X-ray evidence for two structural states of the actomyosin cross-bridge in muscle fibers

(x-ray diffraction/myosin layer lines/skinned muscle fibers/rigor cross-bridges/low ionic strength)

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ABSTRACT Biochemical data, stiffness measurements, and equatorial x-ray diffraction patterns provide evidence that actomyosin cross-bridges form in relaxed skinned rabbit fibers at low ionic strength (20 mM). In the present study we examined the structure of these cross-bridges by using two-dimensional x-ray diffraction. In contrast to rigor cross-bridges, which significantly weaken the myosin-based reflections characteristic of relaxed fibers at 120 mM ionic strength (notably the 86-Å and 108-Å layer lines and the 72-Å and 143-Å meridians), the formation of low ionic strength cross-bridges produced only small changes in these reflections. In addition, all these cross-bridges did not produce the additional intensity on the 59-Å actin-based layer line near the meridian that is associated with rigor cross-bridges. However, the formation of low ionic strength cross-bridges caused the 215-Å meridional reflection to decrease in intensity, as also is the case when rigor cross-bridges are formed. These observations show that the structure of the low ionic strength cross-bridge is significantly different from that of the rigor cross-bridge, and they raise the possibility that contractile force may be generated by a transition between these two actomyosin configurations.

The idea that the actomyosin cross-bridge† in muscle cells has more than a single configurational state and that contractile force is developed by a transition between two such states figures prominently in theories of muscle contraction (1–5). This possibility seems plausible because the rigor (ATP-free) cross-bridge can be made to change configuration by allowing it to react with adenylyl-5′-yl imidophosphate (p[NH]ppA), a nonhydrolyzable analogue of ATP (6). However, there is as yet no direct evidence that a cross-bridge takes on different configurations during the normal, ATP-driven, activity cycle.

In the present study we used two-dimensional x-ray diffraction to examine the structure of the cross-bridge that forms in rabbit psoas fibers when the ionic strength (μ) of the relaxing solution is lowered from 120 mM to 20 mM (7–9). The equatorial pattern (9) suggests that the structure of this cross-bridge differs from that of the rigor cross-bridge; the intensity ratio I11/I01 shows an intermediate value between rigor and the relaxed state. The kinetic properties of the two types of cross-bridges are also quite different (8); the low μ cross-bridge is in rapid equilibrium with the detached state of S-1, while the rigor cross-bridge is considerably more stable.

We found that the myosin-based layer line pattern of the low μ cross-bridge is almost the same as that of the relaxed fiber at normal ionic strength and significantly different from that of the rigor fiber. This is also the case for the 72-Å and 143-Å meridional reflections. These findings show that the structure of the low μ cross-bridge is markedly different from that of the rigor cross-bridge, possibly because S-1 is more flexibly bound to actin in the low μ cross-bridge than in the rigor cross-bridge.

METHODS

Specimen Preparation. Muscle bundles (1-2 mm in diameter) were prepared from rabbit psoas muscles. They were soaked in a skimming solution that contained 150 mM potassium propionate, 5 mM EGTA, 5 mM potassium phosphate buffer, 3 mM magnesium acetate, and 3 mM ATP (pH 7.0) for 24 hr at 0–4°C (10). The solution was renewed every 8 hr. For data collection, a small bundle of fibers (0.6–0.8 mm thick) was split off from the skinned bundle. Low μ relaxing solution contained 1 mM MgCl2, 2 mM EGTA, 5 mM imidazole, and 3 mM MgATP (Sigma), pH 7.0 at 4°C; μ was about 20 mM. In normal μ relaxing solution, 100 mM potassium propionate was added to low μ relaxing solution. Rigor solution contained 128 mM KCl, 2 mM EGTA, and 5 mM imidazole buffer, pH 7.0 at 4°C. In ATP backup solution, creatine kinase (Sigma) at 200 units/ml and 5 mM creatine phosphate were added to relaxing solution.

X-Ray Camera and Cell. A rotating anode x-ray generator (Elliott GX-13) was used at 36 kV and 60 mA. A mirror-crystal monochromator camera (11) was used for two-dimensional patterns; the pattern was recorded on a stack of three films. The specimen-to-film distance was 34 cm. For the equatorial pattern, a single-mirror Franks camera was used. The pattern was recorded with an electronic position-sensitive detector (12). The specimen-to-detector distance was 45 cm. Sarcomere length was measured by a 2 mW He/Ne laser before and after the experiment.

