order myosin reflection would now have a spacing of 27.2 Å, much closer to the actin reflection (now at approximately 27.38 Å). The centers of the reflections would now be separated by only about 5.5 pixels, and would overlap sufficiently so that only a slight shoulder would be

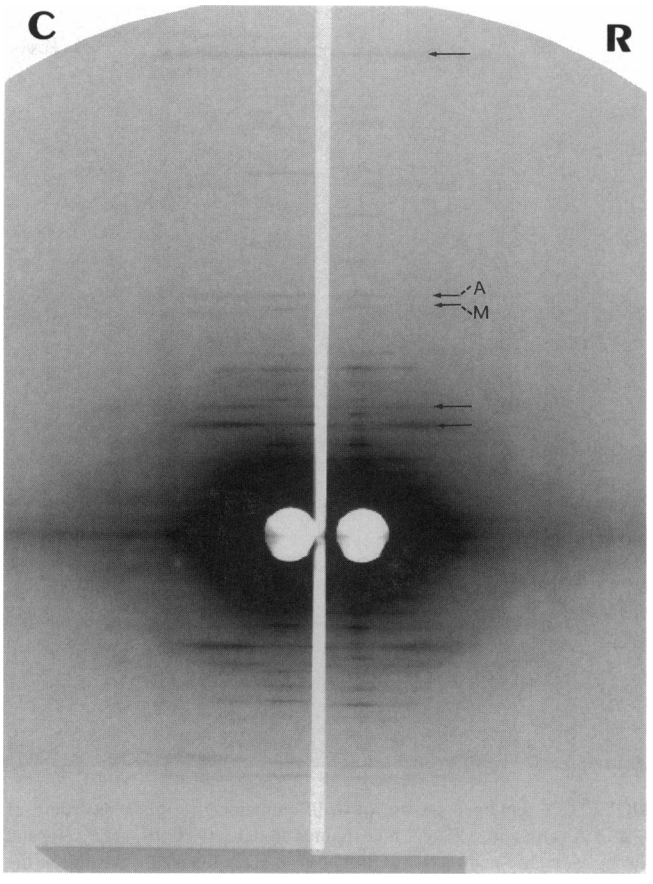


FIGURE 1 Imaging plate record of x-ray diagram for frog sartorius muscle. Alternating exposures at rest (R) (R.H.S.) and during isometric contraction (C) (L.H.S.), with plate displaced laterally between the two conditions. The strong reflection at the top of the picture is the ~13.65 Å meridional actin reflection (*single arrow*): the pair of meridional reflections (*two arrows*) halfway between it and the center of the pattern are the ~27.3 Å first meridional actin reflection and the ~28.6 Å myosin reflection (5th order of ~143 Å). The lowest two arrows mark the positions of the 51 Å (*upper*) and 59 Å (*lower*) off-meridional actin reflections. Total exposure on each side was approximately 3 s.

produced on the actin reflection unless the myosin reflection had undergone a large increase in intensity from its resting value. In fact, a small shoulder does appear on the actin reflection in the expected position. However, we have no way of telling a priori what the intensity of the myosin reflection should now be—myosin meridional reflections in general *decrease* in intensity during contraction except those at the 3*n* orders of the 429 helical repeat—and even in the resting patterns there are traces of a small shoulder on the actin reflection in a similar position. Also, we do not have a priori knowledge of the shape of these reflections. However, if the 16th order myosin peak is indeed present during contractions, it could cause the centroid measurements of the actin peak to *underestimate* the actual movement by between 0.25 and 0.5 pixels. That is, separation of the actin meridional peak in contracting muscle into actin and myosin components has to place the actin component slightly closer to the center of the pattern than the center of gravity of the peak as

