Effects of Pressure on Equatorial X-ray Fiber Diffraction from Skeletal Muscle Fibers

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ABSTRACT When skeletal muscle fibers are subjected to a hydrostatic pressure of 10 MPa (100 atmospheres), reversible changes in tension occur. Passive tension from relaxed muscle is unaffected, rigor tension rises, and active tension falls. The effects of pressure on muscle structure are unknown: therefore a pressure-resistant cell for x-ray diffraction has been built, and this paper reports the first study of the low-angle equatorial patterns of pressurized relaxed, rigor, and active muscle fibers, with direct comparisons from the same chemically skinned rabbit psoas muscle fibers at 0.1 and 10 MPa.

Relaxed and rigor fibers show little change in the intensity of the equatorial reflections when pressurized to 10 MPa, but there is a small, reversible expansion of the lattice of 0.7 and 0.4%, respectively. This shows that the order and stability of the myofilament lattice is undisturbed by this pressure. The rise in rigor tension under pressure is thus probably due to axial shortening of one or more components of the sarcomere.

Initial results from active fibers at 0.1 MPa show that when phosphate is added the lattice spacing and equatorial intensities change toward their relaxed values. This indicates cross-bridge detachment, as expected from the reduction in tension that phosphate induces. 10 MPa in the presence of phosphate at 11°C causes tension to fall by a further 12%, but no change is detected in the relative intensity of the reflections, only a small increase in lattice spacing. Thus pressure appears to increase the proportion of attached cross-bridges in a low-force state.

INTRODUCTION

In living muscle, active tetanic tension is reduced by pressure (1, 2). The same effect occurs with skinned muscle fibers bathed in an activating solution under isometric conditions (3), showing that pressure acts directly on the contractile proteins. Furthermore, tension transients following release of pressure demonstrate that the effects are on the cycling cross-bridges rather than a passive structural element (4). By contrast, the tension in slightly stretched rigor muscle is increased by pressure, in common with the behavior of many other materials (5). We are interested in understanding the structural basis of these different effects of pressure on muscle, because they may help in understanding the mechanism of contraction itself.

In general, pressure acts by changing the equilibrium position in reactions in which a change in volume occurs. Pressure may perturb a reaction which it is difficult to perturb by any other means, and thereby reveal new steps in a reaction pathway and clarify the overall mechanism. Solution studies on actin and myosin during ATPase activity indicate that the conversion between two attached states is a pressure-sensitive reaction (6). Myosin heads are found to bind to actin in two steps (7; reviewed in Ref. 6), the first of which is a rapid equilibrium, with a relatively weak equilibrium constant of about 10^4 M^{-1}. The second step, an isomerization, has an equilibrium constant that increases as the myosin head proceeds along the pathway of ATP hydrolysis. It is this step which is inhibited by pressure (8).

There is good evidence from mechanical studies for two types of actin-myosin interaction in muscle fibers: following the detached state there is an initial attached state that develops little force, and this is followed by a second, force-holding state (reviewed in Refs. 9–11). By comparison with the biochemical data, the low-force state in muscle could be associated with the weakly bound, initial state in the ATPase cycle. In this interpretation the drop in force in pressurized muscle would be due to an accumulation of this intermediate caused by the pressure sensitivity of its conversion to the tighter binding state associated with force production.

Force in active muscle is markedly reduced by phosphate (12, 13), and in the presence of phosphate force is more sensitive to pressure (13). Moreover, phosphate affects the reciprocal relaxation times of tension recovery following rapid pressure release (4). These and other data are consistent with a scheme in which the pressure-sensitive isomerization is in series with phosphate release as follows.

$$\text{Detached} \leftrightarrow \underset{\text{force}}{\text{low}} \overset{\Delta V}{\longleftrightarrow} \underset{\text{force}}{\text{high}} \overset{\text{phosphate}}{\rightleftharpoons} \underset{\text{force}}{\text{high}} \leftrightarrow \text{detached} \quad (1)$$

It is implicit in this scheme that phosphate increases the proportion of cross-bridges involved in the force-generating transition that is inhibited by pressure. By examining the structure of pressurized active muscle by x-ray diffraction, especially with phosphate present, we can hope to learn about the structure of the low-force state.

There have been few x-ray diffraction studies of biological materials under pressure (14–16), and none on muscle. The 0.15-nm x-rays, that are the best compromise between intensity of diffraction from biopolymers and absorption by the

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aqueous medium, are strongly absorbed by the diamond or sapphire windows conventionally used to resist pressure in cells designed for observation in the visible spectrum. Beryllium absorbs less, but is opaque, precluding simultaneous optical observation, and it is very toxic. We have therefore constructed a cell that uses plastic windows (17), and in this paper we report our initial work on muscle under pressure.

As a first step the equatorial reflections have been examined, as they can show whether pressure affects the force in rigor and active muscle through modifying lattice spacing or the fraction of attached cross-bridges. The sensitivity of the equatorial pattern to cross-bridge detachment is shown by the reversion of the pattern toward that of the relaxed state when active muscle shortens rapidly (18), the change correlating with a reduction in stiffness from the isometric value (19). Also, when skinned fibers are partially activated, the intensities of the 1,0 and 1,1 reflections change linearly with tension as more cross-bridges attach (20). A preliminary survey of the results was presented at a European Muscle Club Meeting (21).