The muscle bundle was mounted in a cell (5 ml capacity) made of anodized aluminum. The length between the ties (Deknatel braided surgical silk, 5-0) was 13 mm. The distance between the Mylar windows was slightly greater than the thickness of the specimen. The bathing solution (25 ml) was continuously recirculated with a syringe pump (13). The temperature of the solution was kept at 4°C by thermo-electric units (Cambion) on the cell and by a cooled reservoir (Lauda) for the bathing solution.

Recirculation of the bathing solution (about 10 ml/min) was necessary for preservation of the relaxed state, as indicated by the intensity ratio I11/I01 of the inner equatorial reflections. At μ = 120 mM, I11/I01 was 0.9, which is close to the value measured in an isolated single fiber (9). When circulation of the relaxing solution was stopped, the fiber went

Abbreviations: μ, ionic strength; p[NH]ppA, adenylyl-5′-yl imidophosphate; S-1, subfragment 1 of the myosin molecule; I01, intensity of the 10 equatorial reflection; I11, intensity of the 11 equatorial reflection.

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†In this paper a "cross-bridge" is defined as the moiety formed when myosin subfragment 1 (S-1) binds to actin.

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into rigor gradually, usually within 1 hr, although this time was also affected by the gap between the Mylar windows.

The ATP backup system was not used routinely because it increased the ionic strength and the viscosity of the relaxing solution. However, in test experiments it was found that the layer line patterns were the same with and without the backup system.

Exposure time on the mirror-monochromator camera was generally about 20 hrs. Over the 3 or 4 days required to obtain an internally controlled series of patterns, the preparation appeared to be stable, although an increase in the equatorial intensity ratio for the relaxed state of about 10% a day was noted. An exception to this was the 215-Å meridional reflection, which appeared to decrease in intensity at the end of a normal cycle of solution changes. However, the reflection could be stabilized by reducing the exposure time in each solution to 2-5 hr.

Intensity Measurements. Intensities were measured either manually or with an automated system. The manual technique made use of a Joyce–Loebl microdensitometer. The automated system consisted of a Perkin–Elmer microdensitometer and the PIC image analysis program (14).

In all, 18 preparations were examined, 14 at sarcomere length 2.3 μm and 4 at sarcomere length 4.0 μm. The myosin-based layer lines were visible in 8 preparations (and measurable in 5 of these) at 2.3 μm; they were measurable in every preparation at 4.0 μm.

RESULTS

Sarcomere Length 2.3 μm

Myosin-Based Reflections. Fig. 1 shows a typical set of diffraction patterns for the same preparation in normal μ (120 mM) and low μ (20 mM) relaxing solutions in a rigor solution (μ = 136 mM). In normal μ (Fig. 1c) the 3rd- (143-Å), 4th- (108-Å), and 5th- (86-Å) order layer lines of the myosin 430-Å repeat are clearly visible. The 143-Å and 72-Å meridional reflections are very intense.

In rigor solution (Fig. 1a) the 4th- and 5th-order myosin layer lines become less intense while the 3rd-order is unchanged. The 143-Å meridional is reduced to about half the intensity in normal μ, while the 72-Å meridional is reduced to about one-fourth (Table 1). Similar changes on passing into the rigor state have been reported previously (11, 15).

Table 1. Intensities of the myosin-based reflections normalized to the intensity of the 59-Å layer line at a distance of 1/120 Å⁻¹ from the meridian and relative to normal μ

<table>
<thead>
<tr>
<th>Reflection, Å</th>
<th>Rigor</th>
<th>Low μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>86⁺</td>
<td>0.25  (0.04)</td>
<td>0.84 (0.05)</td>
</tr>
<tr>
<td>108⁺</td>
<td>0.30 (0.05)</td>
<td>1.08 (0.06)</td>
</tr>
<tr>
<td>72₄₀</td>
<td>0.27 (0.03)</td>
<td>1.15 (0.12)</td>
</tr>
<tr>
<td>143₄₀</td>
<td>0.50 (0.11)</td>
<td>0.92 (0.14)</td>
</tr>
</tbody>
</table>

The Joyce–Loebl densitometer was used. Sarcomere length 2.3 μm. Numbers in parentheses are SEM.

*Subscript m denotes meridional reflection and subscript l denotes layer line.

Average value between 1/140 and 1/190 Å⁻¹.

These effects could be reversed by returning the fibers to normal μ. In low μ the intensities of the myosin layer lines [normalized relative to the 59-Å layer line at a distance of 1/120 Å⁻¹ from the meridian (16)] are almost the same as in normal μ (Fig. 1b, Table 1). The 72-Å meridional is enhanced slightly, relative to normal μ, while the 143-Å reflection is the same (Table 1).