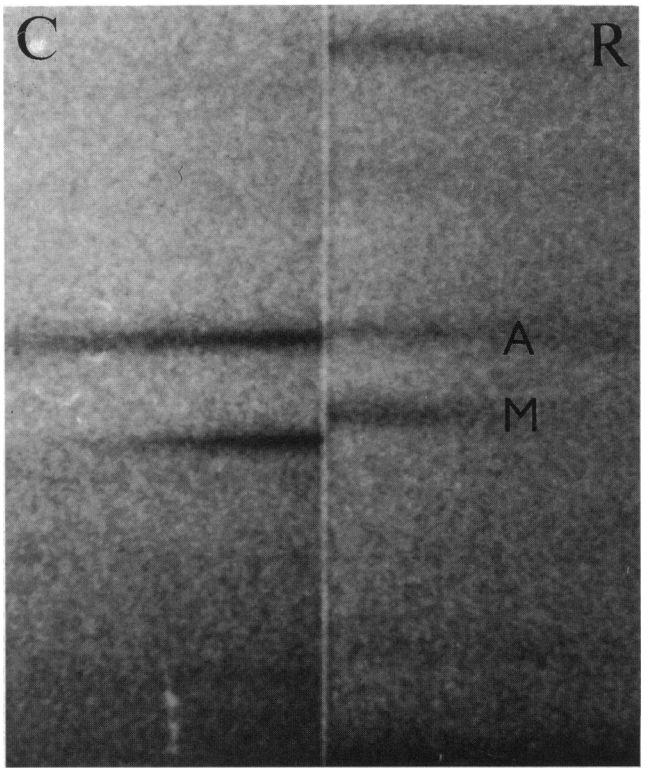
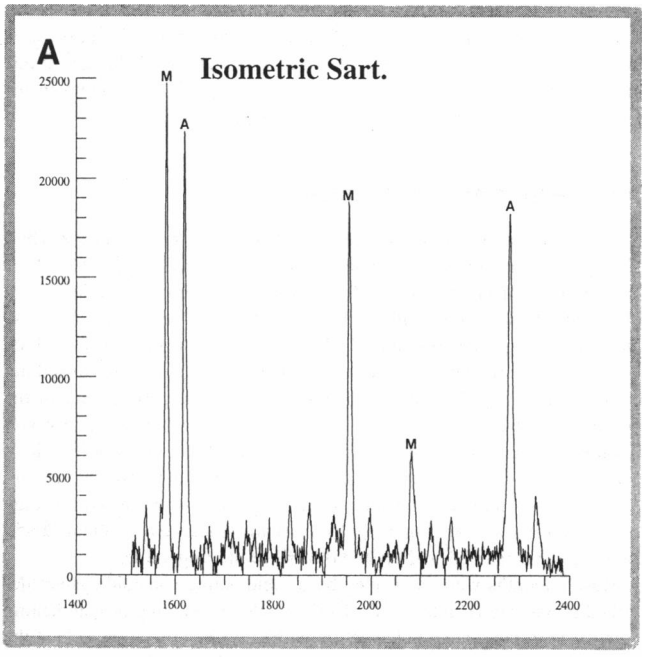


FIGURE 2 Enlarged picture of a different imaging plate recording of ~27.3 Å actin and ~28.6 Å myosin meridional reflections, at rest (R) and during isometric contraction (C). Alternate sides blanked off between the two conditions, but plate held stationary. Note the large increase in intensity in the contracting pattern, the prominent shift of the myosin reflection toward the center of the pattern (below region shown), and the visible shift of the actin reflection in the same direction, i.e., to a slightly longer spacing.



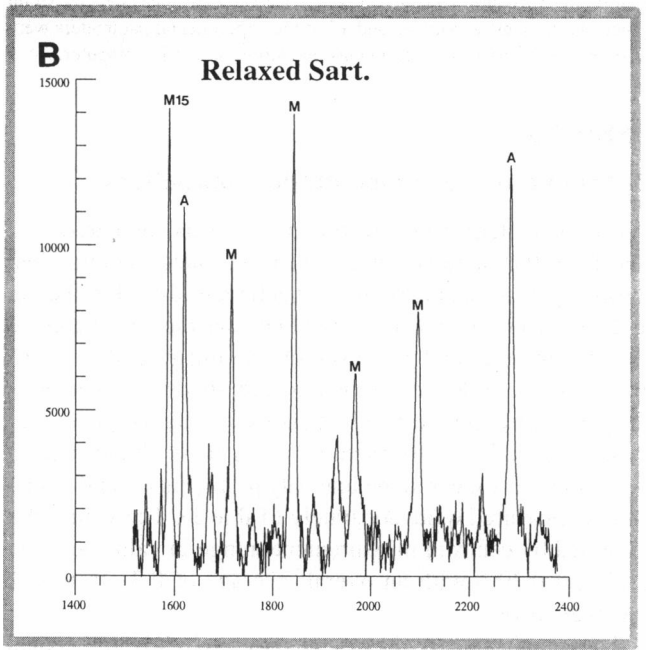


FIGURE 3 Densitometer tracing of meridional region of x-ray pattern from frog sartorius muscle after background subtraction, showing the reflections between about 30 and 13 Å, (*A*) during isometric contraction and (*B*) at rest. The pair of reflections on the L.H.S. of the diagrams are the ~28.6 Å myosin and ~27.3 Å actin meridional reflections. The prominent reflection on the R.H.S. of the diagrams is the ~13.65 Å actin meridional reflection. Note the change of vertical scale (intensity) between the two diagrams and the increase in intensity of the actin and certain of the myosin reflections.

a whole. Thus, it is possible that we are underestimating the change in spacing, and rather than having an average value of about 0.31% it could be as large as 0.36%. However, we prefer to take the more conservative value of the spacing change, and we have outlined these complications to show that any effect of interfering myosin reflections would be to make the true value larger.