MATERIALS AND METHODS

Muscle specimens

Bundles of prerigor rabbit psoas muscle, with 1-mm diameter and tied onto sticks, were chemically skinned for 2 h at 4°C in a solution comprising 70 mM propionic acid, 8 mM magnesium acetate, 5 mM EGTA, 7 mM Na_2ATP, 6 mM imidazole, adjusted to pH 6.80 at 20°C with KOH, and supplemented with 0.5% Brij 58. This medium was replaced with the same medium (lacking Brij) mixed 1:1 v/v with glycerol, and, after 24 h at 4°C, stored for upto 10 weeks at −20°C. X-ray patterns of this preparation in relaxed conditions (Table 1) show a high 1,0/1,1 intensity ratio (see Figs. 1 (*inset*) and 3 *a*), and strong myosin layer lines. Smaller, ribbon-shaped bundles, containing about 6–10 fibers, were prepared from this material in relaxing buffer (Table 1), and attached to the specimen holder of the pressure cell (see below) at a sarcomere length, measured by laser diffraction, of 2.0–2.4 μm (i.e., full filament overlap). When required, the rigor state was induced by a thorough exchange of rigor solution (Table 1) in the cell; it generally took about 15 min for the equatorial reflections to change fully from the relaxed to the rigor values. Sarcomere length was remeasured at the end of the experiment. In some cases the muscle, still in the holder, was fixed for light microscopy by adding 2.5% glutaraldehyde to the final solution used.

TABLE 1 Composition of experimental solutions

	Relaxing solution	Pre-activating solution	Activating solution	Rigor solution
Imidazole	50	50	50	50
Potassium phosphate			20	
Magnesium acetate	13	12	12	
Na_2-ATP	10	10	10	
Potassium propionate	61	106	23	175
EGTA	15	0.5		
Ca-EGTA			15	

Concentrations given in millimolar; solutions prepared from concentrated stocks that were adjusted to pH 7 with KOH or acetic acid; pH finally adjusted to be 7.0 at the temperature of use using KOH. The concentration of magnesium acetate was calculated to give a free Mg^{2+} concentration of 2 mM, of Ca-EGTA to give a free Ca^{2+} concentration of 30 μM, and of propionate to give a total ionic strength of 200 mM, using a computer program from Dr. Y. E. Goldman. When phosphate was omitted from the activating solution, propionate was increased to 62 mM.

To avoid the risk of ATP depletion causing rigor within the active preparation, the muscle fibers were bathed in several milliliters containing a high concentration of ATP (10 mM), as this should give a better resistance to local depletion than, for instance, a combination of 1 mM ATP plus 10 mM phosphocreatine + creatine kinase. Fortune et al. (13) showed that addition of such a backup to the present activating solution has no effect on the tension responses of single fibers. As a further precaution to ensure an adequate supply of ATP to the muscle ribbon during activation, the pressure cell windows were kept about 1.7 mm apart. The x-ray patterns provide clear evidence that the strategy was successful: whereas for rigor the 1,1/1,0 intensity ratio was about 3.3, for active muscle in the absence of phosphate, it was about 1.3 (see Fig. 5), similar to the value of 1.6 found for this muscle in similar conditions by Brenner and Yu (20).

Pressure cell

The pressure cell (17) was machined from a block of stainless steel. The removable specimen holder was a ring with two lengths of wire forming curved chords across it. The specimen was attached, using nitrocellulose dissolved in acetone, between the points of closest approach of the wires. The specimen holder was inserted into the cell, taking care that the fiber axis was horizontal, so that it coincided with the long axis of the x-ray beam profile and was at right angles to the axis of the x-ray detector. The x-ray beam passed through the block via a sheet of Kapton V (Du Pont, Kensulat Ltd., UK), nominally 0.125-mm thick, on either side of the muscle ribbon. These sheets were each stretched over a domed window holder containing a 3-mm diameter aperture on the optical axis, the dome shape serving both to reduce window distortion and to enhance the supply of ATP during muscle activity. Pressure (10 MPa) was applied by pumping water (with a Gilson 302 high-performance liquid chromatography pump with 802C control unit) into a second chamber separated from the specimen cell by a deformable membrane, and released by a valve (22): both devices could be controlled remotely. With the cell mounted on the x-ray beam line, solutions were exchanged manually at atmospheric pressure using syringes plugged into ports on the cell. The cell was mounted on a water-cooled stand which held its temperature at 11 or 20 ± 1°C as monitored by a thermocouple inserted near the specimen chamber.

X-ray measurements

Low-angle equatorial x-ray diffraction patterns were produced on the small angle scattering camera on line 2.1 at the SERC Daresbury Laboratory Synchrotron Radiation Source. The camera was operated at 0.154 nm with a specimen-to-detector distance of about 4.5 m and using in-house software for data acquisition (23). Slits after the focusing mirror were used to reduce the width of the beam to avoid scatter from the windows of the pressure cell, and a lead shutter next to the pressure cell was used to minimize x-ray dose. Both sides of the equatorial pattern were intercepted by a linear position-sensitive detector of 10-cm active length, and recorded into 512 channels. Reflection intensities were normalized to the intensity of the main beam that was measured by an ion chamber that was placed after the pressure cell in order to register the reversible drop in intensity caused by window distortion during pressurization (17). The camera constant was determined using the meridional reflections (arising from a 66.8-nm collagen repeat (24)) from a wet rat tail tendon mounted in the cell. The detector's response to a uniform field of x-rays was recorded, and used to eliminate spurious irregularities in the patterns caused by variations in detection efficiency.