Fig. 2 shows densitometer traces of the diffraction pattern at 1/180 Å⁻¹ off the meridian (broken lines in Fig. 1). The similarity of intensities of the 4th- and 5th-order myosin layer lines in normal and low μ, and their weakening in rigor, is evident (Table 1).

The 215-Å meridional was clear (in the third film) with the fiber in normal μ but was almost absent in low μ and in rigor.

Actin-Based Reflections. The prominent actin layer lines, the 370-, 69-, 59-, and 51-Å, are seen in Fig. 1. For the same exposure time, these reflections were enhanced slightly when the ionic strength was reduced from 120 to 20 mM and more so when the fiber was put into rigor (11). The intensity increase in rigor (measured at a distance of 1/120 Å⁻¹ from the meridian) was not the same for all the actin-based reflections: the 370-, 69-, and 51-Å reflections increased more than the 59-Å reflection, which changed, at its maximum, by less than 20%.

The intensity distribution along the 59-Å layer line (Fig. 3) was analyzed because this distribution is related to the scattering diameter of the actin filament (11, 17). In normal μ,

![Fig. 1. X-ray diffraction patterns for three states: (a) rigor (μ = 136 mM), (b) low μ relaxed (20 mM), and (c) normal μ relaxed (120 mM). Sarcomere length was 2.3 μm. Exposure time was about 20 hr for each state. No significant change was observed in sarcomere length before and after experiment. The sequence of exposure was normal μ, low μ, and then rigor. The strong 59- and 143-Å layer lines, and the 72-Å meridional, are identified. The 69- and 51-Å layer lines, which flank the 59-Å layer line, are weak but clearly visible. The numbers 2 through 6 locate the orders of the basic 430-Å myosin repeat.](image)
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Fig. 2. Densitometer tracing (Perkin-Elmer) along line 1/180 Å⁻¹ from the meridian for normal μ relaxed (top trace), low μ relaxed (middle trace), and rigor (bottom trace) states. The line along which the intensity was measured is indicated by the broken line in Fig. 1. The intensities of the 4th- and 5th-order myosin layer lines are about equal for the two relaxed states at normal and low ionic strength, but they are considerably reduced in rigor. The intensity of the 3rd-order myosin layer line is similar in the three states.

The intensity maximum of the 59-Å layer line was at about 1/110 Å. When the ionic strength was reduced to 20 mM, the intensity distribution was almost same as that in normal μ. In rigor a broad additional peak appeared around 1/250 Å⁻¹. This additional peak is probably caused by an increase in the effective diameter of the actin filament due to decoration by S1 [11, 17].

Sarcomere Length 4.0 μm

To distinguish between a direct effect of ionic strength on the structure of the myosin filament and effects due to cross-bridge formation, experiments were made at a sarcomere length of 4.0 μm, where the actin and myosin filaments no longer overlap. The lattice spacing (d₁₀) was 30% less than at 2.3 μm, the intensities of the actin-based layer lines were similar to those at 2.3 μm, and the myosin-based layer lines were more intense (15).

At this sarcomere length the patterns in normal and low μ were almost the same. In contrast to the result at 2.3 μm, the intensity of the 215-Å meridional did not appear to be affected by the decrease in ionic strength. In rigor the 4th- and 5th-order myosin layer lines were about 30% less intense than in normal μ. This change in intensity was considerably less than the 70% decrease seen at sarcomere length 2.3 μm. It could have been due to heterogeneity in sarcomere length of the preparation (a few sarcomeres with myofilaments in overlap might have been present) or possibly to a direct effect of ATP absence on the myosin helix.

DISCUSSION

One of the main findings of the present study is that in a low μ relaxing solution several of the myosin-based reflections are significantly different from those in the rigor solution. Since the stiffness in a low μ relaxing solution, measured during a rapid stretch, is more than half the rigor value (8), presumably because more than half the rigor number of cross-bridges are present, the geometry of S-1 binding to actin for the cross-bridge formed at low μ must be different from that of the binding that takes place when ATP is absent.

A possible explanation for this difference is that in the absence of ATP the binding of S-1 to actin is strong enough to perturb the myosin helix and thereby attenuate the myosin-based reflections. On the other hand, the interaction of S-1 with actin is much weaker in the presence of ATP at low μ.

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2 In the present study the ionic strength of the rigor solution was 136 mM. The rigor diffraction pattern was also recorded at μ = 20 mM. This pattern was similar to the 136 mM pattern, except that at the lower ionic strength the actin-based layer lines were more intense and the maximum of the 59-Å layer line was closer to the meridian.