We also examined the changes in spacing that occur in the 59 and 51 Å actin off-meridional layer line reflections. Very accurate measurement of these spacings is much more problematic than in the case of the meridional reflections. The layer line reflections may be 1.5 times or more wider in an axial direction, caused at least in part by the broadening effect of angular disorder on off-meridional reflections and, in practice, the measured value of the spacing varies significantly depending on the distance from the meridian at which the measurement is made. Although these reflections sometimes appear to have weak meridional components (which should be forbidden if the structure is perfectly helical), the spacing of these does not follow that of the rest of the layer line and we suspect that they contain components from other diffracting structures in the sarcomere. If measurements are made by integrating the layer lines between radial distances from 0.0015 nm^{-1} to 0.003 or 0.0045 nm^{-1} (avoiding the meridional spots but minimizing the effect of "arcing" of the reflections by measuring close to the meridian), then both the 59 and the 51 Å reflections can be seen to increase in spacing by approximately equal amounts (on a percentage basis) during isometric contraction (Table 2). The increases

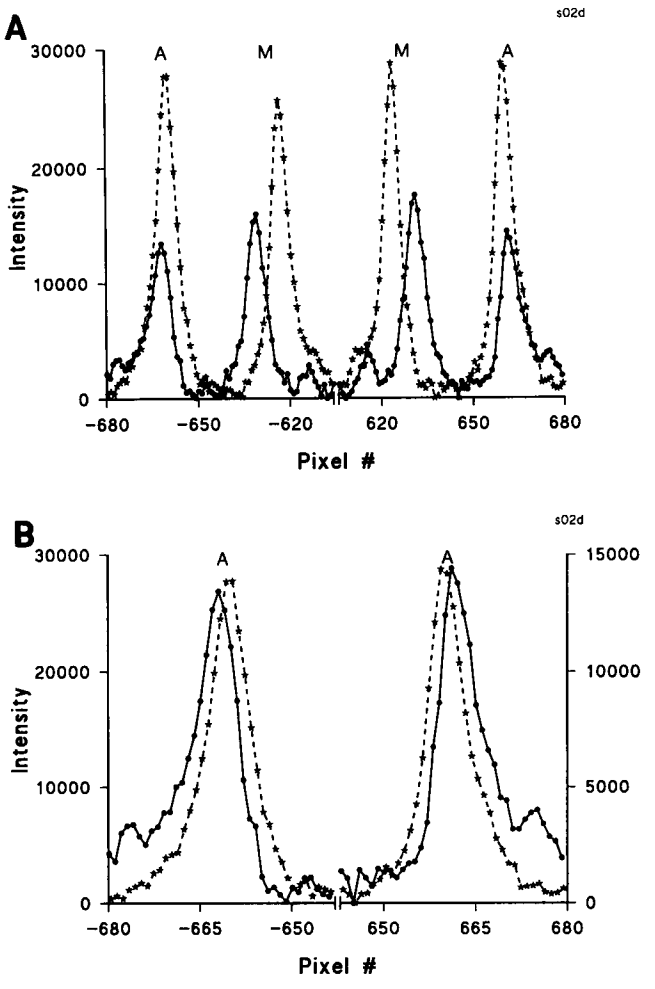


FIGURE 4 (*A*) Densitometer tracing of meridional regions of x-ray patterns from relaxed (——) and contracting (- - - -) muscles, showing enlargement of regions containing ~28.6 Å myosin and ~27.3 Å actin reflections on either side of center of pattern at Pixel #0, but all inner regions of pattern omitted. The large inward movement of the myosin reflections and the smaller movement of the actin reflections, as well as their substantial increase in intensity, are clearly visible. (*B*) Enlarged view of actin ~27.3 Å meridional reflection, on either side of origin, showing inward shift between resting (——) and contracting (- - - -) conditions, with change of intensity scale to make shift of position more readily visible (left-hand scale, contraction, right-hand scale, rest). The small peaks visible in the resting patterns just outside the actin peaks correspond to another myosin meridional reflection (16th order of 429 Å, at ~26.8 Å). In the contracting patterns, the corresponding reflection would have moved closer in to the actin reflection, and may possibly contribute to its slightly asymmetrical shape. If it has sufficient intensity to contribute, it would cause our measurements of centroid position to underestimate the shift in position of the actin reflection, so that the actual change in spacing would be larger than the measured values.

are on average about 10% less than the changes in the 27.3 Å meridional reflection, but because of the problems we have mentioned above and the fact that the spread of orientations changes somewhat between rest and contraction, we attach much greater significance to the meridional measurements. The changes in the layer line reflections simply illustrate the occurrence of the change in spacing in a less accurate way, but one that is easier to measure in images with shorter exposure times.

TABLE 1 Isometric changes in actin axial repeat

	Expt.	27 Direct	27 from 51/59
December	8a	0.39	0.27
	9c	0.31	0.24
	10b	0.33	0.24
	11a	0.35	0.37
	12c	0.35	0.22
	13a	0.31	0.34
Av.		0.34	0.28
July	2a	0.32	0.21
	2d	0.28	0.20
	8a	0.33	0.30
	8c	0.26	0.24
	11c	0.25	0.35
	15a	0.30	0.16
	T2a	0.30	0.28
Av.		0.29	0.25

% Increase in spacing between rest and isometric contraction.