The reduction of beam size, the plastic windows, and the liquid path length in the cell combined to reduce the x-ray beam intensity by a factor of at least 20. This, together with the small number of muscle fibers, resulted in an exposure time per pattern of typically 10 s at 0.1 MPa, and 20 s at 10 MPa. The longer time compensated for the reduction in count rate under pressure that was due to an increased liquid path length caused by window distortion. Typical counts in each peak were 1 to 5 × 10^4. In some experiments we recorded a series of patterns under constant conditions, in order to verify whether the preparation was structurally stable.

Data analysis

Following established procedures, x-ray patterns were normalized to main beam intensity using the OTOKO package (Koch and Bendall, EMBL, Heidelberg, Germany). The positions of the reflections on each side of the backstop were then determined using an in-house package written by Dr J. V. Seymour. This employs an iterative least squares fitting procedure in which the background is taken to be an exponential plus a second order polynomial and the reflections are taken to be Gaussians (25). For rigor and active preparations, the lattice spacing (i.e., center-to-center spacing of thick filaments) was calculated independently for the two sides of the pattern from the separation of the 1,0 and 1,1 reflections. This eliminated the need to correct for the small spatial offset between the two sides of the recorded pattern introduced by the detector electronics. For relaxed preparations, where the position of the 1,1 reflection was generally less well defined, the lattice spacing was calculated from the separation of the two 1,0 reflections which was corrected for the spatial offset by subtraction of its value as estimated by analysis of the collagen meridional pattern.

Initial analysis of intensity changes was done by inspection of difference patterns that were generated by subtracting a pattern recorded at 10 MPa from appropriate ones at 0.1 MPa, after subtracting appropriate cell blanks from each pattern. This method was chosen in preference to a comparison of the reflection intensities determined by the fitting procedure, due to the fact that slight differences in the shape of the fitted background produced spurious differences between patterns. Alternatively, the total intensity of a reflection, including the underlying background, was obtained by summation of a segment of a pattern after subtraction of the cell blank.

RESULTS

X-ray diffraction under hydrostatic pressure

X-ray data have been collected from a muscle specimen that is under a hydrostatic pressure of up to 15 MPa, by using a stainless steel cell fitted with Kapton plastic windows (17). X-ray reflections arising from spacings as small as about 1 nm can be recorded with the cell used in the present work, but because of the geometry of the pressure-resistant window holders, even the first order diffraction of visible wavelengths from the sarcomere repeat cannot emerge from the cell, so the sarcomere length cannot be continuously monitored. The two Kapton windows absorb about 20% of the beam, but they do not contribute reflections in the low-angle region. However, they do bow outward reversibly under pressure, increasing the path length through liquid and consequently reducing the count rate by a factor of about two when 10 MPa is applied. This effect can be accounted for by normalizing counts to the intensity of x-rays emerging from the cell. Although Kapton is remarkably resistant to pressure and x-rays, windows do occasionally fail, but because pressure is applied via liquid rather than gas there is no explosion hazard. Using this cell we have begun to explore whether changes of structure occur when muscle is perturbed by pressure.

Rigor muscle

The tension held by an isometric rigor fiber increases linearly with increased pressure over the range 0.1–10 MPa (5). The increase is readily reversible, and a fast pressure release (complete in about 1 ms) shows the tension change to occur in phase with the pressure change. Similar results with stretched filaments of collagen, hair, silk, nylon, and glass lead to the interpretation of a pressure-induced change in the length of an elastic element in the cross-bridges or in series with the cross-bridges (5). However, changes in lattice spacing or the fraction of attached cross-bridges could not be eliminated. Therefore we examined the influence of pressure on the equatorial reflections from muscle fibers under rigor conditions.

A bundle of muscle fibers was mounted in the cell under relaxing conditions, and the low-angle equatorial diffraction pattern was collected at 0.1 MPa. This showed the expected strong 1,0 and weaker 1,1 reflections (Fig. 1, *inset*). Following a change of the bathing medium to rigor solution, the classical reversal of the intensities of these two reflections occurred, showing that rigor had developed. When the bundle was pressurized, the diffraction pattern still showed the characteristic rigor pattern of strong 1,1 and weak 1,0 reflections (Fig. 1 *a*). A typical plot of the arithmetic difference between the high and low pressure patterns (Fig. 1 *b*) shows that only subtle changes were induced by pressure: the absence of strong positive or negative peaks indicates that there has not been a gross redistribution of mass associated with the thick and thin filaments. Because of the mismatch of the periodicities of myosin and actin in the thick and thin filaments, rigor muscle even when slack contains cross-bridges that are strained and therefore generating local positive and negative axial forces; thus it was possible that the increase in rigor force caused by pressure (5), was due to preferential dissociation of those strained bridges generating negative force. The x-ray pattern shows no evidence for this.