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Fig. 3. Intensity distribution along the 59-Å actin layer line. The intensity was normalized to the peak intensity for each distribution. The measurements were made by scanning the film across the layer lines at various distances from the meridian and plotting intensity on the layer line against distance. The data were averaged for four quadrant components. The error bar is the SEM.
(7). If this corresponds to more flexible binding, the myosin head could remain ordered according to the thick filament backbone repeat. This loose binding is consistent with the kinetic liability of the bridge observed in quick-retch (8) and biochemical (18) experiments. It could come about in the fiber if only one of the two S-1 moieties of myosin were bound to the actin filament, or perhaps if each S-1 were bound to the actin filament at only one of the two binding sites (19).

59-Å Layer Line. The distribution of intensity along this layer line peaks closer to the meridian in rigor than in normal μ relaxing solution, presumably because the scattering units are larger in rigor due to decoration of actin by S-1 (11, 17). In low μ relaxing solution the intensity distribution is close to that in normal μ, even though more than half the rigor number of cross-bridges are thought to be present. This result is additional evidence that binding of S-1 to actin is more flexible in low μ than in rigor, in which case the bound S-1 and actin would not be expected to scatter coherently.

215-Å Reflection. Although the origin of this “forbidden” reflection is uncertain (20, 21), its intensity is decreased when calcium-activated cross-bridges (22) or rigor cross-bridges are formed. The present study shows that the intensity also decreases reversibly in low μ relaxing solution when the myofilaments overlap. Lowering μ when the myofilaments are out of overlap does not affect the intensity of this reflection. These results indicate that cross-bridges are formed in low μ relaxing solution, in agreement with stiffness (8) and equatorial x-ray diffraction measurements (9).

Order–Disorder Consideration. A possible explanation of the preservation of the myosin layer lines in the presence of low μ cross-bridges, in the face of layer line weakening by rigor cross-bridges, is that low μ has an intrinsic ordering effect (23) that compensates for the disordering effect associated with cross-bridge formation. However, this seems unlikely because fibers with the filaments out of overlap showed no intensification of the myosin layer lines when the ionic strength of the relaxing solution was lowered from 120 to 20 mM.

Relationship to p[NH]ppA. On the basis of insect muscle studies, Tregear et al. (6) suggested that in the presence of p[NH]ppA cross-bridges may be attached to actin but still be stereospecifically related to the myosin filament. The myosin-based layer line reflections from rabbit psosa fibers in low μ relaxing solution point to a similar structure in this case as well. The observations that the 72-Å meridional reflection is strong (relative to rigor) and the 59-A actin-based layer line is relatively weak near the meridian for both kinds of cross-bridges (ref. 24, Table 1, Fig. 3) also indicate that they have structural aspects in common.

Although quantitative differences exist, a biochemical feature common to the p[NH]ppA and low μ cross-bridge is that the binding of S-1 to actin is weaker than in rigor, where nucleotide is absent (25, 26). This suggests that “weak” binding between actin and myosin preserves the myosin-based structure in fibers, while “strong” binding perturbs the myosin helix. Thus there appears to be a correlation between binding strength in solution and cross-bridge structure in the fiber. Binding strength also affects the kinetic properties of the cross-bridge, as both p[NH]ppA (27) and low μ relaxed (8) cross-bridges equilibrate with the detached state more rapidly than the rigor cross-bridge.

Relation to the Contraction Mechanism. In calcium-activated, force-generating muscle fibers the myosin-based features of the diffraction pattern are much less intense than in the relaxed fiber (11). This implies that the structure of the low μ cross-bridge is significantly different from that of the force-generating cross-bridge as well as that of the rigor cross-bridge. Thus we have evidence for two structurally distinguishable cross-bridge states in the presence of ATP. This raises the possibility that force may be generated in the ATP-driven activity cycle by a transition between these structural states. In addition, the fact that one of these structural states is found in a calcium-free solution supports the idea (7) that calcium may activate the contractile process by enabling the force generating transition to proceed at a physiological rate.

Conclusion. Our data and related observations of others discussed above provide evidence for the existence of two significantly different cross-bridge structures: one that preserves the myosin-based features of the diffraction pattern (low μ relaxed, p[NH]ppA) and another that reduces the intensity of these features (rigor, Ca2+-activated). The former are associated with weakly bound cross-bridges; the latter, with strongly bound cross-bridges. The correlation between cross-bridge structure and binding strength is of particular interest because during the hydrolysis of ATP by actomyosin in solution the actin and myosin cycle between weakly and strongly bound states (3, 18). The structural data provided by the present work indicate that these changes in binding strength may be closely related to the mechanism by which the energy of ATP hydrolysis is converted to mechanical motion (28).

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