TABLE 2 Isometric changes in actin helical repeats

	Expt.	Δ51	Δ59	ΔHelix
December	8a	0.28	0.26	0.18
	9c	0.30	0.18	1.08
	10b	0.30	0.16	1.14
	11a	0.44	0.30	1.3
	11c	0.39	0.31	0.89
	12c	0.27	0.17	0.88
	13a	0.29	0.40	-0.4
Av.		0.31	0.245	
July	2a	0.22	0.20	0.37
	2d	0.20	0.21	0.17
	8a	0.26	0.35	-0.31
	8c	0.23	0.25	0.14
	11c	0.39	0.31	0.89
	15a	0.16	0.16	0.17
	T2a	0.22	0.34	-0.53
Av.		0.24	0.26	

% Increase in spacing between rest and isometric contraction.

The reflections also give some information about the behavior of the pitch of the actin helix. The 51 and 59 Å layer lines can be thought of as being spaced at equal distances (in reciprocal space) on either side of an absent reflection occurring at twice the axial repeat (i.e., $2 \times 27.3 = 54.6$ Å), which enables a value for this repeat to be determined from the layer-line measurements. They are separated from this center point by a distance whose reciprocal corresponds to the pitch of the long pitch helix (approximately 720 Å) followed by each of the two chains of monomers forming the actin filament. However, measurements of the spacing between these two fairly close-spaced reflections is necessarily subject to a much larger percentage error than the spacing of the reflections themselves. We do not see any evidence for very large changes in the pitch of the actin helix, i.e., changes many times larger than the ~0.3% change in the spacing of the measured reflections. In terms of the ~360 Å helical repeat of the two-chain actin structure, the changes we measure correspond to shifts of a few angstroms only (i.e., up to about 1%) with a scatter that obscures the direction of any systematic change. The patterns show clearly, however, that

changes from a pitch of ~360 Å to 370 or 380 Å during contraction can be excluded, at least for the major part of the diffracting structure.

In the case of the myosin axial reflections, the increase in spacing during isometric contraction of the 143 Å cross-bridge repeat has been well documented for many years, although its cause has remained unclear. We have observed this change again in the present work, as can be seen very clearly in Fig. 1, and note that the very strong myosin meridional reflections at approximately 28.6 Å (5th order of 143 Å) changes by an approximately equivalent amount, as do other strong myosin meridionals (Table 3). For reasons that we will discuss later, we believe that at least a large part of this substantial change (1.5%) is associated with events other than tension-induced stretch of the thick filament backbone.

The question then naturally arises whether the changes in the axial periodicity of the actin filaments arise from the effect of tension on the actin filaments or from other structural changes in actin associated either with the activation mechanism in the thin filaments or with the attachment of myosin heads, or with both types of interaction. We have not yet been able to carry out a sufficient number of experiments on semitendinous muscles at zero overlap to settle this question (although the changes we see are certainly greatly reduced), but experiments at different levels of tension produced by steady shortening or lengthening indicate that much of the actin stretch is tension-related; these experiments are described below.

Changes in actin and myosin periodicities during slow stretch and moderate speed shortening

Stretch

When these muscles were stretched at a constant speed of approximately 0.1 muscle lengths per second during tetanic stimulation, the tension in them rose to 40–60% above the isometric value and remained at that level during the period of stretch, usually 300–400 ms. By repeating such a stretch (a length change of about 3%) during 10–12 separate contractions with the muscle being allowed to return to its original length each time during the recovery period and alternating with isometric contractions, it was possible to make detailed recordings of the changes in pattern. In these experiments, the spacing measurements were in principle somewhat easier to make, because the extent of overlap of the ~27.3 Å actin reflection and the possible 16th order myosin reflection (at ~27.16 Å) was approximately constant between the isometric contraction and the slow stretch. In practice, the "shoulder" on the actin meridional reflection was often more prominent in the stretched patterns, perhaps because the intensity of the main actin reflection was reduced. Our measurements of the spacing change using the centroid method would therefore again tend to underestimate its true value, and the figures we give are again conservative ones. The 59 and 51 Å actin off-meridional reflections were relatively free of interference because of the large decrease in intensity of all the off-meridional myosin reflections during contraction.

When the tension was increased in this way (usually by about 40–60%), a further increase in spacing of the ~27.2 Å actin meridional reflection was observed (Table 4). The changes were small, generally in the range of 0.10–0.15% at the tensions we employed, but they were very consistent and, when scaled up by linear extrapolation to the values expected for a 100% tension increase over isometric, gave an average increase of about 0.23%. This is less than the initial isometric increase (0.3%), but we cannot tell at present whether the difference represents an activation component or whether the extension is nonlinear with tension.