Some difference plots, including Fig. 1 *b*, show pairs of small positive and negative peaks, located at the positions of the reflections, with the positive peaks further from the center of the plot. This indicates that there was an increase in lattice

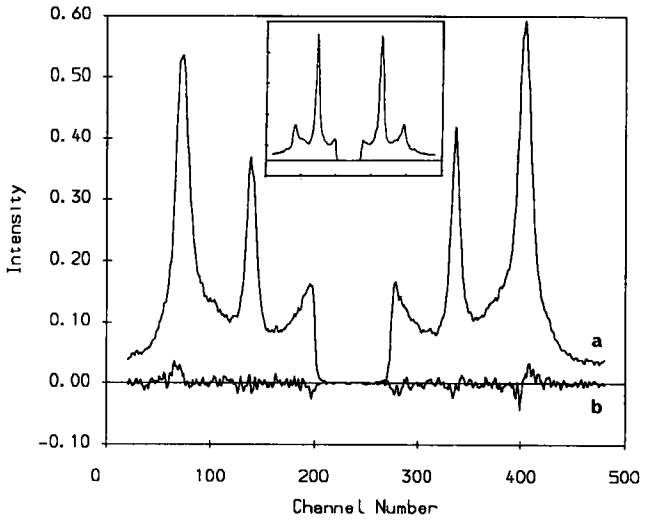

FIGURE 1 Equatorial x-ray intensities from rigor muscle. (*a*) Pattern at 10 MPa. (*b*) Pattern at 0.1 MPa minus pattern from the same specimen at 10 MPa. Inset: pattern from the same bundle in relaxing solution before irrigation with rigor solution. Temperature, 20°C; data collected for 10 s at both pressures; intensities normalized as described in Methods.

spacing under pressure. This conclusion was confirmed and quantitated by using a computed fit to locate the positions of the reflections in each of the patterns. Fig. 2 shows the result obtained in an experiment where a series of patterns was recorded during a pressurization cycle. A very small but significant increase in spacing was produced by pressure, which was reversed when pressure dropped. There was variation in the magnitude of the increase between experiments, in part because the changes were on the limit of detection, given the width of the reflections and statistical noise in the data. The rigor lattice spacing was about 46 nm, and it increased under pressure by 0.160 ± 0.041 nm (mean ± SE, $n = 8$), i.e., 0.35 ± 0.089%. The effects of high pressure on lattice spacing and reflection intensities were similar at 11 and 20°C.

Relaxed muscle

A relaxed muscle fiber either at rest length or holding a passive tension shows no measurable change in tension when exposed to pressures of up to 10 MPa (5). This property is shared by materials showing rubber-like elasticity, and shows that the elastic element holding the load in the relaxed fiber is quite different in character from that in the rigor fiber. Examination of the equatorial pattern of the relaxed fiber provides information on the effect of pressure on the myofilament lattice in the absence of stabilizing cross-bridges, and on the mass associated with the two primary filaments.

The equatorial diffraction from bundles of muscle fibers mounted at rest length under relaxing conditions showed strong 1,0 reflections and weaker, broad 1,1 reflections, as expected, and this pattern was retained under 10 MPa pressure (Fig. 3 *a*). Difference plots (Fig. 3 *b*) showed small positive peaks lying slightly further from the center of the pattern than the main reflections. As with rigor muscle, this indicates a small increase in lattice spacing under pressure, but no major change in mass distribution. Computer fitting showed that at 0.1 MPa the lattice spacing was about 51.5 nm (thus about 5.5 nm greater than in rigor) and at 10 MPa it increased by 0.35 ± 0.048 nm (mean ± SE, $n = 4$), i.e., about 0.68 ± 0.093%.

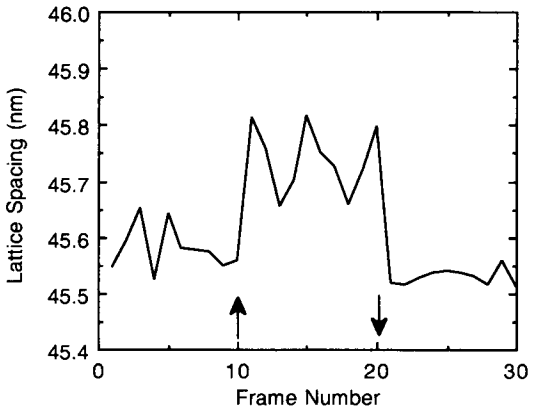

FIGURE 2 Effect of pressure on the lattice spacing in rigor muscle. Pressure was held at 10 MPa between the arrows, and 0.1 MPa elsewhere. Temperature, 20°C; 10-s time frames.

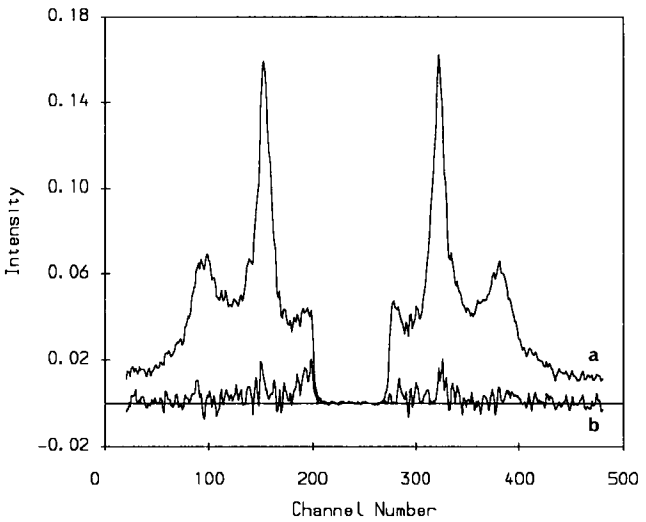

FIGURE 3 Equatorial x-ray patterns from relaxed muscle. (*a*) Pattern at 10 MPa. (*b*) Pattern at 0.1 MPa minus pattern from the same specimen at 10 MPa. Temperature, 11°C; 20-s data collection at 10 MPa, 10 s at 0.1 MPa; intensities normalized as described in Methods.