Similar changes can be seen in the 59 and 51 Å actin layer-line reflections. As mentioned previously, measurements of the spacing of these reflections is less accurate than of the meridionals, but providing that corresponding regions of a layer line are used, consistent results are obtained (Table 5). The two layer lines appear to increase in spacing by approximately equal percentage amounts and give an increase in the axial period by about 0.19% when scaled up to a 100% tension increase. Changes in the pitch of the actin helix are

TABLE 3 Myosin spacing changes, isometrics

	Expt.	143 Å	72 Å	39 Å	28 Å
December	8a	1.7%	1.75%	1.90%	1.48%
	9c	1.63	1.57	1.78	1.38
	10b	1.65	1.80	1.89	1.47
	11a	1.20	1.32	1.9	1.38
	12c	1.66	1.77	1.83	1.37
	13a	1.65	1.81	1.9	1.48
Av.		1.58	1.67	1.87	1.43
July	2b	1.59%	1.59%	1.85%	1.51%
	2d		1.22	1.61	1.24
	8a	1.46	1.12	1.56	1.22
	8c	1.29	1.01	1.29	0.93
	10d	1.20	1.25	1.37	1.23
	11c	1.43	1.22	1.59	1.16
	15a	1.51	1.22	1.64	1.29
Av.		1.41	1.23	1.56	1.22

% Increase in axial spacings between rest and isometric contraction for some of stronger myosin meridional reflections.

TABLE 4 Slow stretch: 27 vs. 51/59 changes

	27 Direct (before scaling to T_0)	51/59 (before scaling to T_0)	ΔT	27 Scaled	51/59 Scaled
9b	0.11	0.10	0.59	0.19	0.17
10a	0.146	0.13	0.56	0.26	0.23
11b	0.10	0.086	0.50	0.20	0.17
11c	0.15	0.094	0.52	0.29	0.18
12a	0.125	0.13	0.66	0.19	0.19
12b	0.18	0.13	0.57	0.31	0.23
13b	0.13	0.07	0.60	0.22	0.12
13c	0.10	0.11	0.47	0.21	0.23
				Av. 0.23	Av. 0.19
19a	0.105	0.106	0.49	0.21	0.22
20a	0.108	0.012	0.47	0.23	0.25

% Increase in axial spacing between isometric contraction (T_0) and stretch (tension increase ΔT as fraction of T_0).

TABLE 5 Slow stretch: 51/59 layer line changes (scaled)

	Expt.	Δ51	Δ59
December	9b	0.16	0.17
	10a	0.26	0.19
	11b	0.16	0.19
	11c	0.21	0.18
	12a	0.18	0.21
	12b	0.23	0.23
	13b	0.14	0.10
	13c	0.27	0.20
Av.		0.20	0.18
July	19a	0.21	0.23
	20a	0.25	0.25

% Increase in axial spacing of actin off-meridional layer-line reflections between isometric contraction and slow stretch (scaled to tension change of T_0).

of a similar magnitude, but cannot be estimated with great reliability from these reflections.

We have also made some measurements of the change in spacing of the second order actin meridional reflection at ~13.7 Å during stretch (and also between rest and isometric contraction). However, this reflection occurs at a greater distance from the sphere of reflection of a normally oriented muscle, and its apparent position is therefore much more affected by changes in angular spread than the other reflections we have studied. Also, because of camera limitations, we could only record this reflection on one side of the origin. The spacing changes we see are similar to those in the 27.3 Å actin reflection, but with a larger scatter. During isometric contraction, the 13.7 Å reflection increases in intensity between 1.2 and 1.5 times (e.g., Fig. 3).

The axial periodicity in the myosin filaments also shows a significant increase during these slow stretches (Table 6) by an amount quite similar to that seen in actin. Scaled up to a 100% tension increase, the change represented an average increase of about 0.2%, in addition to the approximately 1.5% increase present in isometric contraction.

Shortening

To give rise to sizable decreases of tension below the isometric value, velocities of shortening need to be much larger than those used during stretch, and the values we used were in the range 0.7–1.5 muscle lengths per second, giving tensions less than 50% of the isometric value during exposures of 20–50 ms. Satisfactory patterns could be collected with 20–50 repetitions of the complete cycle, with isometric contractions alternating with those in which shortening took place. Most of the experiments yielded uniform results and showed consistent decreases in both the actin and myosin axial periodicities, but in two otherwise apparently normal runs practically no changes were seen in either periodicity. Although the muscles that we used were seen to contract evenly and without change in orientation, as far as could be determined by eye, when they were initially mounted, it is possible that nonuniformities developed occasionally in the course of these shortening experiments and led to the anomalous results. We therefore discarded the results of these two runs in the final average.

In the rest of the moderate speed shortening runs, the ~27.3 Å actin meridional reflection decreased in spacing toward its resting value, the decrease corresponding to an average length change of approximately 0.22% when scaled up to a 100% tension change (Table 7). Again, this is significantly less than the ~0.3% change between zero tension at rest, and isometric contraction, but again we cannot tell at present whether this is caused by the presence of an activation-related component in the overall change or whether there might be a relatively larger change at very low levels of tension. The actin layer line reflections at 59 and 51 Å again changed by approximately similar amounts (Table 8), giving average changes in the axial repeat of approximately 0.24% when scaled to 100% tension change. Again, values for the change in the pitch of the actin helix show a considerable scatter, with changes considerably less than 1% in nearly all cases.

The meridional myosin reflections decreased in spacing during shortening by amounts that ranged from 0.2 to 0.5% when scaled to a tension change of 100%. We have observed previously (Huxley et al. 1989) that during more extended periods of rapid shortening, decreases of up to 0.7% took place, but that these were delayed some 20–30 ms behind the onset of the shortening period. In the present experiments, we were only able to observe the average change in spacing during the whole shortening period. We believe that the larger values of the change are associated with cross-bridge

TABLE 6 Slow stretches: myosin spacing changes (after scaling to T_0)

	Expt.	143	72	48	39	28
December	9b		0.16	0.20	0.17	0.16
	10a	0.27	0.20	0.23	0.19	0.16
	11b	0.19	0.04	0.17	0.16	0.19
	11c	0.15	0.11	0.15	0.11	0.17
	12a	0.23	0.17	0.18	0.14	0.20
	12b	0.22	0.18	0.22	0.13	0.14
	13b	0.20	0.18	0.23	0.15	0.17
	13c	0.26	0.20	0.22	0.23	0.18
Av.		0.22%	0.15%	0.20%	0.16%	0.17%
July	19a		0.30	0.27	0.26	0.25
	20a		0.13	0.20	0.17	0.16

% Increase in spacing of some of stronger myosin meridional reflections between isometric contraction and stretch.

TABLE 7 Fast shortening

	27 Direct (before scaling to T_0)	27 from 51/59 (before scaling to T_0)	27 Direct scaled	27 from 51/59 scaled	Tension (fraction of T_0)
2E	0.218	0.208	0.35	0.33	0.369
5B	0.082	0.109	0.14	0.18	0.402
7C	0.21	0.265	0.27	0.34	0.233
9A	0.18	0.238	0.22	0.29	0.187
13C	0.109	0.084	0.20	0.16	0.467
13E	0.214	0.152	0.26	0.18	0.18
14A	0.194	0.214	0.23	0.26	0.16
16A	0.16	0.166	0.17	0.18	0.09
17A	0.105	0.19	0.11	0.21	0.08
Av.			0.22	0.24	

% Decrease in actin axial repeat between isometric contraction and rapid shortening from direct measurements of 27 Å meridional reflection and from measurements of off-meridional layer lines.

TABLE 8 Fast shortening: 51/59 layer line changes (scaled)

Expt.	Δ51	Δ59
2E	0.34	0.32
5B	0.19	0.17
7C	0.37	0.31
9Aa	0.37	0.20
13C	0.10	0.22
13E	0.19	0.18
14A	0.27	0.24
16A	0.21	0.15
17A	0.24	0.16
Av.	0.25	0.22

% Decrease in axial spacing of actin off-meridional layer-line reflections between isometric contraction and rapid shortening, scaled up to 100% tension change.

detachment rather than elastic changes in the backbone of the myosin filaments and that the values that we measure in the current experiments probably represent a variable mixture of the two effects. We therefore do not believe that they provide any independent evidence for an elastic change, but they are obviously consistent with an elastic change of 0.2% for a 100% change in tension from the isometric value.

Changes associated with a very rapid release

We have carried out only a small number of such experiments so far because of time limitations, but have found it quite feasible to obtain informative two-dimensional x-ray diagrams during 2 ms time slots occurring 2 or 3 ms after a quick release (of a whole muscle) from an isometric contraction when the tension fell to zero in less than 1 ms and remained at zero during the exposure (release of about 2%). The total exposure times that we have obtained have been insufficient to allow very accurate spacing measurements of the 27.3 Å meridional actin reflection, but we have been able to make adequate measurements of the 59 and 51 Å off-meridional actin reflections (following the same protocol that we used in the other experiments described above, when spacing changes of a similar magnitude to those in the 27.3 Å actin reflection were observed) and of the 71.5 and 143 Å myosin meridional reflections. In the technically best experiment that we have done so far, the 59 and 51 Å actin reflections decreased in spacing by 0.25 and 0.345%, respectively, corresponding to a change in axial periodicity of 0.29% accompanying the quick release. At the same time, the 71.5 and 143 Å myosin reflections each decreased in spacing by approximately 0.1%. Thus, the changes we observe take place very rapidly.

DISCUSSION

The results that we have described show that there is a significant degree of extensibility in the actin filaments in muscle, and probably in the myosin filaments too. Between rest and isometric contraction, the observed actin axial repeat increases by more than 0.3%, and in a fully active muscle moderate changes in the applied tension produce further alterations in the axial repeat that scale up to an additional change of about 0.22% for a 100% isometric tension change. The situation in the myosin filaments is more complicated because of the relatively large (~1.5%) spacing increase on activation, probably mainly associated with cross-bridge attachment rather than actual tension development, but a further increase can be produced when increased tension is applied to a muscle (by a slow stretch), scaling up to a change of about 0.2% for an increase of 100% of isometric tension. To a first approximation, these results imply that when the load on an isometrically contracting muscle is suddenly removed, at least 20 Å per half-sarcomere of the resultant length change could be accounted for by decreases in length of the actin and myosin filaments themselves. From the few quick release experiments that we have carried out, these changes appear to be completed within 2 or 3 ms or less and, because the extent of the length change would amount, for example, to only 0.11 Å for each actin monomer, the response could very well be a purely elastic one. Such a length change in the filaments will introduce considerable complications into the interpretation of detailed mechanical measurements on muscle fibers, where it has usually been thought that most of the elasticity resides in the cross-bridges (Ford et al. 1981).