Active muscle

Initial experiments on active muscle have mostly been performed in the presence of phosphate and at 11°C. These conditions have two significant advantages. First, the activation and recording of the x-ray pattern at both high and low pressure requires the fibers to be activated for periods of up to 2 min. It is our experience that the presence of 20 mM phosphate, which reduces the isometric force developed by 40% (12, 13) and, to a lesser extent, the fiber ATPase rate (26), results in a more stable preparation. This is judged both by the stability of the observed tension and the ability to repeatedly activate a fiber to the same tension level. A background of 20 mM phosphate also reduces the sensitivity of the preparation to the effects of phosphate released by ATP hydrolysis.

The second advantage is that the fall in steady tension induced by a hydrostatic pressure of 10 MPa, 7.9 ± 2.3% in control conditions, is increased to 11.7 ± 4.6% in the presence of 20 mM phosphate (13). Thus, as mentioned in the Introduction, any pressure-induced changes in the x-ray pattern should be more apparent in the presence of phosphate. The range of observations is relatively wide but in paired measurements on the same fiber the fall in tension was consistently greater (by a factor of about 1.5) in the presence of phosphate compared to the control. These detailed observations were made at 20°C but the same phenomenon is apparent at the lower temperature used here (4).

The stability of the mechanical responses of the bundles under our experimental conditions is demonstrated in Fig. 4, which also shows a typical protocol for solution exchange. A bundle of five fibers was mounted in the apparatus described by Geeves et al. (27), except that the AKERS strain gauge described by Ranatunga et al. (5) replaced the optical strain gauge. The apparatus allows tension to be measured as the hydrostatic pressure is varied. The relaxed bundle was

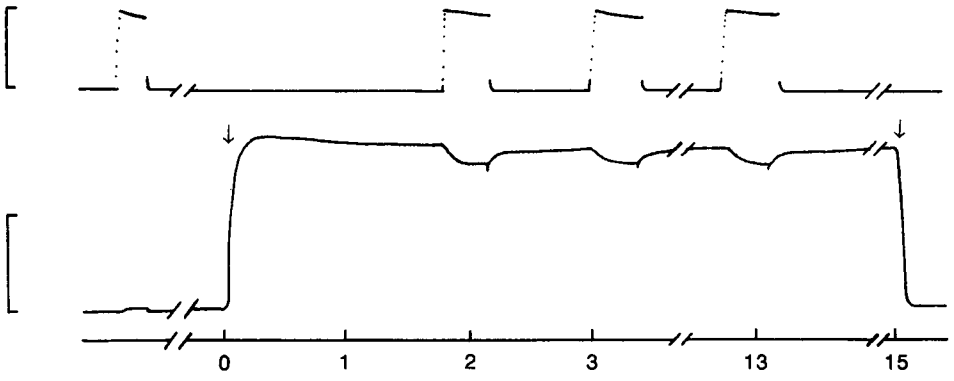

FIGURE 4 Stability of force response of a fiber bundle. The upper trace is pressure; scale bar indicates 10 MPa; middle trace is tension, scale bar is 1 mN; bottom line represents time; the tick marks are positioned at 1-min intervals. Bathing solution was changed from preactivating to activating solution (containing phosphate) at the first arrow, and to relaxing solution at the second arrow. Temperature, 12°C.

washed with preactivating solution (Table 1) to reduce the EGTA concentration, then exposed to cycles of pressure increase and release. The tension trace shows that pressure causes a small, reversible increase in signal, but this was also seen for the unloaded force transducer, and derives from a pressure sensitivity of the transducer itself. The bundle was then activated by flushing the cell with activating solution containing 20 mM phosphate. The tension rose quickly (10 s), fell by 4% over the next minute, and thereafter remained stable for 14 min, when the bundle was relaxed. During this extended period of activation, several cycles of pressure increase and release all produced similar responses in which the tension fell reversibly by 12%.

Effect of phosphate on the equatorial pattern

A series of patterns was recorded at 0.1 MPa from small muscle bundles at about 11°C, activated first in the presence of phosphate, then in its absence, then in its presence once again. It was generally the case (Fig. 5) that the presence of phosphate changed the positions and intensities of the 1,1 and 1,0 reflections, as can be seen by comparing Fig. 5, *a* and *b*. The difference plot (Fig. 5 *c*) shows prominent positive and negative peaks, with the negative peaks closer to the origin, indicating an increase in lattice spacing in the presence of phosphate, while the predominance of the negative peak under the 1,0 reflection and the positive peak under the 1,1 reflection indicates that the 1,0 reflection is strengthened and the 1,1 weakened in the presence of phosphate.

Quantitative analysis of a series of patterns (Fig. 6) indicates that in the presence of phosphate both the 1,1/1,0 intensity ratio and the lattice spacing are intermediate between the values for relaxed and fully active muscle. It is also apparent that the changes due to activation and to phosphate are reversible. Both of the changes in the pattern induced by phosphate indicate that the decrease in tension it causes is associated with a reduction in the fraction of attached cross-bridges (20, 28), in agreement with stiffness data (29). In one experiment, the effect of added phosphate at 10 MPa was no

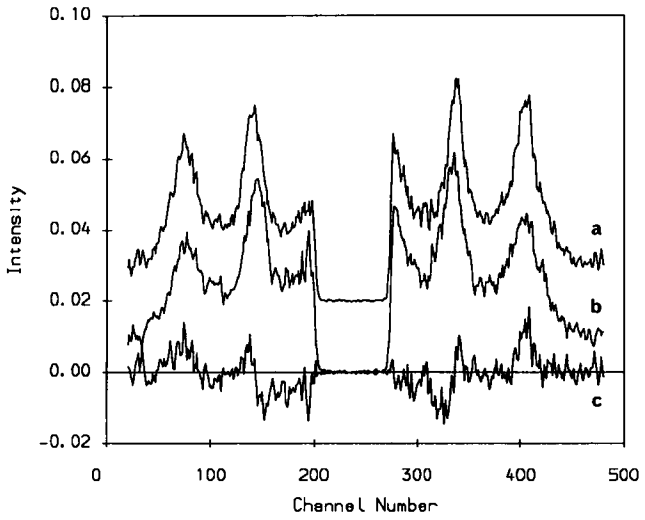

FIGURE 5 Effect of phosphate on the equatorial pattern from active muscle. (*a*) Active with no added phosphate. The pattern has been displaced along the intensity axis by 0.02 intensity units to avoid overlap. (*b*) 20 mM phosphate present in the activating solution. (*c*) Pattern *a* minus pattern *b*. Temperature, 11°C; 10-s count periods; intensities were normalized as described in Methods.

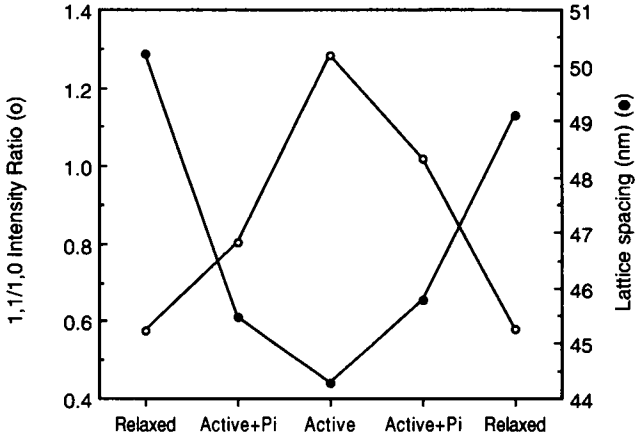

FIGURE 6 Effect of phosphate on the lattice spacing (●) and ratio of intensities of the 1,1 and 1,0 reflections (○) of active muscle. A bundle of fibers was taken through the series of states indicated on the abscissa, at 0.1 MPa and 11°C, and patterns were recorded for 10 s each. Lattice spacing was calculated from the computed best fit for the position of the 1,0 reflections in each pattern. Intensities of the reflections were obtained from prints of the patterns by drawing in the background under the peaks and then measuring their areas. The ratio plotted is the average of the values from the two sides of each pattern.

more marked than at 0.1 MPa. At 20°C, in the presence of phosphate, the 1,1/1,0 intensity ratio was higher than at 11°C, suggesting that the higher force developed at 20°C originates at least in part from a larger fraction of attached cross-bridges.

Computer fitting of patterns from active muscle in the presence of phosphate at 0.1 MPa showed that the lattice spacing was about 45 nm, a shrinkage of 12% compared to the average relaxed value, and thus intermediate between the shrinkages of 8 and 14% previously reported for skinned mammalian muscle preparations (20, 28).

Effect of hydrostatic pressure on the equatorial pattern

Hydrostatic pressure had very little effect on the equatorial pattern of active muscle in the presence of phosphate. Where difference plots from patterns collected at 0.1 and 10 MPa showed visible peaks, these were also visible in the difference plots from the two patterns collected at 0.1 MPa before and after exposure to high pressure, and thus were due to instability in the structure of the fibers rather than an effect of hydrostatic pressure. Difference plots from stable fibers showed little evidence of intensity changes (Fig. 7 *b*). The local noise in Fig. 7 *b* is about 10% of the peak intensities in *a*, but since the peak of intensity of the reflections is spread over many channels, we would expect to be able to detect a difference in intensity between patterns that is less than the local noise as a shift in the noise "baseline."