The observation of a 0.2% (or greater) change in the actin axial periodicity does not necessarily mean that all of the actin periods change by the same amount. The spacing change is considerably less than the width of the reflections

themselves, and a mixed population of changes ranging from 0 to 0.4% would not be distinguishable from a uniform one in our present data. One would expect that the tension on an actin filament in the overlap zone would increase linearly from zero at the free end to a maximum at the end of the A-band, and then have that same value through the I-band and up to the Z-line. At a sarcomere length of 2.3 μ, with actin filaments 1.0-μ-long and myosin filaments 1.6-μ-long, giving an I-band length of approximately 0.35 μ per half-sarcomere, 35% of the actin would show the larger spacing change and 65% would give rise to a reflection showing half of the spacing shift. If actin monomers in the overlap and nonoverlap regions contributed equally to the intensities in the two parts of the reflection, the average spacing change measured would be only 0.675 of the actual extension in the I-band region. However, the total change in length of the actin filament would still be 0.2% of its original value. This would no longer be the case, however, if the actin monomers in the overlap zone were to diffract more strongly than in the I-band. If their individual intensity contributions were doubled, e.g., by the attachment of myosin heads, it can readily be calculated that the average spacing change measured would be 0.606 of the maximum extension, and the overall change in length of the actin filament would be approximately 0.22% for a 0.2% apparent spacing change. The actin filament length change would reach a maximum of approximately 0.27% for a measured change of 0.2% if all the scattering were from the overlap region. Thus, the total change in actin filament length that will be experienced by mechanical experiments is likely to be somewhat larger, if anything, than the minimum value of about 0.2% that we have estimated from the x-ray measurements.

Similarly, if the 0.2% additional increase in the myosin periodicity associated with doubling the load on the muscle arises from extensibility of the myosin filament backbone, then the extension would also be expected to be nonuniform, and to vary from a maximum of 0.4% near the center of the A-band where the load on the myosin filaments is a maximum to a very small value at the end of the A-band where only the last row of cross-bridges are contributing. This is the region where the load on the actin filaments is maximal, so the mechanical situation is a very complicated one whose full analysis lies outside the scope of this paper. However, it is clear that when the load on a muscle is quickly reduced to zero, the actin and myosin filaments in the A-band region must each decrease in length by approximately 0.2%, and the parts of the actin filaments in the I-band must decrease by a similar proportion. If the total elastic change were 40 Å per half-sarcomere, then only an average of about 20 Å could be attributed to an elastic response in the cross-bridges. This could imply that the cross-bridge steps were of a similar magnitude and that rapid tension recovery involved more than one step.

The figures we have used in the above discussion are conservative ones, based on the changes we see when changing the load on a muscle, rather than the larger ones (0.3% or more in the actin filaments) that occur when going from the relaxed state to full isometric tension. From our own results alone, these latter values could contain a component associated with activation itself, e.g., an extension by 0.1% of the actin filaments, which might not contribute to the observed extensibility of a fully active muscle. However, measurements of the length change in the actin filaments when activated at zero overlap and reported in the accompanying paper (Wakabayashi et al., 1994) indicate that activation may even produce a change in the reverse direction, i.e., a shortening of the actin filaments, in which case the load-associated extension would need to be even larger than the apparent value. So, it is possible that 0.3% or more is a true figure for the load-associated extension of the actin filaments when going from zero load to maximum, and that the elasticity is nonlinear, and the first 0.1% or more has already taken place at very low loads. The measurements we have been able to do so far on muscles released to zero tension are insufficient to resolve this problem. In this connection, however, it is worth mentioning that in a limited number of experiments in which we have compared the actin spacings in resting muscles at rest length with those from muscles stretched so that they were developing significant resting tension (about 20% P_0), we consistently observed an extension of about 0.1%. If this represents an initial extension of the actin filaments at relatively low levels of tension, it would also of course require that some of the resting tension is indeed carried by the actin filaments and that some cytoskeletal connection links their ends, directly or indirectly, across the center of the A-band.

The behavior of the helical pitch of the actin filaments during activation and tension development is a matter of some interest, because well known structural changes do take place in the actin-troponin-tropomyosin complex during activation. It is difficult to measure this pitch very accurately, because the value depends on the separation of the 59 and 51 Å actin reflections from each other, so that measurement errors are at least 6 times larger than in absolute measurements of the 59 and 51 Å reflections themselves, made from one side of the pattern to the other. The different shape and radial position of these reflections also makes measurements of their separation more problematical. Our measurements show a considerable scatter and give both positive and negative values for the changes in pitch during activation and during changes of load. Therefore, we have not listed any average values, but individual ones lie within the range of approximately $\pm 1\%$ or less, with the resting value of the helical repeat being close to 360 Å, close to that derived from previous measurements of actin second layer line spacings (Kress et al., 1986).