A more sensitive test for cross-bridge detachment is to examine the ratio (1,1/1,0) of intensities of the 1,1 and 1,0 reflections. Since the intensities of the two reflections move in opposite directions, this is a sensitive indicator of detachment, and it is less affected than are difference plots by instabilities in the fibers. At low temperature in the presence of 20 mM phosphate, 10 MPa causes a 12% fall in steady tension. Yet Fig. 8 shows that over a series of data frames the intensity ratio is unperturbed by the application of 10 MPa, in contrast to the marked fall that accompanies relaxation. The same result was obtained at 20°C. In control experiments, single fibers were isolated from muscle fiber bundles prepared at the same time as those used for the x-ray diffraction studies, and their tension responses examined in our standard cell (13). The results were similar to those shown for the fiber bundle in Fig. 4.

Changes in lattice spacing were slight: analysis of the patterns showed that there tended to be a very small increase in spacing under pressure, and the values at 11 and 20°C were indistinguishable. At 10 MPa the lattice spacing was higher on average by 0.17 ± 0.11 nm (mean ± SE, $n = 7$), i.e., 0.38 ± 0.24%.

DISCUSSION

Causes of changes in lattice spacing

The cause of the small increase in lattice spacing in relaxed muscle under pressure is not known. Relaxed muscle shows no change in its passive tension on pressurization, even when it has been stretched enough at low pressure to produce a passive tension similar to that in rigor muscle (5). The compression of water (0.46% decrease in volume for 10 MPa (30)) would not be expected to affect the lattice dimensions, as water molecules can flow freely between the filaments. The effects of hydrostatic pressure on protein structure are not yet well characterized. Two detailed studies have been made on crystalline proteins: there was an overall compression of 0.47% in lysozyme under 101 MPa, but, while some parts of the molecule shrank, others were unchanged, and one

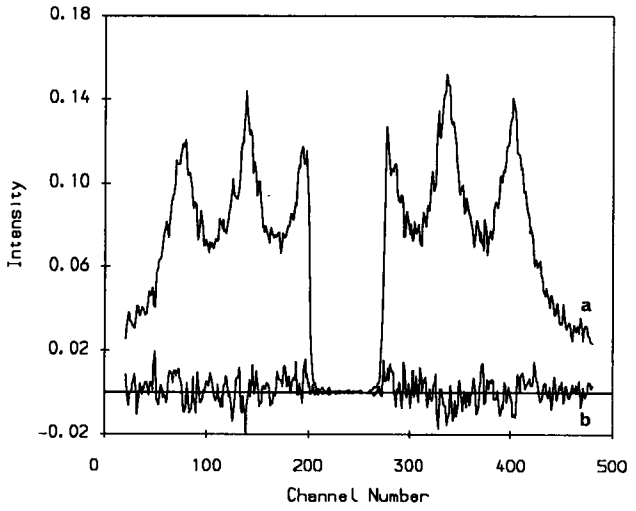

FIGURE 7 Equatorial x-ray intensities from active muscle in the presence of phosphate. (*a*) Pattern at 10 MPa. (*b*) Pattern at 0.1 MPa minus pattern from the same specimen at 10 MPa. Temperature 11°C; 10-s count periods; intensities normalized as described in Methods.

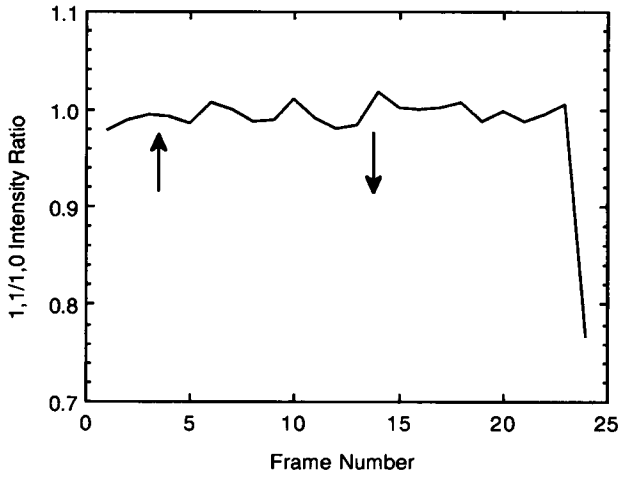

FIGURE 8 Effect of pressure on the ratio of intensities of the 1,1 and 1,0 reflections from active muscle in the presence of phosphate. The ratio was calculated independently for the two sides of the pattern (see Methods) and averaged. The arrows indicate that the pressure was raised from 0.1 to 10 MPa after the third frame and returned to 0.1 MPa after the 13th. The final frame was recorded under relaxing conditions. Temperature 11°C; 10-s count periods.

helix actually swelled by 0.17% (14). The crystals were bathed in a mother liquor containing 1.4 M NaCl, because at 0.83 M the crystals cracked on pressurization, suggesting that a more pronounced change in structure occurred when ionic screening was reduced. For myoglobin crystals grown in 3 M ammonium sulfate, there was no detectable volume change at 15 MPa, but some internal rearrangement occurred (16). At our comparatively low ionic strength (0.2 M) small changes in the structure of myofibrillar proteins therefore seem possible, and consequentially some structure, such as the Z disc or M line, might expand transversely under pressure to cause the increase in lattice spacing. Measurements of the position of the Z disc equatorial reflection would test this idea, but in our data the reflection was not well resolved from the 1,1 reflection from the filament lattice. Alternatively, the myosin heads could move to a slightly higher radius from the thick filament axis under pressure, increasing the electrostatic repulsive force between the filaments and thereby causing a lattice expansion: charge radius rather than total charge determines lattice dimensions at the filament charge densities present under our conditions (31).

The amounts of lattice expansion found in active and rigor muscle were similar, and about half the expansion seen in relaxed muscle. The same mechanism may underlie the expansion in each case, but be opposed by cross-bridges when these are present. In rigor the electrostatic repulsion between myosin heads and the thin filaments no longer exists, because the heads are all bound to the thin filaments (reviewed in Ref. 32), and the thin filaments are tethered to the thick filaments, so the force balance equation that sets the filament lattice spacing is quite different.

In rigor muscle, lattice spacing is linked to axial force when the former is caused to change by osmotic compression or variation in pH or ionic strength (33, 34). From these data we calculate that if the pressure-induced increase in rigor tension (about 25 kN/m^2 for a 10-MPa pressure rise (5)) had this cause, there would need to be a lattice expansion of about 7 nm, over 40 times the expansion we observe; clearly the tension increase in pressurized rigor muscle has a different origin, such as a pressure-induced shortening of the cross-bridge or the thick or thin filaments. The latter would have effects on the cross-bridges similar to stretch at constant pressure. The data of Ranatunga et al. (5) indicate that pressurization to 10 MPa is equivalent to a stretch of 3.5 nm per half sarcomere. When rigor muscle is stretched by this amount there is no measurable effect on the 1,0/1,1 intensity ratio (35, 36), though with larger stretches a small fall in the intensity of the 1,1 reflection has been found (37), and the effect on lattice spacing is less than 1% (36). Nevertheless, distortion of cross-bridges in stretch toward a more perpendicular orientation might be expected to increase the lattice spacing (38), and may contribute to the small increase we have found.

Interpretation of data from active muscle

Our initial results from active muscle are less satisfactory than those from relaxed and rigor muscle due to the instability of the preparation. Nevertheless some preliminary conclusions may be drawn. The 40% reduction in tension caused by phosphate is accompanied by a shift in the intensities and positions of the reflections in the equatorial pattern toward the values in relaxed muscle. For the following reasons these changes indicate detachment rather than simply isomerization of cross-bridges to a low force state without detachment. Low force attached states have a 1,1 reflection intensity hardly different from high force states, as shown by the increased intensity of this reflection both ahead of force development following stimulation (39–41), and in a low ionic strength relaxing solution where cross-bridges attach without producing force (42). They also exert a compressive radial force on the filament lattice (43).

In contrast with the effects of phosphate, the marked reduction in active tension under pressure seems not to be accompanied by cross-bridge dissociation, or indeed any major redistribution of mass within the unit cell, since there is no detectable change in the intensities of the equatorial reflections, or in their ratio. There was no evidence that depolymerization of either type of filament was the cause of tension fall: the x-ray pattern was essentially unchanged, whereas depolymerization of either filament type, to the extent required to produce a 12% fall in tension, would be expected to affect the reflection intensities. Moreover, measurements of tension under pressure (3) do not show the progressive, irreversible, downward trend that would be expected from breakage in the filaments followed by rapid translocation of filament fragments at zero load. Higher pressures than those we have used are required to cause significant depolymerization of synthetic filaments of either myosin or muscle actin under our conditions of pH and ionic strength (44–46).

Further evidence against detachment under pressure is the near constancy of the lattice spacing. Matsubara et al. (28) and Brenner and Yu (20) have shown that the transition between the active and relaxed states is accompanied by lattice expansion, and we have seen the same behavior under our conditions. Although the relationship between lattice spacing and force is nonlinear, such that there is little increase in the spacing when force falls from 100 to 80% of its maximum value (20), our data show that the amount of detachment associated with the 40% reduction in tension in the presence of phosphate is enough to allow expansion. The lack of further significant expansion under pressure therefore indicates that there has been no more detachment.

Although there was on average a small change in lattice spacing, it was an expansion, whereas it is a large compression that is known to reduce active tension which is otherwise independent of lattice spacing over a wide range of values (47); changes in lattice spacing cannot, then, explain the fall in active tension under pressure.

Our results thus favor the alternative that the reduction of tension under pressure is caused by a shift in the population from high-force cross-bridge states toward attached low-force states. This conclusion is broadly consistent with the results from the solution studies and tension transient studies outlined in the Introduction.

The apparently different effects of phosphate and pressure on cross-bridge detachment are, however, surprising. The equatorial diffraction data indicate that some detachment accompanies the addition of phosphate. Since phosphate induces an increase in the proportion of cross-bridges bearing ATP or ADP plus phosphate (see Ref. 6), it appears that the two-step actin binding equilibria for these cross-bridges favor detachment under the conditions of effective protein concentration present in the filament lattice. It is therefore unexpected that pressure, which perturbs the equilibrium between the two attached states in favor of the weaker binding state (see Introduction and Ref. 6), caused no detectable detachment. If this result is confirmed in our future studies, revision of the reaction pathway outlined in the Introduction may be required.

This initial study has shown that useful results can come from fiber x-ray diffraction under hydrostatic pressure. For the structurally stable states of relaxed and rigor muscle good data can be obtained from small bundles of skinned fibers. A second generation pressure cell has now been constructed that allows intact muscle fibers to be electrically stimulated under pressure, with simultaneous force measurement, which will enable us to test and extend our observations using live muscle (27).

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