The average value of the percentage changes in the 51 and 59 Å layer line spacings has to be approximately equal to the percentage change in the actin ~27 Å meridional reflection. Given an increase of 0.3% in the 27 Å spacing, then obviously in principle both the 51 and 59 Å could each also increase by 0.3%, in which case the pitch of the helix would increase by 0.3%. However, a constant value of the 59 Å spacing and an increase by 0.56% in the 51 Å spacing would

also be compatible with an increase in the meridional 27 Å spacing by 0.3%, as would a constant 51 Å spacing and an increase by 0.54% in the 59 Å spacing. In these cases, there would need to be a change of 4% in the pitch of the helix, an increase in the first case and a decrease in the second. The measured range of values is much less than this, because the 59 and 51 Å reflections always show somewhat similar spacing changes, both in the same direction. As we have mentioned earlier, our measurements of these off-meridional layer-line spacings are inherently less reliable than those of the meridional 27 Å reflection that provide the stronger evidence of the extent of the change. We regard the layer-line measurements as supportive evidence that also serves as an indicator that only relatively small changes in helical pitch occur under the conditions we have studied.

The changes in the axial repeat of the myosin reflections produced by increasing the load on an active muscle are very much smaller than those associated with the change between rest and isometric contraction (0.2% vs. ~1.5% for a 100% tension change). This supports our belief that the latter change arises predominantly as a consequence of the structural changes that are a precondition of tension development, rather than as a consequence of tension development itself. During activation the increase in spacing precedes the rise in tension (Huxley 1979), and when the tension on an active muscle is decreased substantially to allow rapid shortening to take place, there is a delayed decrease in spacing by as much as 0.6% or more (Huxley et al. 1989), i.e., by a much larger amount than the change seen here at much shorter times in the quick release experiments. Thus, these larger spacing changes in the myosin reflections are not caused by a purely elastic response to changes in tension. Indeed, a spacing increase of about 1% is also seen when a muscle goes into rigor, even in the case of a frog semitendinosus muscle stretched so that the actin and myosin filaments no longer overlap (Haselgrove 1975). In this latter case, the regular helical arrangement of cross-bridges around the thick filament backbone is lost when ATP is no longer present, and in electron micrographs they appear to be splayed further out in an irregular way, as compared with the relaxed arrangement (Padron and Craig, 1989). We therefore suspect that it is a major change in the radial position of the cross-bridges that is responsible for the larger changes in spacing of the myosin axial repeat, and that this spacing change accompanies attachment to actin during activation and rigor at normal degrees of overlap. The rapid elastic changes in a quick release take place too rapidly, it is believed, for significant overall cross-bridge detachment to be involved, and so the changes in spacing accompanying radial position changes would not occur, only the elastic ones.

If we treat the actin filaments and myosin filaments as solid cylinders of appropriate diameter (taken as ~80 and 130 Å), we can derive approximate values for their effective moduli of elasticity. Using a value of 3 kg/cm^2 for isometric tension and assuming that the myofilaments occupy 80% of the cross section of a muscle and that the spacing between myosin filaments is approximately 40 nm, it can be calculated readily that the actin filaments and the myosin filament backbones occupy 5.9 and 7.66%, respectively, of the cross section area of a muscle and that an extension of 0.2% under isometric tension would imply elastic moduli of approximately 2.5×10^9 N/m^2 and 1.9×10^9 N/m^2, respectively. These seem to be quite consistent with measured values for other biological materials, e.g., 2.6×10^9 N/m^2 for actin (Gittes et al., 1993), $5–10 \times 10^9$ N/m^2 for silk, 4×10^9 N/m^2 for keratin, and up to 2.5×10^9 N/m^2 for collagen (Wainwright et al., 1976).

As can be seen from Tables 3 and 8, the change in myosin periodicity upon contraction has slightly different values at the different myosin meridional reflections. These reflections exhibit considerable fine structure, a consequence of the complexities of the A-band structure itself (Huxley and Brown, 1967; Haselgrove, 1975; Sjostrom and Squire, 1977). When the substructure of the reflections is not resolved, sampling effects can give rise to small departures from integral order of a ~429 Å repeat, and the effects we see probably arise from the same mechanism. Analysis of these phenomena lie outside the scope of this paper and would require much higher resolution data than can be obtained at present.

A striking feature of the contracting patterns is the large increase in intensity of both the 27 Å actin meridional reflection and the neighboring 5th order of the 143 Å myosin repeat (15th order of 429 Å helical repeat, so referred to as M15) by amounts between 1.4 and 2 times. This would indicate a regular axial alignment of cross-bridges with the actin monomer repeat, implying a restricted range of axial displacements or tilts, at least in one population of attached cross-bridges. However, the smaller intensity changes in the off-meridional actin layer-lines indicate less precise labeling of the actin helical repeat and imply more azimuthal flexibility of the attached head-actin monomer system.

The most immediate tasks are now to investigate in more detail the behavior of the actin patterns at different degrees of filament overlap, particularly at nonoverlap, and to collect more data on the changes occurring upon release to zero tension. However, our present data under conditions of full overlap do establish that the spacing changes are quite significant in practical terms.

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